Factors Influencing Assisted Reproductive Technology [ART] Outcome: Possible Implications for a Private and Public Sector Fertility Clinic.

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DECLARATION

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ABSTRACT

Infertility treatment, more specifically Assisted Reproductive Technology [ART], is available worldwide, but in many countries and public clinics, this service is not being offered, mostly due to limited resources and funds. Many factors can influence the outcome of ART and insufficient funds can have an effect on ovarian stimulation protocols, assisted reproduction procedures, laboratory procedures and equipment (i.e. CO_2 incubator). Strategies making ART as affordable and accessible as possible is of importance.

The objective of the study was to investigate which factors in ART treatment might have the most significant effect on ART outcome in two ART laboratories – one in the public sector and one in the private sector. Two studies, one retrospective and one prospective were conducted.

The **retrospective study** (2013 - 2014) investigated the effect of two different CO_2 incubators (MINC® benchtop incubator and large conventional Forma® incubator) used at a private fertility clinic, on ART outcome. Fertilization, embryo quality and development, and clinical pregnancy rate [CPR] outcomes were compared. A strict exclusion criteria was applied to eliminate other factors that could have an effect on the outcomes and patients were well paired for the study. Three hundred and eighty five (385) cycles were included. No statistical significant difference was observed between the two incubators for embryo quality on culture days 2 and 5. For day 3, the MINC® incubator showed a significant superiority over the Forma® incubator for the proportion of good quality embryos [GQE]/number of ova aspirated (44.58% vs. 39.31%; $p < 0.05$). There was no statistical significant difference in CPR between the incubators (45.43% vs 47.17%; $p = 0.81$).

The **prospective study** aimed at determining (by means of regression analyses) the possible negative or positive impact of female patient profile (specifically number of oocytes, age, body mass index [BMI], Anti-Mullerian Hormone [AMH] and female diagnosis - tubal factor and endometriosis) in two different ART clinics (public and private fertility clinic) on ART outcome with regard to CPR. Eight hundred and twenty (820) cycles (572 in the private clinic; 248 in the public clinic) were included. Patient profiles in the two clinics were very different. The most common female diagnosis at the private clinic was Advanced Maternal Age compared to Tubal Factor Infertility [TFI] at the public clinic. Patients with a high BMI was also much more prevalent in the public clinic. No statistically significant association, in both clinics (with pooled and separate data), was observed between BMI, AMH, endometriosis or TFI and CPR. The only significant association with CPR in the final regression analysis (pooled data) was the Site (clinic) and the number of metaphase II oocytes available. Data analysis for the two clinics separately, considering all confounding factors investigated, indicated that the number of metaphase II oocytes available was the only factor that showed a significant association with CPR - and only

at the private clinic. For the public clinic, none of the factors had a significant association with CPR when all factors were included in the analysis.

Various factors contribute to ART outcome, and these factors may differ in public and private clinics as shown in this study. Although the results did not show marked differences in outcome between the incubator types, all outcomes were better in the MINC® and its use should be encouraged. The result of an independent, significant association between number of MII oocytes and CPR is linked to specific ovarian stimulation protocols and potential alternative strategies should be investigated to optimize outcome without increasing costs.

OPSOMMING

Infertiliteit behandeling, meer spesifiek geassisteerde reproduktiewe tegnieke [GRT], word wêreldwyd toegepas, maar in baie ontwikkelende lande en staatsklinieke is hierdie diens nie beskikbaar nie. Die rede daarvoor is hoofsaaklik beperkte bronne en befondsing. Alhoewel daar baie faktore is wat die uitkoms van GRT kan beïnvloed kan 'n gebrek aan fondse die ovulasie stimulasie protokolle, GRT prosedures en beskikbaarheid van apparaat (bv. CO_2 inkubator) affekteer. Strategieë wat GRT so bekostigbaar en toeganklik as moontlik maak is dus van uiterste belang.

Die doel van hierdie studie was om te bepaal watter faktore moontlik 'n effek kan hê op die uitkoms van GRT behandeling by twee verskillende GRT laboratoriums – een in die staat- en 'n ander in die privaatsektor. Twee afsonderlike studies, een retrospektief en die ander prospektief, is gedoen.

Die retrospektiewe studie (2013 – 2014) het beoog om te bepaal wat die effek van twee verkillende inkubators, (MINC® inkubator "benchtop" en 'n groot konvensionele Forma® inkubator), op GRT uitkoms by 'n privaat fertiliteitskliniek is. Bevrugting, embrio kwaliteit en ontwikkeling en die kliniese swangerskap uitkoms [KSU] is vergelyk. Om faktore wat moontlik die uitkoms van die studie kon beïnvloed te elimineer, is 'n streng uitsluitingskriteria toegepas en paring van pasiënte was dus voldoende. Drie honderd vyf en tagtig (385) siklusse is ingesluit. Geen statisties beduidende verskil ten opsigte van embrio kwaliteit op kultuurdae 2 en 5 is gevind tussen die twee inkubators nie. Die MINC® inkubator het egter beter gevaar as die Forma® inkubator op kultuurdag 3, en statisties betekenisvol meer goeie kwaliteit embrio's/aantal oösiete geaspireer is gevind (44.58% teen 39.31%; $p < 0.05$). Daar is ook geen statisties betekenisvolle verskil ten opsigte van kliniese swangerskap uitkoms tussen die twee inkubators waargeneem nie (45.43% teen 47.17%; $p = 0.81$).

Die prospektiewe studie het beoog om te bepaal (d.m.v. 'n regressie analise) watter faktore van die vroulike pasiëntprofiel (spesifiek die getal oösiete, ouderdom, liggaamsmassa-indeks, *Anti-Mullerian* hormoon en vroulike diagnose - buisfaktor infertiliteit en endometriose), moontlik 'n positiewe of negatiewe effek kan hê op die GRT uitkomste, veral kliniese swangerskap [KSU], in twee verskillende fertiliteitsklinieke. Agthonderd en twintig (820) siklusse is ingesluit (572 in die privaatkliniek; 248 in die staatskliniek) in die studie. Die resultate het aangedui dat daar wel 'n verskil was in die pasiëntprofiele tussen die twee klinieke. Die algemeenste vroulike diagnose in die privaatkliniek was gevorderde moederlike ouderdom en by die staatskliniek, buisfaktor infertiliteit. Die staatskliniek het ook 'n hoër insidensie van pasiënte met 'n hoë BMI getoon. Geen statisties beduidende assosiasie, in beide klinieke (met saamgevoegde en aparte data), is waargeneem tussen BMI, AMH , endometriose of buisfaktor infertiliteit en KSU nie. Die enigste

statisties beduidende interaksie in die finale regressie model vir die saamgevoegde data met KSU, was die kliniek (“Site”) en die getal metafase II oösiete beskikbaar. Vir die twee klinieke apart, en wanneer al die faktore in ag geneem is, was net aantal metafase II oösiete betekenisvol geassosieer met KSU en ook net vir die privaatkliniek. Vir die staatskliniek het geen faktor wat ondersoek is, ’n statistiese beduidende assosiasie met KSU getoon nie.

Verskeie faktore beïnvloed die uitkomste van ’n GRT siklus en hierdie faktore mag verskil in die staats- en privaatklinieke, soos bewys deur die studie. Alhoewel resultate nie betekenisvolle verskille in uitkomstes vir die twee inkubators gewys het nie, was alle uitkomstes beter in die MINC® en die gebruik daarvan moet aangemoedig word. Die resultaat van ’n onafhanklike, beduidende assosiasie tussen die aantal metafase II oösiete en KSU is afhanklik van die spesifieke ovariale stimulasie protokol en potensiële alternatiewe strategieë moet ondersoek word om uitkomstes te optimaliseer sonder om die kostes te vermeerder.

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LIST OF ABBREVIATIONS

AFC	Antral Follicle Count
AMH	Anti-Müllerian Hormone
ART	Assisted Reproductive Techniques/Technology
BMI	Body Mass Index
BU	Biostatistics Unit
CC	Clomiphene Citrate
CPR	Clinical Pregnancy Rate
EC	Early Compact
ET	Embryo Transfer
EQ	Embryo Quality
FSH	Follicle Stimulating Hormone
FR	Fertilization Rate
GnRH	Gonadotrophin Releasing Hormone
GRT	"*Geassisteerde Reproduktiewe Tegnologie*"
GQE	Good Quality Embryo/s
hCG	Human Chorionic Gonadotrophin
HEPA	High Efficiency Particulate Absorption
HIV	Human Immunodeficiency Virus
hMG	Human Menopausal Gonadotrophin
HREC	Health Research Ethics Committee
HSG	Hysterosalpingogram
ICSI	Intracytoplasmic Sperm Injection
IMSI	Intracytoplasmic Morphologically Selected Sperm Injection
IVC	Intravaginal Culture
IVF	In-vitro Fertilization
IU	International Units
IUI	Intra-uterine Insemination
KSU	"*Kliniese Swangerskap Uitkoms*"
LH	Luteinizing Hormone
MI	Metaphase 1
MII	Metaphase 2
OPR	Ongoing Pregnancy Rate
PCOS	Polycystic Ovarian Syndrome
PGD	Pre-Genetic Diagnosis
PGS	Preimplantation Genetic Screening

PICSI	Physiological Intracytoplasmic Sperm Injection
PID	Pelvic Inflammatory Disease
ROS	Reactive Oxygen Species
SAMRC	South African Medical Research Council
SI	Standard Incubator
SOP	Standard Operating Procedure
STD	Sexually Transmitted Disease
TB	Tuberculosis
TFI	Tubal Factor Infertility
TMS	Time-lapse Monitoring System
UV	Ultra-violet
VAT	Value-added Tax
VOC	Volatile Organic Compound/s
WHO	World Health Organization
ZAR	South African Rand

CHAPTER 1 – BACKGROUND INFORMATION AND LITERATURE REVIEW

1.1 Assisted Reproduction Globally

For more than three decades, In Vitro Fertilization [IVF] has played a critical role in human conception. IVF and other Assisted Reproductive Techniques [ART] have revolutionized the possibility of helping childless couples. There have been estimated that more than 5 million babies have been born following ART treatment (Franklin, 2013).

Infertility has been clinically defined as a reproductive system disease that causes failure to achieve a clinical pregnancy after 12 months or more of unprotected, regular, sexual intercourse (Zegers-Hochschild *et al.*, 2009). In 2015, the World Health Organization [WHO], acknowledged infertility as a global public health issue (Pantoja *et al*., 2015). IVF can be viewed as a test for reproductive potential, allowing for a detailed assessment of oocytes, oocyte-sperm interaction and embryo quality, as well as an effective treatment for most forms of subfertility (Ola *et al.,* 2005).

An estimated 10 – 15% of all couples experience at least one period of infertility during their lifetime (Revonta *et al.,* 2010). Approximately 50% of infertile couples will require treatment with some form of assisted conception in order to achieve a pregnancy and a review article by Dyer *et al.* (2013) reported that 85% of the world's population are living in countries where ART are available (Collins, 2002). Although millions of babies have been born from IVF (Franklin, 2013), ART is not widely used in low-resource environments due to the high cost. In most countries, the public sector offers limited ART services (Hovatta *et al.,* 2006). The overall demand for infertility treatment has been estimated at 56% of the population (Makuch *et al.,* 2011). This could be due to the limited attention infertility has received at global and regional levels. Vayena *et al*. (2009) claims two main reasons for the poor attention infertility receives in developing countries. Firstly, the wide perception that infertility is a problem limited to the developed world and not that of developing countries. The second reason is the belief that ART is technically much too demanding for the capacity and expertise available in developing countries and too expensive, because their resources are already limited.

1.2 Infertility in Developing Countries

Technological progress in the field of ART has produced new medical, ethical, social and economic issues that require attention from health professionals and society at large. Current barriers to reproductive treatment are predominantly financial in nature (Huyser, 2008). In low-resource countries, especially where the prevalence of infertility is high, financial resources

are too scarce to provide affordable services and do not allow for expensive treatment (Pantoja *et al*., 2015). Annual increases in the cost of IVF also put the treatment beyond the reach of the majority of infertile couples (Aleyamma *et al*., 2011).

Although infertility is one of the major health problems individuals are facing in developing countries, healthcare systems in developing countries are more focussed on addressing overall health at lower costs and other health issues that do not include infertility (Habbema, 2008). There are numerous ethical concerns regarding ART in developing countries. One of the biggest concerns is overpopulation in low-resource countries. Other concerns include; the fact that natural resources are extremely limited and the ethical problem of practitioners who are not sufficiently trained, but still offer services to unsuspecting and uninformed patients. Although findings have indicated that these concerns are not unique to developing countries, its prevalence is far greater compared to that of developed countries (Allahbadia, 2013).

Another factor that largely contributes to infertility in developing countries is the prevalence of Sexual Transmitted Diseases [STD] and Human Immunodeficiency Virus [HIV], affecting both the male and female partner (Ombelet, 2014). Infections, especially pregnancy related, abortions that are not performed in a clinical setting or by a healthcare professional, lack of STD and HIV awareness and diagnosis are some of the main causes that contribute to infertility in developing countries (Ombelet, 2014).

Infertility treatment in developing countries should be prioritized and low-cost options drastically needs to be explored to address this problem (Vayena *et al.,* 2009). With available lowered cost ART treatment options, governments could be motivated to allocate public funds for ART but with the implementation of these services quality control measures should be standardised practice to ensure the delivery of appropriate maternal and neonatal health services (Dyer & Pennings, 2010).

1.3 Cost of Assisted Reproduction and the South African Context

It is commonly known that ART procedures are expensive, due to various contributing factors (Johnson, 2014). Not only are equipment and ovarian stimulation protocols expensive, but highly specialized clinicians and scientists/technologists, also essential for this treatment, are not available in many hospitals and clinics (Mahajan, 2013).

Similar to other countries, South Africa has limited risk protection against the costs involved with regard to ART and assisted reproduction services offered in the private and public sector.

The costs of ART treatment are in general not covered by medical aid schemes in the private sector (Dyer *et al.,* 2013). In 2012, Huyser and Boyd published an article providing a breakdown of the cost per IVF cycle in a large private clinic, where 35% of the cost was for laboratory purposes, 29% for clinicians' fees and consultations, 28% for medication used during the cycle and 8% for clinic fees. The cost of ART procedures, in South Africa, in 2012, ranged from R7 000 – R14 000 in the public sector and R25 000 – R50 000 in the private sector (Huyser & Boyd, 2012). Fee structures have since increased.

Although ART treatment is available in South Africa in the public sector, a limited number of hospitals and clinics across the country offer good quality ART treatment (Huyser & Boyd, 2013). A possible reason for this could be the limited resources allocated to public sector ART treatment, with related concerns regarding shortcomings of other healthcare systems, such as HIV and tuberculosis [TB] treatment. Another reason could be the fact that, on a national and international level, health strategies have been more focussed on contraception and on lowering fertility rates overall (Ombelet *et al.,* 2008). The government subsidizes public sector ART treatment, but only to a certain extent, where a segment of the funds have to be provided by the patients. Thus, a substantially lower cost option is available when compared to private clinics, but by lowering the cost, adaptions have to be made to standard protocols exploring options where treatment costs can be reduced (Dyer *et al.,* 2013).

Various studies have explored strategies to lower the cost of ART treatment. Examples of these strategies include simplification of standard ART procedures, with adaptions to the ART laboratory, application of milder ovarian stimulation strategies and non-IVF ART, which include fertility awareness programmes (Ombelet *et al.,* 2008).

Due to the fact that one of the most expensive components of ART is ovarian stimulation medication, various experts have proposed milder stimulation protocols, as previously mentioned (Mahajan, 2013). Low-cost cycles created in this way, may provide accessibility to ART treatment for patients in lower socio-economic environments, who still have the right, according to the South African Bill of Rights, to "make decisions concerning reproduction" (Constitution of the Republic of South Africa Act, No. 108 of 1996 Chapter 2. Bill of Rights), and to be provided with the opportunity to at least one ART treatment cycle (Mahajan, 2013).

As mentioned before, the main contributors to ART costs are; a) ovarian stimulation drugs and protocols, b) expensive specialized equipment needed and c) highly specialized and skilled clinicians and scientists/technologists.

1.4 Role players in ART Outcome

Various factors influence the outcome of an ART cycle. They range from the ART laboratory and equipment to the method of female stimulation and also the patient's fertility profile - with female age being a significant factor (Eijkemans *et al.,* 2014; Klitzman, 2016). Patient profile specific factors are often related to lifestyle choices or behaviour and include; BMI (Luke *et al.,* 2011), smoking habits (Fuentes *et al.*, 2010) and tubal factor infertility (Dun & Nezhat, 2012). Equipment - specifically the incubators used for gamete/embryo culturing (Gardner *et al.,* 2008:4) - as well as ovarian stimulation (Bosch *et al.*, 2016) – with a wide variety of approaches available, not only affect ART outcome but also have a significant impact on the final cost of the cycle.

1.4.1 Laboratory Equipment, Culture Conditions & Incubators

Embryo culturing is one of the most important aspects of ART (Gruber & Klein, 2011). For optimum embryo culturing, various key environmental variables need to be considered within the culture system. The most important variables are; appropriate regulation of culture media (especially with regard to pH, temperature and osmolality), air quality inside the laboratory and overall sterility (Swain, 2014). As all of these variables can be influenced by the type of culture incubator, it can be regarded as the most crucial component of an ART laboratory (Swain, 2014). One of the most important functions of an embryo culture incubator is to regulate and maintain environmental variables such as gas concentrations, specifically carbon dioxide [CO_2] and oxygen [O_2] (Guarneri *et al.,* 2015). Regulation of CO_2 is crucial as the concentration of the gas plays a vital role in the pH regulation of embryo culture medium and the pH of the culture medium is one of the most important variables since it can significantly influence gamete function and embryo development (Swain, 2012).

Various types of embryo culture incubators are commercially available. CO_2 incubators differ mainly in terms of; size, temperature maintenance and recovery, CO_2 and O_2 monitoring and regulation and lastly, pH and gas supply requirements. A few examples of major culture incubator differences can be seen in Table 1. Over the last decade incubator functions have revolutionized and the most advanced type of incubator can now also provide time-lapse images of embryos as they develop over time (Rubio *et al.,* 2015, Kirkegaard *et al.,* 2015, Goodman *et al.,* 2016). Regardless of modern modifications, all of these incubators are still dependent on a power supply and a trustworthy gas or gas mix supply, thus incubator management, maintenance and quality control remains crucial for optimal ART outcomes (Higdon *et al.,* 2008)

1.4.1.1 Oxygen Concentration

As mentioned previously, gas concentrations play a crucial role with regard to optimal embryo culturing and development. In *in vivo* conditions, embryos are exposed to an O_2 concentration of 2 to 8% (Ciray *et al.,* 2009). In the older ART incubators, the O_2 was provided by ambient air and was therefore 20%. Numerous studies have however demonstrated that embryo culture *in vitro* should occur in the same O_2 range as they would physiologically (± 5%), contradicting earlier embryo culturing protocols that made use of 20% O_2 concentration (Meintjies *et al.,* 2009; Nanassy *et al.,* 2010, Nastri *et al.*, 2016).

The damaging effect of O_2 at atmospheric concentration (20%) on embryo development has been reported widely in previous studies (Thompson et al., 1990; Catt & Henman, 2000; Karagenc *et al.,* 2004). The accumulation of reactive oxygen species [ROS] in the cytoplasm is most likely the mechanism through which high O_2 concentration reduces developmental ability of embryos during in vitro culture (Guarneri *et al.,* 2015). A beneficial effect of lowering O_2 concentration in incubators to 5% has been observed for both embryo quality and pregnancy rates, mostly in trials where embryos were cultured and transferred at blastocyst stage (Guarneri *et al.,* 2015). Other reports also showed that atmospheric O_2 is injurious through the generation of free oxygen radicals (Guérin *et al.,* 2001). Atmospheric O_2 concentrations preferentially damage the inner cell mass (ICM) of blastocysts, while the trophectoderm is less affected or even stays well developed. Experimental evidence however, also demonstrates that the ability of embryos to develop into blastocysts does not necessarily indicate an absence of O_2 toxicity and its associated anomalies in cell properties, such as altered metabolism and gene expression (Bavister, 2004).

In 2008, Kovačič *et al.* conducted a prospective study to determine the effect of 5% and 20% O_2 on prolonged development of embryos. The study reported the effects of the differing O_2 concentrations on fertilization rate, proportion of morphologically optimal embryos, blastocysts and optimal blastocyst development on day 5. The study was conducted using sibling oocytes from routine consecutive stimulated IVF and ICSI cycles. The results for IVF (n=988 oocytes) and ICSI (n=928 oocytes) were analysed separately. The results indicated that lower O_2 did not influence fertilization rate, however 20% O_2 resulted in a significantly higher proportion of optimal quality embryos on day 3 after IVF. In both procedures, IVF and ICSI, the lower O_2 concentration improved blastulation rate and increased the proportion of embryos reaching the expanded blastocyst stage with a normal inner cell mass on day 5. The conclusion was that a lower O_2 concentration in the incubator atmosphere contributed to better embryo

morphology and higher blastulation rates (Kovačič *et al.,* 2008). Guarneri *et al.* (2015) conducted a similar retrospective analysis comparing two routine IVF culture strategies. The first culture system consisted of atmospheric O_2 concentration (± 20%) until insemination on Day 0 for ICSI cycles or until denuding on Day 1 for standard IVF, followed by the use of a low (±5%) O_2 concentration for the rest of the culture period to the blastocyst stage until embryo transfer. The second culture system consisted of exclusive use of low O_2 concentration. The main outcome of the study was determined by the utilization rate defined as the number of transferred plus vitrified embryos per inseminated cycle. Other outcomes of the study included pregnancy and live birth rates. The results of the study indicated that of the 701 IVF/ICSI cycles that were performed, the utilization rate for IVF (38% and 37%; p=0.78) and ICSI (37% and 41%; p=0.40) was similar between the two culture systems.

Another study conducted by Kovačič *et al.* (2010) aimed to assess whether embryo culture at different O_2 concentrations had any effect on ICSI outcome. This prospective randomized trial's first outcome was to assess on-going pregnancy rate (OPR) and secondly, the cumulative pregnancy rate, implantation and embryo quality for two treatment groups and the clinical outcomes for sub-groups (which included; optimal cycles, poor responders and older women). The two treatment groups consisted of embryos cultured either at 6% CO_2, 5% O_2, and 89% N_2 mix or at 6% CO_2 in air. The findings indicated that although a low O_2 concentration resulted in a higher incidence of good quality day 2 embryos and blastocysts, the on-going pregnancy rate and implantation rate were similar in both O_2 concentration groups. Low O_2 concentration resulted in a higher cumulative pregnancy rate in the main group (high O_2 concentration vs. low O_2 concentration) and a higher pregnancy rate in the poor responder subgroup with embryo transfers performed mostly on Day 3. The conclusion was that the use of reduced O_2 concentration in IVF is reasonable, irrespective of the duration of embryo culture. Reduced O_2 concentration not only improved embryo development and cumulative pregnancy, but was also recommended for poor responder patients. A very recent study by Nastri *et al.* (2016) conducted a meta-analysis that included 21 studies that compared low and atmospheric O_2 concentration for embryo culture. From the results obtained the researchers concluded that although a small improvement of approximately 5% in live birth/ongoing pregnancy and clinical pregnancy rates was observed. The evidence was of very low quality and the best interpretation of the study was that uncertainty remains about differences in the comparison between the two O_2 concentrations.

Although the effect of atmospheric O_2 on embryo development is well-known, conventional large box incubators using CO_2 in air (20% O_2) are significantly cheaper

compared to modern benchtop incubators (5% CO_2 and 5% O_2 gas mix) and the replacement of incubators remain a very expensive exercise for low resource laboratories. According to Guarneri *et al.* (2015) scientists at ART clinics tend to overload available low O_2 incubators causing an increase in the frequency of the opening and closing of the incubator door, with the potential rebound effect for culture conditions and micro-environment maintenance. Since the conventional evaluation of embryo morphology at different embryo development stages under a microscope necessitates the frequent opening of the incubator door, it results in fluctuating O_2 and CO_2 concentrations. Kovačič *et al.* (2008) suggested an embryo culture system should be developed that will maintain a constant atmosphere in the best way possible.

1.4.1.2 pH

Internal pH homeostasis is essential for normal development in a preimplantation embryo as it plays a crucial role in cellular communication, protein synthesis, cellular division and enzyme activity (Lane *et al.,* 1998). During the later stages of embryo development robust regulatory mechanisms are in place to regulate internal pH, which does not seem to be the case for preimplantation embryos and exposure to culture conditions without optimal intercellular pH have shown to result in developmental delay or even arrest (Squirrell *et al.* 2001; Lane & Gardner, 2005). Due to the fact that the pH of embryo culture medium is regulated by the balance between bicarbonate and CO_2 (based on the Henderson-Hasselbalch equation), the slightest fluctuation in the CO_2 concentration inside the culture incubator can induce significant changes within the culture medium, directly influencing the embryo (Lane *et al.,* 2008). The optimal pH for embryo culturing is 7.2 to 7.3 (Kelly & Cho, 2014).

Zander-Fox *et al.* (2010) conducted a study investigating the effect of intracellular pH fluctuations on preimplantation embryos. The results concluded that a lowered intracellular pH resulted in embryos with a decreased cell number and inner cell mass and increased apoptosis. This study contributed to the field of knowledge that intracellular pH fluctuations, directly affected by culture media composition, can have detrimental effects on embryo development (Zander *et al.,* 2006; Rooke *et al.,* 2007), thus, highlighting the importance of pH maintenance and regulation within embryo culture media.

1.4.1.3 Temperature

Temperature plays a pivotal role during embryo development. The fine regulation of temperature inside an ART incubator is extremely important when trying to maximize embryo development, implantation and pregnancy (Walker *et al.,* 2013). The optimal temperature for

culturing embryos has, since 1969, been determined at 37°C (Kelly & Cho, 2014). Depending on the number of patients, every ART laboratory needs at least 2 – 3 CO_2 incubators that need to be monitored daily for proper maintenance of correct temperature regulation. Frequent opening/closing of the incubator door, overnight power outages or failures as well as temperature differences at different locations within the same incubator contribute to increased difficulty to maintain a stable temperature (Anifandis, 2013).

Heating systems in incubators differ widely but there are three main options available for CO_2 incubators. The three systems include; water-jacketed, contact heat (not to be confused with direct heat) and air-jacketed, also known as direct heat (Swain, 2014; Kelly & Cho, 2014; Meintjies, 2014). In a water-jacketed incubator, temperature is maintained through heated water (with high heat capacity) within the incubator's chamber walls giving a consistent interior temperature (Kelly & Cho, 2014). The water-jacketed heating system is especially prevalent in larger CO_2 incubators and have shown to maintain the interior temperature within the incubator chamber four to five times longer (Kelly & Cho, 2014; Swain, 2014) compared to the direct heat system in cases of incubator opening or power failure (Meintjies, 2014). Although this aspect of a water-jacketed incubator is beneficial, these incubators tend to have high power consumption (Swain, 2014). The contact heat heating system, mostly adopted in benchtop incubators, consists of heat being transmitted from the warmed incubator surface directly onto the culture dish (Meintjies, 2014). Another heating system, direct heat (air-jacketed) consists of warm air originating from mounted chamber heaters being circulated inside the incubator, sometimes with assistance of an internal fan (Meintjies, 2014). With evaluation of these different heating systems in terms if temperature recovery, contact heat shows superiority (Swain, 2014). Cooke *et al.* (2002) reported that a benchtop contact heat incubator (MINC) showed superior temperature recovery time of 5.5 minutes (from 35°C to 37°C) compared to > 20 minutes in a conventional water-jacketed incubator.

With regard to optimal temperature regulation and maintenance, several studies have indicated that prolonged exposure of embryo culture to temperatures other than the optimal 37°C, reduces fertilization ability and also the ability of cell division/cleavage, growth, implantation potential and subsequently pregnancy rate (Wang *et al.,* 2002; Hong *et al.,* 2014). One study indicated that a 1°C drop from optimal temperature reduces the ability of embryos to cleave but allows division of nuclei (McCulloh, 2004). This indicates that cytokinesis is more temperature sensitive than mitosis. Thus, prolonged exposure of embryos to higher temperatures than optimal, has a deleterious effect on cytokinesis in all embryos (normal and abnormally fertilized; 1, >2 pronuclei). Human embryos are therefore very sensitive to any fluctuation in temperature (Anifandis, 2013).

1.4.1.4 Incubator Types and Specifications

As multiple incubator types are available, the decision of selecting a one should be dependent on the needs of the specific laboratory. Culture incubators differ in various aspects (Table 1), as previously mentioned. Not only are different capacities and sizes available, but the type of gas monitoring systems, gas supply and temperature control can differ significantly from one incubator to another.

Benchtop incubators are gaining favour among ART laboratories since stringent control and recovery of temperature and gas concentrations and therefore pH is possible (Hong *et al.,* 2014). When considering temperature, benchtop type incubators are capable of direct heat transfer compared to larger box-type incubators, which maintain temperature mostly by means of a water-jacket (Swain *et al.,* 2016). In 2007, Fujiwara *et al.,* conducted a study to determine the effect of micro-environment maintenance on embryo culture and clinical results using two types of incubators. They used a benchtop incubator (K-MINC-1000, COOK) and a conventional large incubator with a water-jacketed heating system (Personal Multi Gas CO_2 incubator, APM-30D, ASTEC). Both incubators used standard O_2 concentrations, 5% was used in this specific study. The temperature and O_2 concentration in both incubators were compared following a 5 second door opening/closing procedure. Embryos of 30 IVF cases were selected randomly and assigned to either one of the incubators. The early-stage good quality embryo formation rate and the good blastocyst formation rate were compared as indicators for micro-environment maintenance ability. The results indicated that, after the 5 second door opening/closing, the temperature recovery for the benchtop was approximately 5 minutes compared to 30 minutes for the conventional incubator. The O_2 concentration recovery was significantly better in the benchtop (3.0 minutes ± 0 minutes) compared to the conventional incubator (7.8 minutes ± 0.9 minutes). The early-stage good quality embryo rate and good blastocyst formation rate for the benchtop (39.5% and 15.1%) were significantly higher than the conventional incubator (28.4% and 7.8%). The results confirmed that the micro-environment maintenance ability of an incubator influences the rate of successful formation of good embryos and the micro-environment can be improved by replacing culture equipment (Fujiwara *et al.,* 2007).

Since the bicarbonate in the media and the CO_2 concentration in the culture incubator determine media pH, maintenance and stability of CO_2 extremely important. Although various published studies show that embryos can still develop in a medium with fluctuating pH values, these variations influence the quality and development of embryos (Zander-Fox *et al.,* 2010).

Table 1: Comparison of specifications of different incubator types/brands.

Specifications	Incubator Type/Brand						
	K-MINC™	***Planer (BT37)***	***Miri®***	***Forma Series II (Model 3110)***	***Forma Scientific (Model 3164)***	***Miri® TL***	***EMBRYOSCOPE®***
Manufacturing Company	COOK Medical (Marcus Medical*)	ORIGIO (Harrilabs*)	ESCO Medical	Labotec	Labotec	ESCO Medical	Illex South Africa
Price ZAR (Incl. VAT)	± 480 000	± 221 000	± 435 480	± 100 000	*Not Available - Discontinued*	± 993 000	± 1 300 000
Design	Benchtop	Benchtop	Benchtop	Large Box	Large Box	Benchtop time-lapse Imaging	Benchtop time-lapse Imaging
Gas	Premixed gas (CO_2; N_2; O_2)	Premixed gas (CO_2; N_2; O_2)	Premixed gas (CO_2; N_2; O_2) **OR** Built-in gas mixer (CO_2; N_2; ambient air)	CO_2 only	CO_2 only	Built-in gas mixer (CO_2; N_2; O_2)	Built-in gas mixer (CO_2; N_2; O_2)
Humidification	Yes (Disposable flask)	Yes (Disposable flask)	Yes (Water reservoir) **OR** No	Yes (Internal water reservoir/pan)	Yes (Internal water reservoir/pan)	No	No
CO_2 Sensor		Infra-red	Infra-red	Thermal Conductivity (Other models Infra-red)	Infra-red OR Thermal Conductivity	Infra-red	
Temperature Control	Direct heat transfer	Direct heat transfer & cooling fan	Direct heat transfer (PT1000 sensors)	Water jacket	Water jacket (Precision thermistor sensor)	Direct heat transfer (PT1000 sensors)	Direct heat transfer
Air Filter	Hydrophobic filter	HEPA	HEPA / VOC / UV	HEPA	0.22 µm filter	HEPA and VOC 254 nm UV-C with 185nm filter	HEPA and VOC
Capacity (Volume In Litres)	0.43 (2 Chambers)	0.43 (2 Chambers)	0.886 (6 chambers)	184	184.1	n/a - 6 chambers (holds 12 embryos each)	n/a - 6 chambers (holds 12 embryos each)
Recovery Time Temperature/CO_2	± 5 min / ± 3 min	± 5 min / ± 3 min	< 1 min / < 3 min	± 20 min	± 20 min	< 1 min < 3 min	< 0.2 min / < 5 min

*South African supplier

Therefore, small benchtop incubators with individual chambers are now the incubators of choice since various studies showed a decreased recovery time for both temperature and CO_2 compared to the conventional incubators (Fujiwara *et al.,* 2007).

Embryo selection for transfer remains one of the most important aspects of ART. Currently, morphological assessment of the embryo remains the method of choice in many ART laboratories, which conventionally requires inspection outside the controlled environment of the incubator. This leads to exposure of the embryos to undesirable changes in critical parameters such as; temperature, pH, humidity and gas concentrations (Meseguer *et al.,* 2012). A relatively new, but very expensive addition to ART incubators is the time-lapse monitoring system (TMS). Time-lapse monitoring overcomes the obstacle of removing embryos from the incubator, thus not exposing them to environmental changes. By using time-lapse imaging of the developing embryo, one also increases the number of morphologic observations available to the embryologist for assessing embryo quality. The use of an automated time-lapse monitoring system with continuous embryo surveillance provides comprehensive data on embryo development kinetics. This system allows the precise determination of the onset, duration and intervals between cell divisions (Meseguer *et al.,* 2012). Results from time-lapse incubator culture are controversial, since comparison with other types of incubators is difficult and large randomised controlled studies are lacking. Rubio *et al.* (2014) conducted a prospective study focusing on whether embryo culture in the integrated EmbryoScope® time lapse monitoring system [TMS] and selection supported by the use of a multivariable morphokinetic model, would improve ART outcome when compared to embryo culture in a standard incubator [SI] (conventional large incubator). The results indicated that the pregnancy rate per treated cycle was not statistically significant (65.2% and 61.1% respectively). The only statistical significant difference found were for ongoing pregnancy rate (54.5% and 45.3% respectively $p = 0.01$]. The conclusion was that culturing and selecting embryos in the TMS improves ART outcome. A similar study by Kirkegaard *et al.* (2012) aimed to evaluate the development of sibling embryos from oocytes randomized to be cultured in either a SI or a TMS The results indicated no significant difference between the outcomes of the two incubators and both supported embryonic development equally.

Even though the above-mentioned studies indicate otherwise, comparing the TMS to SI and more specifically the larger SI remains a difficult task. A fairer competitor would be a small benchtop incubator, as characteristics of the two incubators are similar. Goodman (2016) conducted a prospective randomized controlled study to determine whether the addition of morphokinetic data would improve ART outcomes in a closed culture system. This was the first randomized controlled trial where all the embryos were cultured in a similar manner in the

closed TMS. (Meseguer, 2016). The results of the Goodman study indicated that the time-lapse morphokinetic data did not significantly improve clinical ART outcomes (Goodman *et al.,* 2016).

Laboratory equipment, specifically CO_2 incubators in an ART clinic, contributes largely to the high cost of the service (Ombelet, 2007). Currently, due to the high cost of incubators specifically, other *alternatives* have been investigated to possibly lower the cost associated with gamete and embryo incubation (Ombelet, 2013). Therefore, at the other end of the spectrum of CO_2 incubators, a recent study showed comparable IVF outcomes using a "simplified embryo culture system" consisting of a very simple and cost effective "single tube method" incubator system when compared with the traditional box type incubator (Ombelet, 2014, Van Blerkom et al., 2014). In the "single tube method" incubator, the correct concentration of CO_2 is produced by a controlled chemical reaction between specific amounts of sodium bicarbonate and citric acid. The CO_2 gas is produced in a sterile glass test tube and relayed via a sterile connection to another sterile test tube containing the embryo culture medium – ensuring an optimal pH during embryo development. Van Blerkom *et al.* (2014) conducted a pilot clinical trial using this simplified and cost effective laboratory culture method (single tube method incubator) for IVF and found that the fertilization and implantation rates were similar to those reported using a high resource and expensive incubator in the same IVF program. They observed an embryo implantation rate of 34.8% (8/23), a live birth rate of 30.4% (7/23) and one miscarriage at 8 weeks gestation. The results were compared with the Belgian IVF registration data (BELRAP) (high resource IVF laboratories using conventional incubators and culture strategies). The clinical pregnancy rate per transfer was 34.2% (3403/9929) and live birth rate was 29.0% (2808/9680). This indicated that the results obtained by Van Blerkom *et al.* (2014) were almost identical to that of BELRAP.

Thus, there is clearly room and reason for investigation into simplified culture systems. This incubation method is not only a simpler incubation method, but also less expensive when compared to standard incubation methods and has shown similar outcomes. However, ICSI cycles and therefor treatment for patients with severe male factor, are not possible with the use of this incubation method.

Another initiative aiming at reducing laboratory costs, the INVOcell® device, consists of fertilization of oocytes and early embryo development in a capsule, which is placed into the maternal vaginal cavity for incubation, replacing the use of a standard incubator (Frydman & Ranoux, 2008). A study conducted by Mitri *et al.* (2015) aimed to determine if an intravaginal culture device [IVC] could provide acceptable embryo development rates compare to standard

IVF. The study consisted of 10 women aged 27 to 37 years with an indication for IVF treatment. Oocytes were randomized for fertilization using conventional IVF or the IVC device. The results indicated that the fertilization rates in the standard IVF group (68.7% ± 36%) were higher compared to the IVC device (40.7% ± 27%). The clinical pregnancy rate for the IVC device was 30% compared to 43% in the standard IVF group. Although the IVC device produced reasonable pregnancy rates, psychological factors were not investigated (Mitri *et al.,* 2015).

Doody *et al.* (2016) recently conducted a study to compare the efficacy of intravaginal culture [IVC] of embryos in INVOcell™ to standard IVF incubation. The results of this prospective randomised study indicated that there was no significant difference in the percentage of quality blastocysts transferred or live birth rate. A difference was observed in the percentage of total good quality embryos, where standard IVF incubators showed superiority compared to IVC (50.6% vs. 30.7%; $p < 0.05$). The researchers concluded that standard IVF culturing resulted in higher quality blastocysts compared to IVC, however, both produced identical blastocysts for transfer, resulting in similar live birth-rates (Doody *et al.* 2016). The results of this study shows that IVC can be effective and may broaden access to fertility care in selected patient populations (Doody *et al.* 2016).

1.4.1.5 Incubator Management and Quality Control

As previously discussed, the equipment used within an ART laboratory, especially the CO_2 incubators, contributes largely to the success rates of the treatment (Higdon *et al.,* 2008). As variations among laboratories exist, standard quality control recommendations can be considered in optimising ART outcomes. One of the most important means of quality control is record keeping of all variables that could influence ART outcomes. These variables include regular measurements of temperature, culture media pH and CO_2 and O_2 concentrations (Swain, 2014). Other factors to monitor, since they could potentially influence ART outcomes, are air quality, humidity and decontamination within the laboratory and CO_2 incubator (Boone & Higdon, 2014).

As temperature plays a pivotal role with regard to embryo culturing, temperature maintenance, regulation and quality control is of vital importance within the CO_2 incubator. Frequent opening and closing of the CO_2 incubator doors result in temperature fluctuations which should be avoided. Incubators offering a contact heat system has been shown to have the most superior recovery rates (Cooke *et al.,* 2002; Fujiwara *et al.,* 2007). The use of lids for culturing dishes and smaller volumes of culturing medium and oil have also shown to positively influence

temperature maintenance and recovery times (Cooke *et al.,* 2002). Temperature within the CO_2 incubator should be monitored on a daily basis and records of the measurements should be kept. Temperature calibration of an incubator should be ensured before the use thereof and must take place within the laboratory the incubator will be used (Meintjies, 2012).

Due to the fact that the culture media pH is regulated by the CO_2 concentration, the gas supply of the CO_2 incubator should be adapted for the specific culture media used, as different brands/types of culture media require different gas concentrations for the same pH. The assumption that the pH is a direct result of the percentage of CO_2 should not be made. The pH of culture medium is however, a direct function of the CO_2 partial pressure within the culture medium and is affected by the height above sea level, which differs from one laboratory to another (Meintjies, 2012). The pH should be verified for each batch of medium and should be consistent between incubators used within the same laboratory. pH should be monitored regularly and calibration should take place at 37°C (Gardner *et al.,* 2012). Since culture medium pH is determined by the CO_2 concentration maintenance and regulation thereof is extremely important. Equipment controlling gas concentrations should be sensitive to the specific range and monitored on a daily basis (Gardner *et al.,* 2012). CO_2 is mostly supplied in gas cylinders, and to ensure that the cylinder does not run empty without a timely switch to a new cylinder, it is recommended that the changeover pressure set on the manifold is 50% of the original tank pressure (Meintjies, 2014). The cylinder pressure and CO_2 concentration within the CO_2 incubator should also be monitored on a daily basis and records thereof should be kept. Various measuring tools exist to determine the CO_2 concentration within the CO_2 incubator, but an infrared instrument is recommended for improved accuracy (Boone & Higdon, 2014).

ART CO_2 incubators, that make use of 20% O_2, obtain their internal air from the external environment, which contains particles and volatile organic compounds [VOCs] that need to be considered when it comes to incubator management as these compounds can influence embryo development (Boone & Higdon, 2014). Due to the fact that the relevant concentrations of VOCs within an ART laboratory has not yet been determined, most laboratories have implemented air handling systems to increase the air quality. Even though these filtration systems can be beneficial for ART outcomes, the air quality inside the culture incubator is also of high importance as VOCs have been detected in the gas supply for culture incubators (Hall *et al.,* 1998). Filtering of the ART incubator gas supply has mostly become a norm and most incubators are supplied with inline filters as it has shown increased ART outcomes (Merton *et al.,* 2007). These filters usually contains high efficiency particulate absorption [HEPA] filtration

to improve air quality but some incubators also include active carbon filters and an ultra-violet [UV] light to remove and ensure degeneration of VOCs (Sharmin & Ray, 2012).

Increased humidity in a culture incubator is important as it decreases evaporation, which is directly proportional to the osmolality of the culture media. Osmolality can be increased if an increase in evaporation of water within the culture incubator occurs, with potential detrimental effect on the embryos (Ozawa *et al.,* 2006). This effect can be overcome with the use of tissue culture oil overlay (Swain, 2014). Humidity within a conventional culture incubator can be increased with the use of a water pan but maintenance and regular replacement of the water supply and water pan is crucial as bacterial and fungal growth can occur (Boone & Higdon, 2014). An alternative to the water pan is a supplied water bottle/reservoir.

It is clear that maintenance, monitoring and management of culture incubators is extremely important when it comes to embryo culture and ultimately pregnancy outcome. Various variables need to be regulated and monitored to ensure optimal embryo development and the laboratory staff should be adequately trained and informed in terms of quality control and incubator management.

Taking all the above mentioned information into consideration, cost analysis remains an important factor. Although various studies have indicated a slightly improved outcome in ART results when a benchtop incubator was used compared to a conventional large box incubator (Fujiwara *et al.,* 2007), various clinics, especially public clinics, do not always have the financial capacity to replace conventional incubators with new and improved incubators. This is not the case for large private clinics with adequate finances where new improved laboratory equipment (i.e. incubators) and the latest developments can easily be implemented.-With on-going advances in technology, multiple CO_2 incubator types exist - with varying capabilities, costs and different methods of regulating the internal environment. The selection of a CO_2 incubator has become a complex, expensive, but crucial process.

1.5 Female Stimulation in an Assisted Reproductive Treatment Cycle

Conventional standard ovarian stimulation can lead to high cost and the transfer of two or more embryos in IVF exhibit a high probability of multiple pregnancies (Polinder *et al.* 2008). Following a mild stimulation protocol is not only less expensive (Matsaseng & Kruger, 2014), but there is evidence of increased safety. Mild stimulation protocols can minimize discomfort and the risk of complications as well as multiple gestations (Siristatidis *et al.,* 2012).

Ferraretti *et al.* (2015) conducted a prospective cohort study to validate the use of clomiphene citrate [CC] in IVF when a mild stimulation protocol is followed to reduce patient risk and minimise the cost. The study included 163 good prognosis patients (≤38 years old, normal ovarian reserve, normal ovulatory cycles, BMI under 29, no previous ART cycles, no severe endometriosis, no history of recurrent miscarriage and no autoimmune or endocrine diseases) undergoing IVF. Patients that had undergone surgical procedures for retrieval of sperm were also excluded from the study. Mild stimulation was administered by using a fixed protocol of CC (100 mg per day from cycle days 3 to 7) in combination with low doses of gonadotropins (150 IU of recombinant FSH on cycle days 5, 7 and 9) and GnRH antagonist. The outcomes were measured as cumulative delivery rate per patient after three embryos transferred (fresh or frozen). After a 2.4 months mean period to pregnancy the cumulative delivery rate per patient was 70%. The study confirmed that mild stimulation could be a realistic option for good prognosis patients undergoing IVF (Ferraretti *et al.,* 2015).

Matsaseng *et al.* (2013) asked the important question, whether the ART field is *ready* for a change from conventional ovarian stimulation to mild ovarian stimulation. They conducted a met analysis study comparing the efficacy of mild ovarian stimulation and conventional stimulation in IVF. The results of the met analysis showed a strong favour towards conventional ovarian stimulation (Matsaseng *et al.,* 2013). On a national scale in South Africa, various strategies with specific regard to ovarian stimulation have been explored. Currently, the Fertility clinic at Tygerberg Academic Hospital (public) in the Western-Cape implements a mild ovarian stimulation protocol that consists of lower doses compared to standard protocols. The specific protocol uses 100mg CC from cycle days 3 to 7 in conjunction with 75 IU of human menopausal gonadotrophin [hMG] on alternative cycle days, 4, 6 and 8. LH levels are tested on day 9 up until administration of human chorionic gonadotrophin [hCG], (Matsaseng & Kruger, 2014).

Research indicated that the reason for the introduction of GnRH antagonist and GnRH agonist was to decrease the frequency of a premature LH surge, since studies claimed that CC/gonadotrophins stimulation increases a premature LH surge with 20 – 25% (Hwang *et al.,* 2003). A recent study, aimed to determine whether the use of prolonged (8 days) CC administration compared to the standard use of 5 days would be an effective method in preventing LH surge. The study was a randomized controlled trial consisting of 227 patients and the results indicated that prolonged CC administration did not supress a premature LH surge compared to the standard administration in the ART program (Matsaseng *et al.,* 2016). The biggest issue with a premature LH surge is the possible repercussions thereof; unplanned oocyte retrieval, high cycle cancellation rate and insufficient time for oocyte maturation

ultimately affecting the pregnancy outcome of a cycle (Matsaseng *et al.,* 2016). To prevent the negative outcomes of a LH surge more expensive stimulation drugs are used and contributes significantly to ART costs. A premature LH surge can potentially result in cancelation of the cycle and starting a new cycle where every step of the treatment has to be repeated with extra cost to the patient.

1.6 Factors Possibly Contributing to Infertility

The pathophysiology of infertility could include a very wide range of contributing factors. Factors ranging from genetic or chromosomal anomalies, endocrine disorders to congenital or infectious malformations throughout the reproductive tract, not only in the female partner but also in the male partner. Female factors include advanced maternal age (Jackson *et al.,* 2015), endometriosis (Mathieu d' Argent *et al.,* 2010), tubal factors (Zou *et al.,* 2014), polycystic ovarian syndrome, diminished ovarian reserve, ovulatory dysfunction, and implantation failure or repeated spontaneous abortion (Phillips, 2015).

The incidence of idiopathic infertility is 10 - 15% (Phillips, 2015), with abnormal findings upon medical examination occurring minimally. The larger portion of modifiable risk factors contributing to idiopathic infertility can be categorized under environmental risk factors (Gormack *et al.,* 2015). These factors include; diet (which directly links to Body Mass Index [BMI]) (Provost *et al.,* 2016), lifestyle habits (Homan *et al.,* 2007) including alcohol use and smoking habits (The Practice Committee of the American Society for Reproductive Medicine, 2008), medical treatments and environmental factors including exposure to radiation, chemicals etc. (Phillips, 2015).

Currently, there are various indications for the use of ART and a number of factors affecting the success have been identified. IVF was first reported as a treatment option for women with severe tubal disease. With improved efficacy after the introduction of gonadotropin stimulation and Intra-cytoplasmic Sperm Injection [ICSI], the indications for IVF have expanded to include severe male factor infertility, diminished ovarian reserve, ovulatory dysfunction, severe endometriosis and idiopathic infertility. IVF also provides a new means of preconception genetic diagnosis and preservation of fertility. IVF is regarded as the most effective treatment option for couples with multifactorial infertility problems (Huang *et al.,* 2012).

1.6.1 Female Factor Infertility

1.6.1.1 Female Age

Fertility is defined as the capacity to produce a baby (Committee of the American Society for Reproductive Medicine, 2013). Relative fertility is approximately halved among women in their 30's compared to women in their 20's. Fertility varies among populations and declines with age in both men and women, but the effects of age are much more pronounced in women. For women, the chance of conception decreases significantly after the age of 35 years. Although semen parameters in men also decline detectably after 35 years of age, male fertility does not appear to be affected before the approximate age of 50 (Committee of American Society for Reproductive Medicine, 2013).

Advanced maternal age has been defined as 35 years or older, given the increased genetic and obstetric risk (Jackson, 2015). A classic report on the effect of female age on fertility found that the percentage of women not using contraception who remained childless rose steadily according to their age at marriage. Six percent (6%) at age 20 – 24, 9% at age 25 – 29, 15% at age 30 – 34, 30% at age 35 - 39 and 64% at age 40 - 44 (Committee of American Society for Reproductive Medicine, 2006).

Globally, in both high- and low-income countries, there has been a drastic increase in the delay of pregnancy among women, especially among those that are more financially secure and educated (Chan & Lao, 2008). Not only has a delay in pregnancy onset been observed, but also over the last few decades, there has been a drastic decline in the average number of offspring born to individual women in developed countries. This can possibly be contributed to changes in their social and emotional behaviour (Ehrlich, 2015). As a result of advances in the field of medicine and ART, couples are starting to postpone pregnancy to a more optimal or convenient time (Sauer, 2015).

Even though obstetric and gynaecological breakthroughs have been made, and the prevalence of ART services has increased, there are still many risk factors associated with advanced maternal age pregnancy (Carolan & Frankowska, 2011). Aneuploidy (Demko *et al.,* 2016), pregnancy loss (Spandorfer *et al.,* 2004) and pre-eclampsia (Tandberg *et al.,* 2014) are some of the risks associated with advanced maternal age pregnancy. Not only is advanced maternal pregnancy a risk for the foetus but there are also various risk factors associated maternally (Crawford & Steiner, 2015).

Advanced maternal age infertility, has been linked to oocyte abnormalities (The Practice Committee of the American Society for Reproductive Medicine, 2006). A very recent study, aimed to determine how the number of euploid embryos are influenced by maternal age. Results indicated that in the 22599 day 3 and the 15112 day 5 embryos analysed, the average number of euploid embryos per cycle declined from approximately four, for women in their late twenties, to less than one, for women older than 42 years (Demko *et al.,* 2016).). Aneuploid embryos are more prevalent in older women and have a higher risk for miscarriage according to a retrospective study conducted by Spandorfer *et al.* (2004). This study aimed to determine the relationship between maternal age and foetal loss in IVF pregnancies and results showed that there was a significant increase in foetal loss with advanced maternal age. Patients 40 years and older had a foetal loss rate of 22.2% compared to patients 35 to 39 years with 12.8%; 31 to 34 years, 7.6% and younger than 30 years, 5.35% (Spandorfer *et al.,* 2004).

The declining of a woman's fecundability is a natural process of reproductive aging. Age-related infertility has a multi-factorial cause and there has been demonstrated that there is a definite decrease in the ovarian reserve and the number of oocytes, as a women's age increases (Crawford & Steiner, 2015).

1.6.1.2 Anti-Müllerian Hormone and Antral Follicle Count

In females, Anti-Müllerian Hormone [AMH] is assumed to be produced exclusively by follicular granulosa cells in the late pre-antral and small antral follicles (Gnoth *et al.,* 2015). AMH seems to be derived only from the ovary, since postmenopausal women are associated with undetectable AMH concentrations. Granulosa cells of primary follicles show homogenous AMH expression, but maximal expression occurs in late pre-antral and small antral follicles (Peluso *et al.,* 2014). AMH plays a crucial role in the regulation of folliculogenesis by inhibiting the sensitivity of dominant antral follicles to FSH and aromatase, thus, limiting the number of primordial follicles that develop (Gnoth *et al.,* 2015). *Normal* AMH values vary from one laboratory to another, thus the establishment of an exact value has been extremely challenging, as many studies have demonstrated. The AMH determination test has also demonstrated inconsistencies in results as the test is relatively new and standardization of protocols are still under investigation. One interpretation of the level of AMH indicates the following; *high* as over 3.0 ng/ml, *normal as* over 1.0 ng/ml, *low normal* between 0.7 – 0.9 ng/ml, *low* as 0. – 0.6 ng/ml and *very low* as any value less than 0.3 ng/ml (*The American Society for Reproductive Medicine,* 2013).

The serum AMH allows indirect estimating whether the number of available oocytes is above, at, or below the expected value for age and therefore attempting to predict reproductive longevity (Peluso *et al.,* 2014). Not only can AMH concentrations be used as a prediction tool of ovarian reserve, the quantity of the ovarian follicle pool or ovarian response to ovarian stimulation (Gnoth *et al.,* 2015), but in clinical practice also as a prognostic tool in differentiating the various causes of secondary oligo-amenorrhea (Peluso *et al.,* 2014). A study conducted by La Marca *et al.* (2007) aimed to evaluate whether serum AMH measurement on any day of the menstrual cycle could predict ovarian response in women undergoing ART. The results demonstrated that women in the lowest AMH quartile (<0.4 ng/mL) were older and required a higher dose of recombinant Follicle Stimulating Hormone [FSH] than women in the highest quartile (>7 ng/mL). This confirmed their hypothesis that AMH levels can serve as a predictable serum marker of ovarian response, which can be measured independently of the day of the menstrual cycle (La Marca *et al.,* 2007).

Seifer *et al.* (2015) conducted a retrospective study on 5087 cycles where AMH was extremely low (≤ 0.16 ng/mL) to determine which factors contribute to the outcome of the cycle, cancelled or otherwise. The study indicated that the total cancellation rate per cycle was 54% with 38.6% of these cycles cancelled before oocyte retrieval and 3.3% of the cycles produced no oocytes during follicle aspiration. 50.7% of the cycles where oocytes were retrieved during the aspiration, only yielded three or less oocytes. Ultimately, the study showed a live birth-rate of 9.5% per cycle and when compared to age matched normal AMH cycles, indicated a twofold lower live birth rate and a fivefold greater cancellation rate (Seifer *et al.,* 2015).

Various methods have been established to predict IVF response and AMH levels and Antra Follicle Count [AFC] are among the most popular tools at the moment, as they could provide a tool for predicting ovarian reserve (Tran *et al.,* 2011). AFC is defined as the number of follicles within the ovaries on cycle days 2, 3, and 5, that measure 2 to 10 mm in greatest diameter, and have been proportionately related to the size of the primordial follicles (Nelson *et al.,* 2013). An advantage of AFC measurement is the immediate availability of the results as it is done with an ultrasound but this could also be a disadvantage as not all clinics have standardized ultrasound equipment and the technique for measuring and determining AFC. This also makes comparison of AFC results difficult as AFC thresholds before starting ART also differ from one clinic/study to another (Broekmans *et al.,* 2010; Jayaprakasan *et al.,* 2012).

1.6.1.3 Tubal Factor Infertility

Tubal factor infertility [TFI] is one of the most common causes of female infertility (Knuttinen *et al.,* 2014), and TFI accounts for 30% of involuntary childlessness in women (Huang *et al.,* 2012). TFI can broadly be defined as obstructed or blocked Fallopian tubes (Grigorescu *et al.,* 2014) contributing to female infertility.

There are various causes for TFI, as the Fallopian tubes can be extremely vulnerable to infection, surgical damage and endometriosis (Kong & Li, 2015). Most commonly, the Fallopian tubes are obstructed due to infections such as pelvic inflammatory disease [PID] (Kawwass *et al.,* 2015). Other infections that could possibly occlude or disable the Fallopian tubes include infections after childbirth or abortions and intra-abdominal infections including appendicitis and peritonitis (Grigorescu *et al.,* 2014). Sexually transmitted diseases [STD's] have also been known to play a significant role in infertility, especially with regard to TFI (Surana *et al.,* 2012). One of the most common STD's is *Chlamydial trachomatis* infection, which is a bacterial infection. There has been shown that *C. trachomatis* may be the direct cause of intraluminal adhesions, hydrosalpinx, pelvic adhesions and fibrosis (Surana *et al.,* 2012). A study conducted in 2012, aimed to demonstrate the correlation between anti-chlamydial antibodies and TFI. The results indicated a 60% seropositivity for the anti-chlamydial IgM antibodies in females that presented with primary infertility and 52% of these females presented with bilateral tubal blockage (Surana *et al.,* 2012).

Routinely, as part of an initial work-up, a hysterosalpingography [HSG] is performed to assess the uterine cavity and Fallopian tube patency, if a female presents with infertility and can indicate the presence of abnormalities such as; hydrosalpinx, adhesions (Asherman's Syndrome, myomas and polyps (Knuttinen *et al.,* 2014). Fallopian tubes that present full of fluid is referred to as a hydrosalpinx (Barroso *et al.,* 2001) and various reports have indicated the negative impact of hydrosalpinges on ART outcome (Strandell & Linhard, 2002). In 1997, an important article was published demonstrating a direct correlation between hydrosalpinx and obstructed embryo implantation (Blazar *et al.,* 1997), creating a platform for numerous research projects to be conducted proving the same statement.

Various studies have aimed at determining pregnancy prognosis of female patients that present with TFI. In a recent study, by Zou *et al.* (2014), 469 TFI patients were scored pre- and post-operatively, classifying TFI (mild, moderate or severe) based on tubal patency, adhesions, morphology and structure to determine the correlation between TFI and pregnancy outcome. The results indicated an intrauterine pregnancy rate of 43.6% for the mild group,

34.0% for the moderate group and 19.4% for the severe group (P < 0.0001), indicating a direct correlation between TFI and pregnancy prognosis (Zou *et al.,* 2014).

Before the introduction of IVF, reconstructive tubal surgery was the only treatment option for women with tubal obstruction (Huang *et al.,* 2012). Currently, IVF is the treatment of choice for women over the age of 35 years with significant tubal disease and those with other co-existing infertility problems (Huang *et al.,* 2012). In 2005, a study was conducted by Omland *et al.* to determine the pregnancy outcome, with regard to three different infertility diagnoses; idiopathic, endometriosis associated and tubal factor after IVF and ICSI. The live birth rate for the idiopathic group (78.8%; n=274) indicated superiority over the endometriosis associated (66.0%; n=212) and tubal factor infertility groups (66.7%; n=540).

If TFI is diagnosed and treated as a first line infertility treatment, the use of ART may not always be necessary, increasing the probability of natural conception and contributing to lowering the cost of infertility treatment (Dun & Nezhat, 2012).

1.6.2 Lifestyle Contributors to Infertility

Recurrent IVF failure has multiple known causes but many which are not routinely considered as part of the post-treatment analysis or initial work-up when first consulting a couple. There are several causes associated with lifestyle and other related pre-existing conditions that have only a tenuous or no apparent connection to fertility (Penzias, 2012).

Lifestyle factors have a dramatic impact on general health and also on the capacity to reproduce. There is an increasing amount of evidence that lifestyle factors can impact on reproductive outcome. Post-Industrial western society has created the potential for increasing the exposure to specific lifestyle factors and behaviours that can alter an individual's reproductive capacity. Lifestyle factors include; an abnormal weight, smoking and exposure to environmental pollutants and chemicals (Homan *et al.,* 2007). Poor lifestyle habits can contribute to infertility in various ways. A recent study by Braga *et al.* (2015) investigated the effect of certain social habits on embryo quality and the likelihood of blastocyst formation. The results indicated that the quality of cleavage stage embryos and the probability of blastocyst formation correlated negatively with smoking and alcohol consumption. Thus, a correlation between social habits and pregnancy outcome becomes more visible.

1.6.2.1 Female Body Mass Index

Being described as a global phenomenon, obesity is associated with a range of health consequences (Rittenberg *et al.,* 2011). Obesity contributes significantly to morbidity and mortality worldwide, increasing the prevalence of cardiovascular disease, decreased mobility and diabetes (Practice Committee of the American Society for Reproductive Medicine, 2015; Goldman *et al.,* 2015). Obesity not only contributes to the above-mentioned health issues but also to hypogonadism and other perturbations of the body's endocrine system that may be associated with infertility (Phillips, 2015). An obese female patient also has an increased risk, during the course of a pregnancy, towards hypertension, gestational diabetes, spontaneous abortion, concomitant delivery complications and foetuses that are larger than the norm for their gestational age (Phillips, 2015).

To be categorized as overweight or obese, a person has to have a BMI of 25 – 29.9 kg/m² for overweight and >30 kg/m² for obesity. BMI is calculated as weight in kilograms divided by the square of height in meters (Orvieto *et al.,* 2009). Compared to women with a normal BMI (18.5 – 24.9 kg/m²), women with a raised BMI are known to have a threefold greater risk of infertility due to disturbances in the hypothalamic-pituitary axis, menstrual cycle alterations and anovulation as well as psychological and social factors (Rittenberg *et al.,* 2011).

Obese female patients generally have poor ART outcomes due to various reasons (Phillips, 2015). Scientists have tried to determine which factors contribute more to this phenomenon. Poor ART outcome among obese females can be due to a poor response to ovarian stimulation (Orvieto *et al.,* 2009), leading to a decreased number of retrieved metaphase II oocytes and ultimately a decreased fertilization rate (Phillips, 2015). A study examining the influence of BMI on IVF outcome found there was a decrease in pregnancy rate among obese (27.9%; n=122) compared to non-obese (15.4%; n=12) females, contributing to their conclusion that the likelihood of poor responders increased among obese women (Orvieto *et al.,* 2009). Decreased ovarian reserve cannot be excluded as a potential contributing factor to infertility in obese patients and need to be considered. Bazzano *et al.* (2015), in an animal study, evaluated the effect of obesity on ovarian function and found that diet induced obesity alters ovarian function by decreasing serum oestradiol concentrations, prolonging dioestrous phases and increasing the number of antral atretic follicles. The results indicated an increase in follicular cysts and overall decrease in ovarian reserve as there was a decrease in the COX-2-positive antral and pre-ovulatory follicles and the number of anti-Mullerian hormone immune-reactive pre-antral follicles. This lead to the conclusion that diet induced obesity induces follicular cyst formation and disrupts ovulation, delaying pregnancy (Bazzano *et al.,* 2015). On

the other hand, a recent study by Seidler *et al.* (2016), showed controversial results, claiming that their mouse model indicated that diet induced obesity does not diminish ovarian reserve, contributing to the fact that more studies are needed on this specific topic.

Alternatively, various studies have indicated the significant impairment of embryo implantation and live birth rates associated with an increasing BMI in female patients (Luke *et al.,* 2011), suggesting that the reason for poor reproductive outcomes in these patients may be due to uterine activity rather than the oocyte (Goldman *et al.,* 2015). In a large study by Provost *et al.* (2016), the objective was to determine the effect of BMI on IVF outcomes. The study included 22317 fresh donor ART cycles (2008 to 2010). Patients with a normal BMI (18.5 to 24.9) were used as the reference group. The results demonstrated that the clinical pregnancy rate decreased as the patient's BMI increased; from 59% in the low and normal BMI group to 53.6% in the 30 to 34.9 BMI group and 48% in the 45 to 49.9 BMI group. The live birth rate also decreased from 51% in the reference group to 40% in the 25 to 34.5 BMI group, demonstrating a correlation between a high BMI and pregnancy outcome (Provost *et al.,* 2016). Rittenberg *et al.* (2011) conducted a systematic review and meta-analysis study to determine the effect of raised BMI on ART outcome and live birth rate [LBR]. The results of the study indicated that women who were overweight or obese (BMI ≥ 30) had significantly lower clinical pregnancy and live birth rates and a significantly higher miscarriage rate compared to women with a BMI of 25 and under. They concluded that raised BMI is associated with adverse pregnancy outcome and lower LBR in women undergoing ART. Controversially, Schliep *et al.* (2015) conducted a prospective cohort study to determine the effect of female BMI on IVF outcome and found that there was no significant correlation between BMI and pregnancy outcome.

Obese female patients are not the only ones at risk for fertility challenges. Underweight female patients may also be less responsive to ART treatment than patients with a normal BMI (Phillips, 2015). These patients have also shown decreased pregnancy rates and fecundity (Gormack *et al.,* 2015). Underweight patients are at an increased risk for anovulation and other ovulatory dysfunctions, and also pregnancy complications such as premature birth (Phillips, 2015) and intrauterine growth retardation (Gormack *et al.,* 2015).

Currently, various research studies have indicated controversial results regarding the ultimate influence of BMI on pregnancy outcomes, and there is room for more studies to clarify this correlation.

1.6.2.2 Smoking

Cigarette smoking is another lifestyle factor that has been associated with reduced pregnancy rates in ART and male infertility (Revonta *et al.,* 2010).

Cigarette smoke consists of a well-established mixture of reproductive toxins for both male and female patients. Pathology of the reproductive tract may result from a generation of reactive oxygen species [ROS] and oxidative stress, vasoconstriction and modifications in endocrine signalling (Phillips, 2015). The follicular fluid of smoking female patients have been analysed in the past and various studies indicated the presence of toxic compounds found in cigarette smoke also to be present in follicular fluid. Some of these compounds include polycyclic hydrocarbons, cadmium, benzo[α]pyrene and nicotine metabolite cotinine. The accumulation of these toxins in the follicular fluid could potentially influence oocyte growth and maturation, induce follicular atresia and interfere with regulation of necessary hormonal processes crucial for the initiation and support of meiotic resumption and ovulation (Phillips, 2015).

Smoking, with regard to female patients, has also been associated with an earlier onset of menopause, infertility, premature ovarian failure and spontaneous abortion. Studies have shown that smoking female patients undergoing ART do not respond to gonadotropin stimulation optimally, produce less oocytes with a decreased developmental potential and present with an overall decreased live birth rate (Phillips, 2015). Camlin *et al.* (2014) stated that the results of human research models in the last decade indicate that cigarette smoke and exposure to smoke constituents have a negative effect on female fertility, with an increase in follicle death and altered hormone output. Results indicated that cessation of smoking can extend the age of natural menopause and potentially improve ovarian quality (Camlin *et al.,* 2014).

A detailed review on the effects of smoking on fertility from gametogenesis to implantation was published in 1982 by Mattison. The study concluded that cigarette smoke had adverse effects on many biological mechanisms required for successful reproduction in both animals and humans. According to Mattison (1982) cigarette smoking is also associated with an increased frequency of menstrual abnormalities and a cigarette dose-related decrease in the age of spontaneous menopause. The study also observed alterations in hypothalamic-pituitary interrelationships, stimulating growth hormone, cortisol, and vasopressin, release of oxytocin and prolactin and inhibition of luteinizing hormone [LH]. Previous findings have indicated that cigarette smoke or nicotine alters the receptivity of the female reproductive tract

and may impair implantation of an embryo. Both maternal and foetal health risks are increased by smoking, including the risk of miscarriage, premature labour, decreased infant birth weight and sudden infant death syndrome (Gormack *et al.,* 2015). Concluding from this large meta-analysis, consisting of 21 studies, the odds of a live birth and clinical pregnancy per cycle are significantly lowered in female patients that smoke. The odds of miscarriage and ectopic pregnancy are also increased (Gormack *et al.,* 2015).

There has been estimated that approximately 37% of men smoke cigarettes during their reproductive age and that toxins from tobacco smoking can potentially affect sperm function and development with a harmful effect on semen parameters (Sharma *et al.,* 2016). A study by Jong *et al.* (2014) aimed at investigating the effect of cigarette smoke on sperm parameters. The results showed that there was no difference in sperm parameters between the fertile and sub-fertile group, indicating that smoking did not affect sperm parameters and ultimately pregnancy outcome.

To the contrary, various studies have demonstrated results indicating that men should be advised to abstain from smoking in order to improve reproductive outcomes (Kovac *et al.,* 2015; Dai *et al.,* 2014). Lotti *et al.* (2015) conducted a retrospective cross-sectional study to determine the impact of smoking on seminal, hormonal and male genital tract ultrasound parameters in patients seeking fertility treatment. The results indicated that male smokers, compared to non-smokers showed a lowered ejaculate and ultrasound derived seminal vesicle volume. Similarly, Sharma *et al.* (2016) conducted a study, including 5865 participants, to determine whether cigarette smoking altered semen parameters. From their results, the author/s concluded that smoking shows a definite overall negative effect on semen parameters, which is directly related to pregnancy outcome.

1.6.2.3 Alcohol Consumption

Severe increased alcohol consumption has generally been associated with poor fertility outcomes. In female patients, alcohol consumption has been associated with possible effects on the hypothalamus, leading to a decrease in Luteinizing Hormone [LH] and anovulation (Phillips, 2015).

In a study by Eggert *et al.* (2004), the effect of long-term alcohol use on female fertility was investigated. The results of the study indicated that out of the 252 study participants, the high alcohol consumers had an increased risk of infertility compared to low consumers. The study concluded that high alcohol consumption was associated with increased infertility

examinations at hospitals and with lower numbers of first and second partus (Eggert *et al.,* 2004). Gormack *et al.* (2015) conducted a study to determine what the effect of lifestyle choices and dietary aspects on women undergoing ART treatment are. The study included 250 women (between 20 - 43 years). The results indicated that women who abstained from drinking alcohol or merely reduced their alcohol consumption were twice as likely to become pregnant than those who maintained their drinking habits prior to fertility treatment.

1.6.3 Male Factor Infertility

Infertility of a couple does not only rely on the female patient profile, but to an equal extent on the male patient profile (Brugh *et al.,* 2003). In approximately 20 to 25% of infertility cases, the male partner is solely responsible for the infertility problem (Morshedi, 2014).

A semen analysis is generally seen as the cornerstone for evaluating men for infertility or subfertility (Kumar & Singh, 2016). The role of the routine semen analysis alone has however been challenged as ineffective and unreliable in predicting a male's fertility status (Barroso *et al.,* 1999; Jungwirth *et al*., 2012). The World Health Organization's *laboratory manual for the examination and processing of human semen* (2010), regard normal semen parameters with Lower References and 95%CI as; semen volume of 1.5 ml (1.4 and 1.7), sperm concentration of 15 million per ml (12 and 16) sperm motility at 32% progressive (31 to 34); normal sperm morphology of 4% (3 to 5) and vitality of 58% (55 to 63) (WHO, 2010).

Abnormal semen parameters may be a contributing factor in up to 30 - 45% of infertile couples (Jungwirth *et al*., 2012). In cases of oligozoospermia (<15 million motile spermatozoa per mL), severe asthenozoospermia (<5% progressive motility) and severe teratozoospermia (<4% normal morphology based on the Tygerberg strict criteria), IVF, or a combination of IVF and ICSI, should be offered. These semen parameters are associated with poor success in artificial insemination (Huang *et al.,* 2012). A study conducted in 1998 by Oehninger *et al.* aimed to investigate the role spermatozoa has on embryo implantation and pregnancy outcome in IVF and ICSI. The results indicated that there is not a significant effect of severe male fertility (oligo- astheno- teratozoospermia) on implantation and pregnancy outcome (Oehninger *et al.,* 1998).

With regard to the prediction of pregnancy and the role the male partner plays, several possible factors can contribute to the outcome (Brugh *et al.,* 2003). A normal semen analysis does not necessarily eliminate a contribution to the fertility problem (Kumar & Singh, 2016) since functional tests and genetic integrity of spermatozoa is not evaluated (Zhang *et al.,*

2015). Although sperm DNA integrity tests generally correlate with semen analysis outcomes and in certain cases, with ultimate pregnancy outcome (Collins *et al.,* 2008) oocyte fertilization and embryo development, possibly rely on the inherent integrity of the spermatozoal DNA (Practice Committee of the American Society for Reproductive Medicine, 2008). There are various causes and contributing factors (intrinsic and external) that play a role in sperm DNA damage, and a large portion of these factors are unknown (Collins *et al.,* 2008). Intrinsic factors that may predispose to sperm DNA damage include; mutations in relation with DNA compaction, oxidative stress caused by leucocytosis or a varicocele, a protamine deficiency and genetic disorders (Practice Committee of the American Society for Reproductive Medicine, 2008).

Multiple assays have been developed to assess DNA damage (Collins *et al.,* 2008). The sperm DNA fragmentation test (DNA Fragmentation Index [DFI]), which evaluates sperm chromatin integrity, has been globally adapted, due to its diagnostic capabilities to evaluate male fertility potential, and ultimately pregnancy outcome (Zhang *et al.,* 2015). The study by Zhang et al. (2015) aimed to estimate and understand the association between the DNA Fragmentation Index [DFI] and Pregnancy outcome after IVF and ICSI. Their results indicated that infertile couples were more likely to become pregnant if the DFI was below the threshold value of 27% DNA fragmentation (Zhang *et al.,* 2015). One could argue that routine semen parameters are not sufficient to predict pregnancy outcomes and could potentially be used in conjunction with sperm DNA tests (Collins *et al.*, 2008).

When deciding how the male factor should be evaluated for its possible contribution to infertility, a semen analysis should be the first line of action, but the test in itself, is not sufficient to determine cause or dictate the means of possible therapy (Brugh *et al.,* 2003). To exclude other likely causes of male factor infertility, the best approach would be a systematic approach, which includes sperm DNA analysis and other necessary characteristics that play a role in successful fertilization and will ultimately lead to a full-term pregnancy (Morshedi, 2014).

With regard to lifestyle factors smoking has shown to influence semen parameters (Kovac *et al.,* 2015; Dai *et al.,* 2014) but other lifestyle factors such as male BMI also needs to be considered. The results from various studies have shown that an increased likelihood of abnormal semen parameters and increased risk for subfertility exist among couples in which the male partner is obese (Du Plessis *et al.,* 2010). Male partner obesity has been associated with a higher prevalence of infertility (Campbell *et al.,* 2015; Nguyen *et al.,* 2007). The increasing prevalence of obesity indicates that there is a definite need for clinical awareness with regard to male partner obesity and its effects on fertility (Du Plessis *et al.,* 2010).

1.7 Infertility in General

As mentioned above, the results obtained by ART depend on diverse factors, not only epidemiological (female age, cause of infertility, etc.) or the clinical practice accepted (pharmacological treatments, the policy regarding embryo transfer, etc.) but also on the social context in which such techniques are practiced. The significance of the role played by each factor however, remains unclear. Social context involves a set of social and cultural factors that influence clinical practice and the results achieved by different healthcare services. One of the most important factors in the field of infertility treatment is the regulation of ART, affecting issues such as restrictions on the number of embryos that may be transferred or the handling of frozen embryos. Other relevant factors in the social context include the existence of competition among clinics and the health care coverage available for infertility treatment, which determines the accessibility to such treatment (Castilla, 2009).

In most countries, prioritizing infertility treatments compared to other treatments in healthcare is a controversial topic. Although the public sector is available for infertility treatment, the private sector predominates in the area of assisted reproduction. Lately, there has been an increase in the public sector facilitating ART, both in terms of the volume and the range of services offered (Castilla, 2009). Differences in public and private ART clinics do exist in terms of the volume of activities, the range of services offered, clinical practice and results achieved. Quantifying the intensity of these differences and determining the factors involved could help and lead to positive changes, in both sectors, in the procedures followed as well as resources available for the management of infertility (Castilla, 2009).

Through analysis of previous applicable literature, findings and study results, it is clear that the ART outcomes in an IVF clinic is dependent on a wide variety of contributing factors. To be considered when interpreting results are: access to the best and latest equipment (i.e. incubators), patient profile (including fertility diagnosis and lifestyle factors) ovarian stimulation practises and clinical and laboratory expertise and procedures. For most of these contributing factors access to sufficient funds for both the laboratory/clinics and the patients, plays a significant role. In the current study, the researchers aimed to investigate some of the possible contributing factors in two different Fertility Clinics. We hypothesise that clinic specific factors may have an effect on outcome and included incubator type, oocyte (female) age, number of oocytes aspirated, embryo quality, female diagnosis (tubal factor and endometriosis), BMI and AMH in the analyses to determine its possible independent or combined effect on clinical pregnancy outcome.

CHAPTER 2 – RESEARCH QUESTIONS

2.1 Research Questions

How does laboratory equipment, specifically two different CO_2 incubator types (COOK MINC™ Benchtop Incubator and Forma Scientific CO_2 Incubator Model 3164 - Water Jacketed) used, contribute to the embryo quality, development and ultimately the CPR?

Which factors (specifically female) have a significant impact on CPR at a public and private fertility clinic?

2.2 Aims & Objectives

The aim of the study was to investigate which factors in ART treatment might have the most significant effect on ART outcome in two ART laboratories – one in the public sector and one in the private sector.

The study consisted of two different analyses, a retrospective and a prospective analysis.

- To **retrospectively** analyse:
    - The effect of two different CO_2 incubators, (COOK MINC™ Benchtop Incubator and Forma Scientific CO_2 Incubator *Model 3164* - Water Jacketed) used at the Drs Aevitas private fertility clinic, on ART outcome (2013-2014).
- To **prospectively** analyse:
    - The possible negative or positive impact of female patient profile (specifically number of oocytes, age, BMI, AMH and female diagnosis - tubal factor and endometriosis) in two different ART clinics public (Tygerberg Hospital Fertility Clinic) and private (Drs Aevitas private Fertility clinic) on ART outcome.

2.3 Hypotheses

Retrospective study: Smaller, triple gas, low O_2 concentration, bench top incubators (COOK MINC™) will result in improved ART outcome compared to large box, high O_2 concentration incubators (Forma Scientific CO_2 Incubator, Water Jacketed - Model 3164).

Prospective study: The female patient profiles will be significantly different for the two clinics. We hypothesize that BMI, tubal factor infertility and female age will be the main factors determining success in the public clinic and the female age will be the main contributor determining success in the private clinic – affecting overall success in the clinics.

Important to Note:

Drs Aevitas Fertility Clinic (Private Clinic) referred to as "*Site 1"* in the Results and Discussion sections.

Tygerberg Fertility Clinic (Public Clinic) will be referred to as "*Site 2"* in the Results and Discussion sections.

CHAPTER 3 – MATERIALS, METHODS & STATISTICS

3.1 Study Population for the *Retrospective Analysis*

The retrospective analysis investigated the effect of two different CO_2 incubators on ART outcome. The study population for this part of the study was patients that had IVF, ICSI, PICSI, IMSI or combinations thereof cycles, during 2013 and 2014, at the private clinic. A total of 1433 ART cycles were performed during the 2013 and 2014 period. Only 385 cycles were included in the final analyses after applying strict exclusion criteria to ensure that results were only due to incubator differences. The Health Research Ethics Committee [HREC] of the University of Stellenbosch approved this study protocol (Ethics Reference number: S15/03/050), for the period 18 June 2015 to 18 June 2016, with a waiver of consent, thus, no consent was needed from the patients for this part of the study.

3.2 Patients included in *Retrospective Analysis*

N = 385 ART cycles

- ART patients, IVF, ICSI, PICSI, IMSI or combinations thereof cycles, at the private clinic (2013 – 2014) - including oocyte donor cycles

Exclusion criteria:

- < 3 MII oocytes aspirated
- < 3 embryos on Day 2
- Females ≥ 37 years
- HIV positive female and male patients
- Oocyte vitrification cycles
- Frozen embryo transfer [FET] cycles
- Cycles with crucial missing data
- Cancelled Cycles

3.3 Data and Information collected *(For Retrospective Analysis Only)*

- Female age
- Specific incubator used (patient specific)
- Number of mature oocytes
- Number of fertilized oocytes
- Day 2, Day 3 and Day 5 embryo quality
- Number of embryos transferred
- Day of transfer

- Quality of the embryo/s transferred
- Pregnancy rate (Clinical – foetal sac/ET)

3.4 Data Collection and Ethical Considerations for the *Retrospective Analysis*

In this study retrospective data from an existing patient data base was used. Patients were not subjected to any additional risks, injury or pain. During collection of data on the password protected spread sheet, no patient identity was entered. Each patient's folder was allocated a number and remain 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. Waiver of consent was granted by the HREC.

3.5 Study Population for the *Prospective Analysis*

The second analysis was prospective and investigated the possible impact (negative or positive) of female patient profile on overall ART outcome. The study population for this part of the study was patients from both the private and public clinics that had an ART cycle, from 23rd of June 2015 until 29th of April 2016. The exclusion criteria for this part of the study were not as strict as for the retrospective study. For the private clinic, 630 patient cycles were captured but only 572 were included in the study. For the public clinic, 275 patient cycles were captured but only 248 were included in the study. Exclusion from the study was mostly due to consent form absence. The HREC of the University of Stellenbosch approved this study protocol (Ethics Reference number: S15/03/050A), for the period 17 June 2015 to 17 June 2016. Each patient that underwent ART treatment at both the private and public clinic during this period was presented with a consent form (see Appendix XV), approved by the HREC of the University of Stellenbosch. The patient could then decide whether they wanted to participate or not. Patients who did not give consent or did not receive the consent form were excluded from the study.

3.6 Patients included in *Prospective Analysis*

N = 572 cycles for the private clinic and N = 248 for the public clinic

- 2015 - 2016 fertility patients receiving treatment at the private and public clinics - including oocyte donor cycles
- IVF, ICSI, PICSI, IMSI or combinations thereof cycles including FET cycles
- All female ages
- All female and male diagnosis

Exclusion criteria:

- Oocyte vitrification cycles
- Cycles with crucial missing data
- Cancelled Cycles
- Cycles without granted consent
- Cycles where a consent form was not presented to the patient

Data and Information collected (Independently for both Fertility Clinics)

- Female and male diagnosis (Male Spermiogram)
- Female patient & oocyte age
- BMI
- AMH
- Number of oocytes (MI and MII)
- Number of fertilized oocytes
- Day 2, Day 3 and Day 5 embryo quality
- Number of embryos transferred
- Day of transfer
- Quality of the embryos transferred
- Pregnancy outcome (Clinical - foetal sac/ET)

3.7 Data Collection and Ethical Considerations for the *Prospective Analysis*

Patients signed informed consent giving permission that their information may be used in the study (see Appendix XV). Patients were not subjected to any additional risks, injury or pain. During collection of 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. Participants did not receive remuneration and there were no extra costs involved.

3.8 Procedures and Methods

Standard IVF, ICSI, PICSI and IMSI protocols for ovarian stimulation, oocyte aspiration, insemination, fertilization, embryo culture, embryo evaluation and grading as well as embryo transfer were followed for both the retrospective and prospective studies. Standard semen preparation was also followed for both studies.

See Appendices I – XIV.

3.9 Methods

3.9.1 Semen Preparation

The preparation of semen samples were conducted according to standard preparation methods, which included wash and swim-up and density gradient centrifugation methods (Appendix I).

3.9.2 ART Procedures

Ovulation Induction and Oocyte Aspiration

Standard ovarian stimulation protocols for each fertility clinic were used.

Private Clinic

Couples were treated according to a standardized stimulation protocol agreed upon by all participating physicians, see Appendix II.

Public Clinic

Standard, modified stimulation protocols were used – 100mg Clomiphene Citrate Clomid®, Letrozole® or Pergonal® for 5 days only on days 3 to day 7 in the cycle and ± 75IU Menopur® on day 8 and 10 **OR** 100 mg Clomiphene Citrate for 8 to 10 days only on days 3 to 12 in the cycle and ± 75IU Menopur® on day 8 and 10. (4, 6, 8). Patients were followed up by doing estradiol determinations as well as serial ultrasonographic measurements of the Graafian follicles. Ovulation was induced by the administration of human chorionic gonadotrophin [HCG] (Ovitrelle®) as soon as the leading follicle reached a diameter of 18mm.

3.9.2.1 Oocyte Retrieval

A standard method (private and public clinic) was followed (Appendix III – public clinic, Appendix IV – private clinic).

3.9.2.2 Insemination Procedure

a) IVF

Metaphase II oocytes were inseminated with 100 000 to 500 000 motile spermatozoa each in NUNC 4-well dishes and then incubated in stage specific mediums, in drops under oil (see Appendix V).

b) ICSI

Prerequisites for successful injection are immobilization of the sperm cells in PVP and mild cytoplasmic aspiration. Each cumulus-free metaphase II oocyte was injected with a single initially motile but immobilized spermatozoon. Oocytes were washed in fresh fertilization medium drops after injection and then incubated individually in 50µl drops of cleavage medium (under oil, 37^0C, 5 % CO_2, Quinn's – Coopersurgical™). Embryos destined for culture to blastocyst stage were transferred into blastocyst medium on day 3 of culture (under oil, 37^0C, 5 % CO_2, (Quinn's – Coopersurgical™), see Appendix VI. Embryos were transferred to fresh medium on day 1, 3 and 5.

c) PICSI

A standard, routine method was followed using commercial PICSI® dishes (Origio, Harrilabs, South Africa), see Appendix VII.

d) IMSI

A standard, routine method was followed of sperm selection under high magnification used for ICSI, see Appendix VIII.

3.9.2.3 Embryo Culture and Evaluation

For all culture procedures the Quinn's Advantage™ (Harrilabs, South Africa) culture medium range was used - including HEPES buffered medium, sperm preparation medium, fertilization/cleavage and blastocyst medium, oil for tissue culture, hyaluronidase, PVP.

Only two CO_2 incubator models were used during the **retrospective study** period (2013-2014) at the private clinic; COOK MINC™ Benchtop Incubator and Forma Scientific CO_2 Incubator Model 3164 - Water Jacketed. During the study period the private clinic laboratory used four MINC™ Benchtop Incubators (purchased in 2010) and four Forma Scientific CO_2 Incubators (purchased before and used since the 90's). Patients were assigned to incubator type according to the availability of space.

During the **prospective study** period (2015 – 2016), the private clinic only used bench top incubators for embryo culturing - four MINC™ incubators and four Planer (BT37) bench top incubators, introduced in January 2015. These are currently still in use in the laboratory. The public clinic laboratory used a Forma Series II Water Jacketed Incubator (model 3111) (purchased in 2012) during the study period, but in July 2016 purchased a Miri® benchtop incubator that is used for embryo culture after fertilization.

A standardised, routine method (for both fertility clinics) was used for all patients.
After incubation for 16-18 hours, oocytes were inspected for fertilization (presence of 2 pronuclei and 2 polar bodies). During further embryo development (Day 2 and Day 3 post-insemination) the embryos were evaluated for embryo quality (blastomere morphology and percentage fragmentation). Culture to the blastocyst stage and subsequent hatching were evaluated from day 4 to 7 post insemination, see Appendix IX & XI.

3.9.2.4 Embryo Quality Evaluation

Embryos were regarded as good quality when they reached the 2 - 4-cell stage at 48h post-injection/fertilization or at the 6 – 8 cell stage, 72h post-injection/fertilization. They should have equal sized blastomeres and minor or no cytoplasmic fragmentation, see Appendix IX.

3.9.2.5 Blastocyst Evaluation

Blastocysts (day 5/6 culture) were graded according to the Gardner and Schoolcraft Blastocyst Grading (Veeck, 2003) method. With this system, blastocysts are given a numerical score from 1-6 on the basis of their degree of expansion and hatching. Additionally the development of inner cell mass and trophectoderm appearance is also evaluated (see Appendix XI).

3.9.2.6 Embryo Transfer

A standard embryo transfer method [private and public clinic] was followed. The transfers were sonar guided and patients presented with a full bladder to facilitate an atraumatic procedure. The number of embryos transferred varied between the two clinics and also from cycle to cycle. In general, no more than three embryos were transferred and each cycle was evaluated individually to determine the number of embryos to be transferred considering various factors (see Appendix XIV).

3.9.2.7 Cryopreservation and Thawing

All supernumerary embryos of accepted quality were cryopreserved using an open system vitrification protocol (Fertipro VitriFreeze™ - VitriThaw™ kits). The protocol suggested by the supplier was used (Appendix XII & XIII).

3.9.2.8 Laboratory Quality Control

Both the private and public clinic laboratories make use of standard quality control measures. The temperature of the incubators and fridges used in both laboratories are checked and documented daily with the use of external digital (Temp-Chex Digital II – *Streck Inc.*) thermometers. The CO_2, N_2 and tri –gas mix levels of gas cylinders are also checked and

documented daily. The pH of culture medium (Sage cleavage medium) is tested weekly using gas tight syringes and a blood gas machine (Gem Premier 3500, Illex South Africa). Maintenance for culture incubators and all other laboratory equipment occur annually at both laboratories. The private clinic laboratory is equipped with a HEPA filter air system and positive pressure. All incubators are also equipped with inline active carbon filters (Gen X – supplied by *Delfran Pharmaceuticals*). The public clinic unfortunately does not have any of these measures (positive pressure, HEPA filters and inline filters).

3.9.2.9 Pregnancy

Pregnancy evaluations are defined as:

- A *biochemical pregnancy* - a positive βHCG blood serum value on day 10 or 14 after embryo transfer – but no foetal sac or heartbeat.
- A *clinical pregnancy* - any product of conception 7 weeks (including a gestational sac, a positive heartbeat and ectopic pregnancy) post embryo transfer. *Note:* Multiple gestational sacs are counted as one clinical pregnancy per cycle.
- An *ongoing pregnancy* - positive foetal heartbeat after 12 weeks.
- An *ectopic pregnancy* - a pregnancy where implantation occurs outside the uterine cavity.
- A *perinatal death* - death of the foetus after 20 weeks.
- A *miscarriage* - the premature loss of a foetus up to 23 weeks. A miscarriage will have three different sub-categories:
    - 1 - If the miscarriage occurred in the first seven weeks
    - 2 - If the miscarriage occurred between 7 – 14 weeks
    - 3 - If the miscarriage occurred after 14 weeks
- a *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 heart beat, 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).

For both the retrospective and prospective data analysis for this study, clinical pregnancy was defined as a gestational foetal sac/s at 7 weeks.

3.10 Statistics

Statistical analysis of results were conducted at the Biostatistics Unit [BU] of The South African Medical Research Council [SAMRC] by Prof Carl Lombard. Prof Lombard was consulted to discuss power analysis to ascertain the number of patients to include. All statistical analysis, for both retrospective and prospective studies, was performed by prof. C. Lombard.

3.10.1 Statistical Analysis - Retrospective Study

All of the outcomes are categorical in nature and therefore categorical regression analysis was best suitable. Two types of categorical models were thus needed.

For the outcomes: fertilization, embryo quality rate (Day 2 and 3); there are multiple outcomes within the same cycle of the participating patients. This correlation or dependence had to be accounted for. A mixed effects logistic regression analysis was used to account for the clustering.

For the outcomes: clinical pregnancy, live birth and miscarriage, standard logistic regression analysis was conducted since the outcome observed is at the cycle level. If there are multiple cycles nested within a patient this hierarchical dependence structure was taken into account, considering each cycle independently.

In each of the regression models the incubator type was included in the model via two indicator variables. Since the variable consists of two types, an overall test, for the significance of the variables, was conducted and reported. The odds ratios of the specific types in relation to the reference type were also reported together with 95% confidence intervals.

Since the study was retrospective and observational, the models had to take into account possible confounders and effect modifications especially with regard to incubator type. Possible confounders considered were: maternal age at the time point of the cycle, ART procedures used, and male reproductive factors.

A sample size of 385 cycles was adequate for the type of analysis used. Basic descriptive statistical analysis of the outcome was also divided per incubator type.

3.10.2 Statistical Analysis - Prospective Study

For the CPR, which is at patient level, analysis was a binomial regression model of the binary outcome. The association between clinical pregnancy rate and a set of clinical factors (age of oocytes, number of MII oocytes, TFI, endometriosis, BMI and AMH) was investigated.

A pooled analysis was performed on the data of the two study Sites. This was necessary to improve the precision of the analysis. To facilitate any Site differences all the factors in the model was evaluated for a significant interaction with Site in the regression model. No significant interactions were found and Site was included in the main effects model as an adjustment factor. The linearity assumption of the continuous factors was investigated by graphical plots and by fitting non-linear terms in the model. The linear assumptions were confirmed (models not reported). Lowess smooth graphs, which creates a smooth line through a scatter plot to indicate the relationship between the variables investigated, were frequently used to illustrate and decide which factors should be investigated through regression analyses.

CHAPTER 4 – RESULTS

4.1 Results - Retrospective Study

Objective: To determine the effect of two different incubators, Forma Scientific CO_2 Incubator and the MINC™ Benchtop Incubator, on embryo quality and pregnancy outcome during the period January 2013 – December 2014.

The incubators used were the MINC™ Benchtop Incubator and the Forma Scientific CO_2 Incubator respectively. The private laboratory made use of four MINC™ Benchtop Incubators and four Forma Scientific CO_2 Incubators. Important to note is that the incubator used was unknown in only three of the total of 388 cycles, and those were excluded from the statistical analysis.

The specific grading criteria for good quality embryos used for the purposes of this study can be seen in Appendix X.

A limitation of the retrospective results was that – during this specific study period - the Forma Scientific CO_2 Incubator was used in more cycles than the MINC™ Benchtop Incubator, 327 cycles versus 58 cycles, respectively. This is due to the design of the study, which was retrospective and observational. Although the cycle numbers significantly differed in the two incubators investigated, the statistical significance of the results was not influenced.

4.1.1 Descriptive Data

Table 2 presents the descriptive statistics for the variables; procedure, number of ova, age of ova (female age), male and female diagnosis, sperm morphology, proportion of fertilized MII ova, fertilization rate, quality of embryos and pregnancy outcome.

Only the % of GQE/number of ova on day 3 was significantly increased in the MINC incubator compared to the Forma incubator, p = 0.03.

Table 2: Descriptive data of patient cycles for the two respective incubator types investigated.

Variable	Forma Scientific CO_2 Incubator	MINC™ Benchtop Incubator	P Value
Number of Cycles	327	58	-
Age of Ova (years - female age)	30.25 (±4.88)	30.70 (±4.64)	0.29
Total Ova per Cycle	13.28 (±7.58)	12.59 (±7.77)	0.52
Sperm Morphology (% normal)	8.41 (±4.60)	9.146 (±4.74)	0.35
Fertilization rate of MII Oocytes (%)	79.40	79.06	0.88
Good Quality Embryos (GQE/Number of Ova) Day 2 (%)	55.89	57.44	0.76
Good Quality Embryos (GQE/Number of Ova) Day 3 (%)	39.31	44.58	0.03
Good Quality Embryos (GQE/Number of Ova) Day 5 (%)	23.60	21.76	0.90
Clinical Pregnancy/transfer (%)	45.43	47.17	0.81

Values are presented as number, percentage or mean (± Standard Deviation)

4.1.1.1 Insemination Procedure

According to Fisher's Exact test, which had a value of p = 0.43, there was also no difference in the distribution of the different ART procedures (ICSI, IVF and combinations thereof) between the two incubator types. Although the number of cycles per incubator differs largely, the ratio was approximately equal between the two incubators. The results are presented in Table 3.

Table 3: Distribution of all ART procedures in incubators under investigation.

Procedure	Forma Scientific CO_2 Incubator	MINC™ Benchtop Incubator	Total
IVF	10.40 (34)	10.34 (6)	10.39 (40)
ICSI	64.22 (210)	63.79 (37)	64.16 (247)
PICSI	7.34 (24)	8.62 (5)	7.53 (29)
IVF / ICSI	13.76 (45)	12.07 (7)	13.51 (52)
IVF / PICSI	3.36 (11)	5.17 (3)	3.64 (14)
FROZEN OVA ICSI	0.61 (2)	0 (0)	0.52 (2)
ICSI / PGD	0.31 (1)	0 (0)	0.26 (1)
Total	**100 (327)**	**100 (58)**	**100 (385)**

Values are presented as percentage (n – number of patients)

4.1.1.2 Oocyte Age

Figure 1 presents the distribution of the age of oocytes in the Forma Scientific CO_2 Incubator and the MINC™ Benchtop Incubator, respectively. The average age of oocytes in the Forma Scientific CO_2 Incubator was 30.24 years (SD = 4.88) and 30.96 years (SD = 4.64) in the MINC™ Benchtop Incubator. A t-test indicated no significant difference among the distribution of the age of oocytes between the two incubators with a p-value of 0.29.

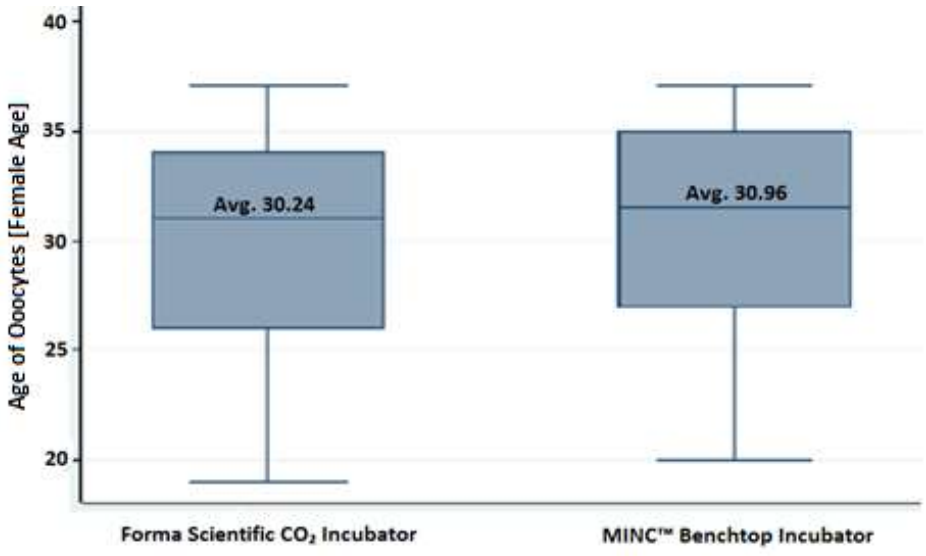


Figure 1: Boxplot showing the distribution of the age of oocytes (female age) in each incubator type the Forma Scientific and the MINC™ Benchtop.

4.1.1.3 Oocyte Distribution per Cycle

The boxplot in Figure 2 demonstrates the distribution of the number of oocytes per cycle between the two incubator types under investigation. The average number of oocytes cultured in the Forma Scientific CO_2 Incubator was 13.28 (SD = 7.58) compared to 12.59 (SD = 7.77) in the MINC™ Benchtop Incubator. The p-value of 0.52 indicated no significant difference in the distribution of oocytes between the incubators.

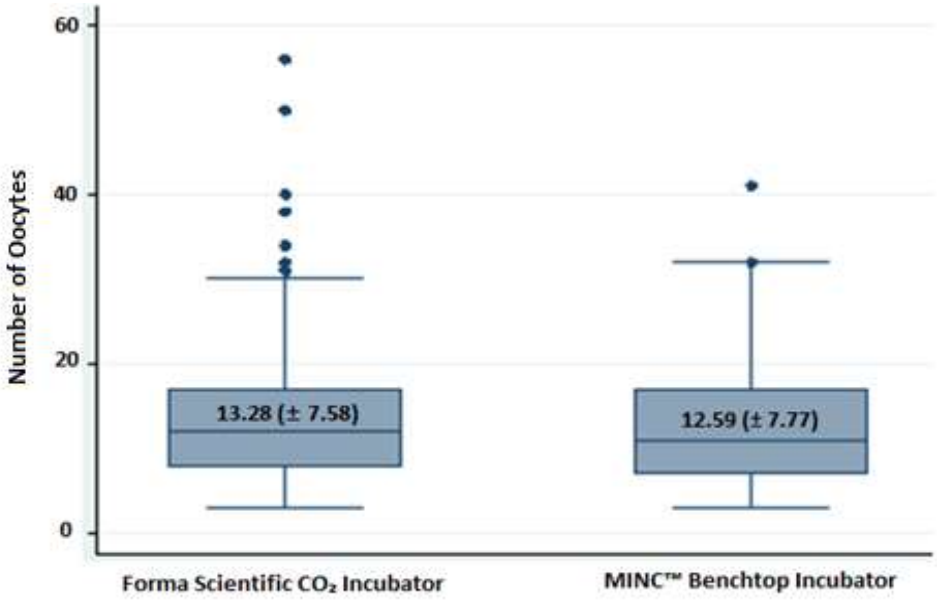


Figure 2: Boxplot showing the distribution of the number of oocytes per cycle cultured in each incubator type: the Forma Scientific and the MINC™ Benchtop.

4.1.1.4 Male and Female Diagnosis

A Fisher's Exact Test was also performed to determine whether there was a difference in the male and female diagnosis profile between the two incubators investigated.

Table 4 presents the different **male diagnoses** and their distribution in the Forma Scientific CO_2 Incubator and the MINC™ Benchtop Incubator, respectively. According to the Fisher's Exact Test, which demonstrated a p value of 0.51, the male diagnosis was distributed evenly between the two incubators.

Table 4: Distribution of male diagnosis among the two incubator types investigated.

Male Diagnosis	Forma Scientific CO_2 Incubator	MINC™ Benchtop Incubator	Total
Normozoospermia	42.57 (126)	37.04 (20)	41.71 (146)
Oligozoospermia	6.08 (18)	3.70 (2)	5.71 (20)
Oligo- & Teratozoospermia	2.70 (8)	1.85 (1)	2.57 (9)
Oligozoospermia (G-Pattern)	1.35 (4)	0 (0)	1.01 (4)
Oligo- Terato- & Asthenozoospermia	4.73 (14)	5.56 (3)	4.86 (17)
Oligo- & Asthenozoospermia (G-Pattern)	0.68 (2)	3.70 (2)	1.14 (4)
Teratozoospermia	8.45 (25)	9.26 (5)	8.57 (30)
G-Pattern	15.54 (46)	24.07 (13)	16.86 (59)
Immunological Factor	1.01 (3)	0 (0)	0.86 (3)
Donor Sperm Used	2.70 (8)	0 (0)	2.29 (8)
Other	6.76 (20)	5.56 (3)	6.57 (23)
Astheno- & Teratozoospermia	0.34 (1)	0 (0)	0.29 (1)
Testis Biopsy	6.76 (20)	7.41 (4)	6.86 (24)
Donor & Own Sperm Used	0.34 (1)	0 (0)	0.29 (1)
HIV Positive	0 (0)	1.85 (1)	0.29 (1)
Total	**100 (296)**	**100 (54)**	**100 (350)**

Values are presented as percentage (n – number of patients)

Table 5 presents the different **female diagnoses** and their distribution in the Forma Scientific CO_2 Incubator and the MINC™ Benchtop Incubator, respectively. A Fisher's Exact Test demonstrated a p value of 0.28, illustrating that the female diagnosis was distributed evenly between the two incubators.

Table 5: Distribution of female diagnosis among the two incubator types investigated.

Female Diagnosis	Forma Scientific CO_2 Incubator	MINC™ Benchtop Incubator	Total
Idiopathic	61.51 (187)	57.14 (32)	60.83 (219)
Endometriosis	5.92 (18)	3.57 (2)	5.56 (20)
Miomas	0.99 (3)	1.79 (1)	1.11 (4)
Tubal Factor	6.91 (21)	1.79 (1)	6.11 (22)
Anovulation	7.24 (22)	16.07 (9)	8.61 (31)
Other	13.49 (41)	19.64 (11)	14.44 (52)
Premature Ovarian Failure	2.63 (8)	0 (0)	2.22 (8)
Cervical Factor	0.33 (1)	0 (0)	0.28 (1)
HIV Positive	0.66 (2)	0 (0)	0.56 (2)
Normal - Male Factor	0.33 (1)	0 (0)	0.28 (1)
Total	**100 (304)**	**100 (56)**	**100 (360)**

Values are presented as percentage (n – number of patients)

Statistical analysis concluded no significant difference in male diagnosis (Fisher's exact p = 0.51) or in female diagnosis (Fisher's exact p = 0.28) between the two incubator types. These factors can therefore be excluded in the ART outcome, emphasizing the effect of the incubators only.

4.1.1.5 Sperm Morphology

Patient sperm morphology distribution between the two incubators was shown to be similar and did not show a significant difference (p = 0.35) as illustrated in Figure 3. The average normal sperm morphology of patients in the Forma Scientific CO_2 Incubator was 8.41 % (SD ± 0.30) compared to 9.15% (SD ± 0.74) in the MINC™ Benchtop Incubator.

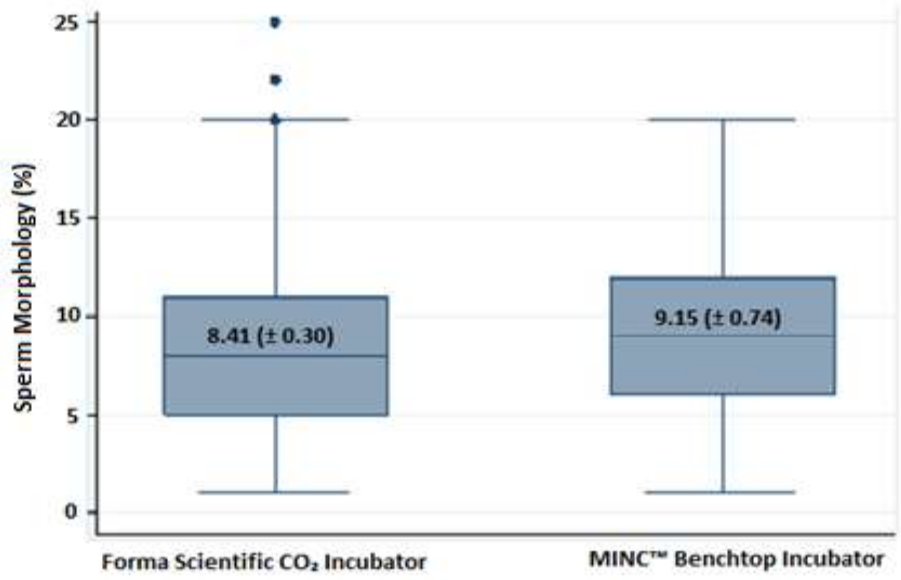


Figure 3: Boxplot showing the distribution of patient normal sperm morphology between incubators investigated.

4.1.1.6 Fertilization

Figure 4 represents the fertilization rate of MI and MII oocytes in the two respective incubator types. The percentage of MI oocytes fertilized in the Forma Scientific CO_2 Incubator was 1.13% (SD = 7.09) and 1.25% (SD = 7.90) in the MINC™ Benchtop Incubator (p = 0.93). As demonstrated in Figure 4, very few patients had fertilization of MI oocytes. *At Aevitas Fertility Clinic there is a policy to inseminate MI oocytes since overnight "maturation" gave similar results – unpublished clinic results.*

MII oocytes had a much higher fertilization rate [as expected] compared to MI oocytes in both the Forma Scientific CO_2 Incubator (79.41% ± 17.94) and the MINC™ Benchtop Incubator (79.06% ± 16.77). No significant difference in the fertilization rate of MII oocytes between the two incubators was observed (p = 0.88).

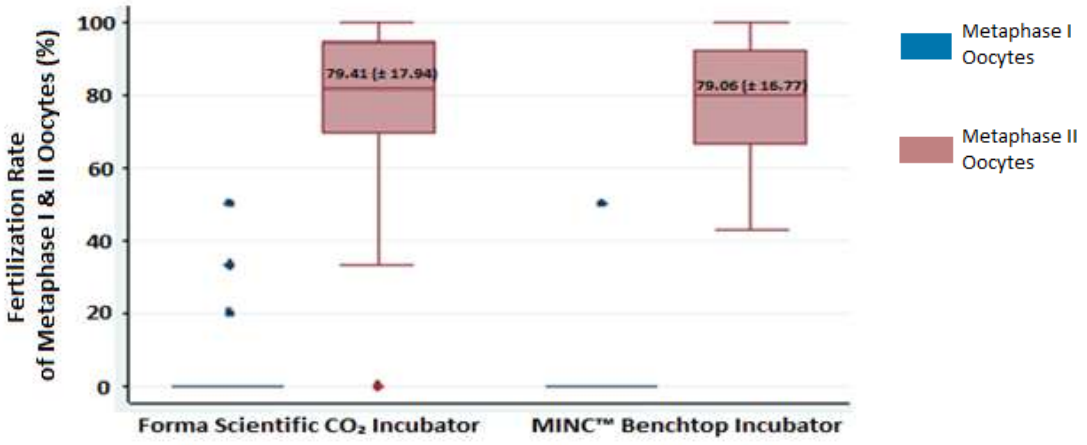


Figure 4: **Boxplot showing the fertilization rates of MI and MII oocytes in the two incubators under investigation.**

4.1.1.7 Embryo Quality - Day 2, 3 and 5

The average number of good quality embryos on **day 2**, as presented in Figure 5, was 6.94 (SD = 3.88) in the Forma Scientific CO_2 Incubator compared to 6.67 (SD ± 3.90) in the MINC™ Benchtop Incubator. No significant difference was shown in the average number of good quality embryos on day 2 (p = 0.62).

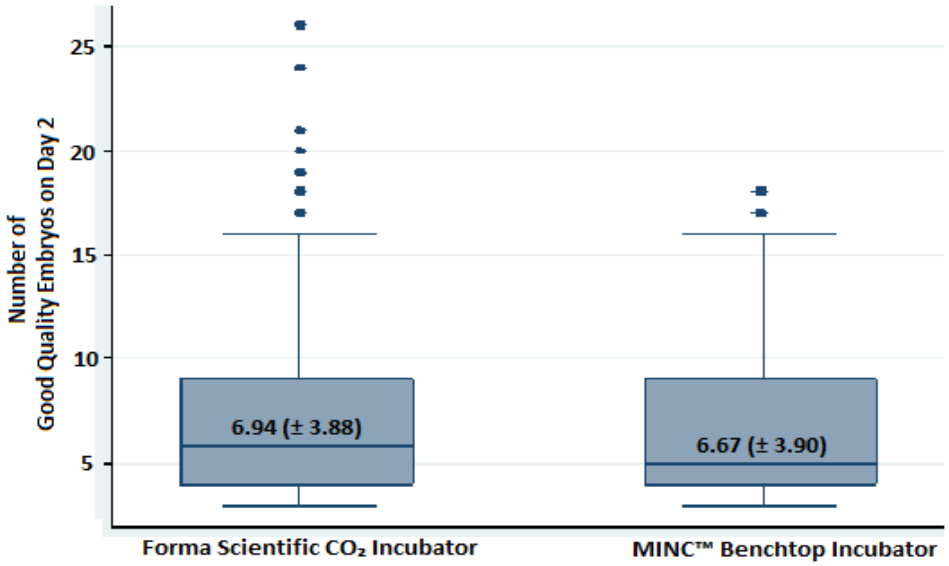


Figure 5: **Boxplot showing the average number of good quality embryos on Day 2 in the two incubators investigated.**

Figure 6 illustrates the average number of good quality embryos on **day 3.** The Forma Scientific CO_2 Incubator had an average of 4.79 (SD ± 3.21) good quality embryos on day 3 compared to 5.29 (SD = 3.51) in the MINC™ Benchtop Incubator. Although there were more good quality day 3 embryos in the MINC incubator compared to the Forma incubator, no significant difference was shown in the average number of good quality embryos, (p = 0.29).

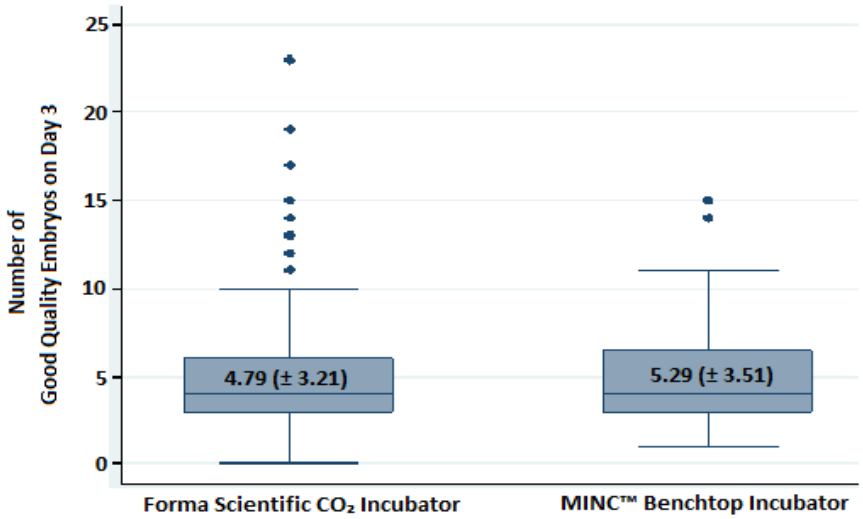


Figure 6: **Boxplot showing the average number of good quality embryos on Day 3 in the two incubators investigated.**

The average number of good quality embryos on **day 5** (Figure 7) also showed no difference between the two incubators (p = 0.85). The Forma Scientific CO_2 Incubator had an average of 3.14 (SD ± 2.47) compared to the MINC™ Benchtop Incubator which had an average of 3.23 (SD = 3.16) good quality embryos on day 5.

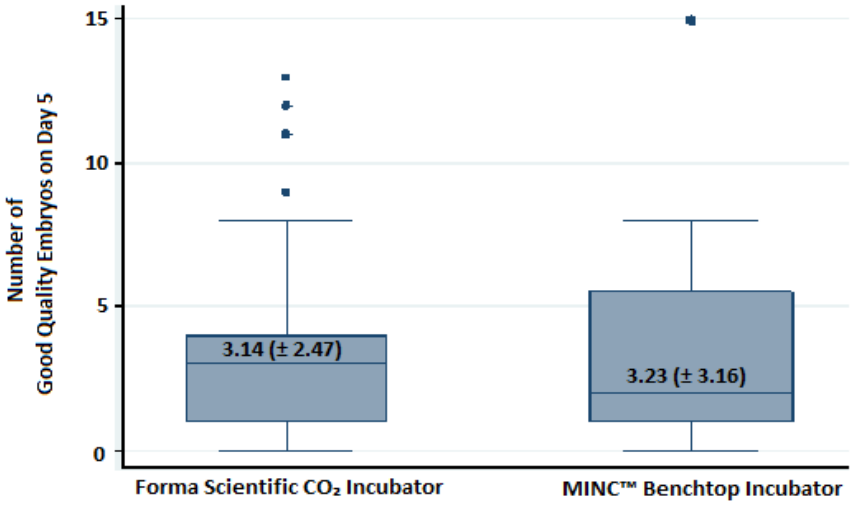


Figure 7: **Boxplot showing the average number of good quality embryos on Day 5 in the two incubators investigated.**

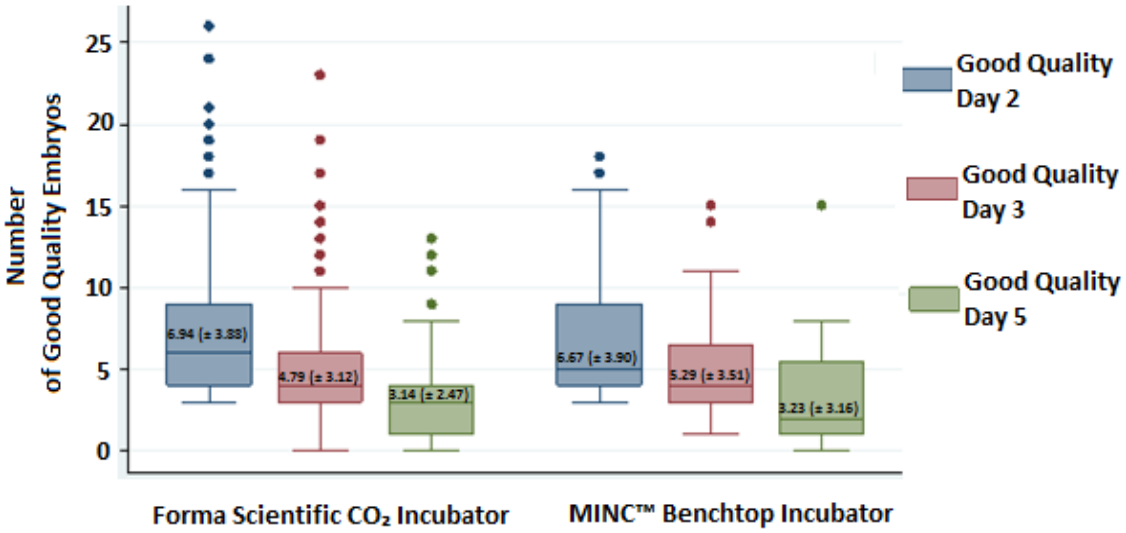


Figure 8: **Boxplot showing a summary of the average number of good quality embryos on Days 2, 3 and 5.**

To determine the probability of good quality embryos on days 2, 3 and 5, modelling was necessary for the two incubators where the denominator was the total number of ova. Figure 9 presents a boxplot of the modelled probability and Table 6 summarizes the outcome.

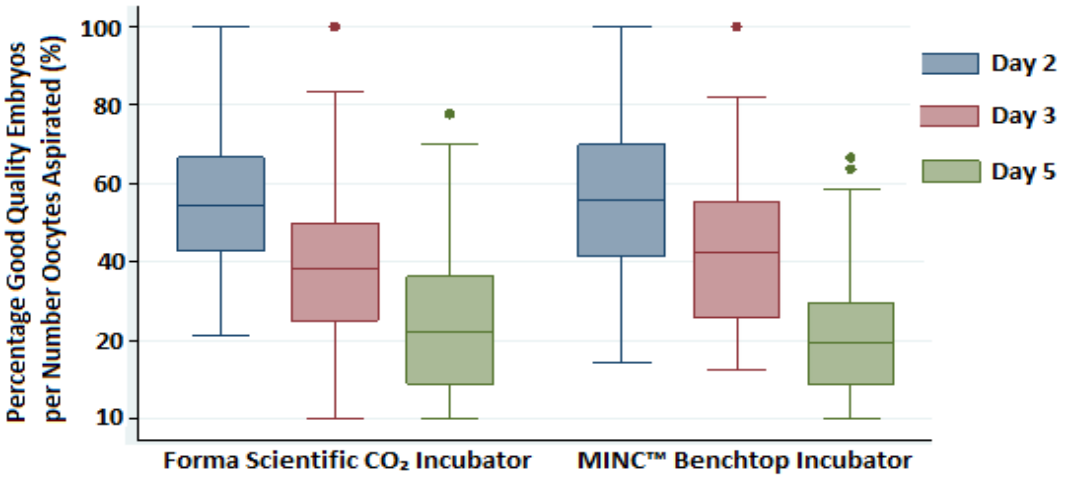


Figure 9: **Boxplot showing the percentage good quality embryos per number of oocytes aspirated for day 2, day 3 and day 5.**

A significant difference in the percentage of GQE/number of ova between the two incubators was shown only for day 3 with the MINC having 44.58% and the Forma 39.31% GQE; p=0.03.

Table 6: Percentage good quality embryos per number of oocytes aspirated between the two different incubators.

Percentage Good Quality Embryos (GQE/Number of Ova)	Forma Scientific CO_2 Incubator	MINC™ Benchtop Incubator	P Value
Day 2	55.89 (318)	57.44 (57)	0.76
Day 3	39.31 (317)	44.58 (56)	0.03
Day 5	23.60 (243)	21.76 (40)	0.90

Binomial Regression Model for Variables (Incubator type, Age of oocytes, Donor oocytes and MII oocytes) Influencing Quality of Embryos on Day 2, 3 and 5.

The model was then also adjusted for the age of the oocytes (female age), use of donor oocytes and the number of MII oocytes available. Binomial regression was applied estimating the risk ratio [rr] for the Forma Scientific CO_2 Incubator compared to the MINC™ Benchtop Incubator on embryo culture days; 2, 3 and 5.

Results are presented in Table 7, showing the risk ratios of the probability of good quality embryos on days 2, 3 and 5 in the two incubators.

For **day 2** there was no significant difference in the probability of good quality embryos between the two incubators (p = 0.76).

From Table 6, there was observed that there was a significant difference on **day 3** in the probability for good quality embryos, rr = 1.15 (95%CI 1.01 to 1.32), p = 0.03. This result indicated that the MINC™ Benchtop Incubator has a 15% (relative) higher probability of having good quality embryos on day 3 compared to the Forma Scientific CO_2 Incubator. From the descriptive statistics (Table 2) there can be observed that the mean proportion of good quality embryos on day 3 is 0.39 in the Forma Scientific CO_2 Incubator compared to 0.45 in the MINC™ Benchtop Incubator. The crude ratio is 1.13, which is close to the adjusted ratio from the model.

There was no difference between the two incubators with regard to the probability of good quality embryos on **day 5** (p = 0.90).

Table 7: Risk ratios of the probability of good quality embryos on Days 2, 3 and 5.

	Day 2	Day 3	Day 5
Incubator	1.01	1.15	1.01
Age of Oocytes	1.00	0.99	0.99
Donor Oocytes	0.98	0.95	0.91
MII Oocytes	0.99	0.98	0.97

4.1.1.8 Pregnancy Outcome

Clinical Pregnancy Outcome (foetal sac/ET)

There was no significant difference observed in the **clinical pregnancy** rate (Table 8) observed in patients whose embryos were cultured in the two respective incubator types (47.17% for the MINC and 45.43% for the Forma respectively), p = 0.81. Of the total of 385 patients, only 370 patients had a known pregnancy outcome; n = 317 patients from the Forma Scientific CO_2 Incubator and n = 53 patients the MINC™ Benchtop Incubator.

Table 8: Clinical pregnancy rate of patients with embryos cultured in the MINC™ Benchtop Incubator and the Forma Scientific CO_2 Incubator.

Incubator	Clinical Pregnancy
Forma Scientific CO_2 Incubator	45.43 (144)
MINC™ Benchtop Incubator	47.17 (25)
Total	**45.68 (169)**

Values represented as percentage (number of patients with a positive clinical pregnancy outcome), p = 0.81

Overall Pregnancy Outcome (All Categories)

No significant difference in any of the other pregnancy outcome categories between the two incubators was indicated by the Fisher's exact test (p = 0.57). All pregnancy category outcomes were similar in both the MINC™ Benchtop Incubator and the Forma Scientific CO_2 Incubator (Table 9). There were a total of 15 patients with missing pregnancy data.

Table 9: Detailed pregnancy outcome between the MINC™ Benchtop Incubator and the Forma Scientific CO_2 Incubator.

Pregnancy Outcome	Forma Scientific CO_2 Incubator	MINC™ Benchtop Incubator	Total
Not Pregnant	40.69 (129)	37.74 (20)	40.27 (149)
Biochemical	7.57 (24)	9.42 (5)	7.84 (29)
Blighted Ovum	0.32 (1)	0 (0)	0.27 (1)
Miscarriage	0.32 (1)	0 (0)	0.27 (1)
Ectopic	0.95 (3)	0 (0)	0.81 (3)
Ongoing	4.73 (15)	7.55 (4)	5.14 (19)
Full Term	35.02 (111)	30.19 (16)	34.32 (127)
Perinatal Death	1.89 (6)	3.77 (2)	2.16 (8)
No Embryo Transfer	3.79 (12)	1.89 (1)	3.51 (13)
No Foetal Heart	0.95 (3)	0 (0)	0.81 (3)
Miscarriage (< 7 weeks)	1.26 (4)	3.77 (2)	1.62 (6)
Miscarriage 2 (7 - 14 weeks)	1.58 (5)	5.66 (3)	2.16 (8)
Miscarriage 3 (> 14 weeks)	0.95 (3)	0 (0)	0.81 (3)
Total	**100 (317)**	**100 (53)**	**100 (370)**

Values are presented as percentage (number of patients)

4.2 Results – Prospective Study

Objective: To determine the possible (negative or positive), independent influence/effect of specifically female patient profile (including oocyte age, BMI, AMH and diagnosis) in two different ART clinics (one in the public sector and one in the private sector) on ART outcome.

The aim of the study was to investigate which factors in ART treatment might have the most significant and independent effect on ART outcome in two ART laboratories. The main outcomes investigated with regard to the different laboratories were oocyte age (female age); number of MII oocytes aspirated; female diagnosis – specifically tubal factor, endometriosis; BMI; AMH and ultimately CPR.

In the private laboratory, the total number of patients during the time of data capturing was 630. Only the 572 consenting patients were included in the analysis. The 58 excluded patients either did not receive a consent form (n = 55) or did not agree to sign consent (n = 3) to participate in the study.

The total number of patients that underwent ART treatment at the public clinic was 275. Of the 275 patients, only 248 could be included in the study. The 27 excluded patients either did not receive a consent form (n = 24) or did not agree to sign consent (n = 3) to participate in the study.

The statistical analysis presented in the first part of the statistical report made use of pooled data from both Sites. Although this was not the initial aim for the prospective data analysis, the statistician, prof. C. Lombard, recommended analysis of the pooled data to identify factors that might have an effect on CPR and to improve the accuracy of the outcomes.

A limitation of the prospective results was the extensive difference in the number of patients between the two Sites although statistical analysis indicated that the results were still statistically valid.

4.2.1 Descriptive Data Results for Site 1 – The Private Fertility Clinic

Table 10 presents the overall descriptive statistics for the variables investigated that could potentially influence ART outcomes at Site 1.

Table 10: Descriptive data of main factors influencing ART outcomes at Site 1.

Variable	N	Min	p25	p50	p75	Max
Patient Age (Years)	572	23	34	37	40	54
Donor Age (Years)	108	19	23	24	28	44
Oocyte Age (Years)	572	19	30	35	39	46
AMH (ng/mL)	195	0.08	0.38	1.20	2.53	12.25
BMI (kg/m²)	468	15.12	20.85	23.31	26.50	45.36
Number of Oocytes Aspirated	463	0	4	8	13	66
Number MII Oocytes	460	0	3	6	11	46
Number of Oocytes Fertilized	472	0	3	5	9	39
Number of Good Quality Embryos on Day 2	415	0	2	4	7	28
Number of Good Quality Embryos on Day 3	366	0	2	4	7	22
Number of Good Quality Embryos on Day 4	44	0	0	1.5	3	10
Number of Good Quality Embryos on Day 5	247	0	1	3	5	17
Number of Good Quality Embryos on Day 6	28	0	0	0	1.5	6
Number of Good Quality Embryos on Day 7	8	0	0	0	0	1
Day of Embryo Transfer	479	2	3	5	5	7
Number of Embryos Transferred	533	0	1	2	2	8
Number of Good Quality Embryos Transferred	478	0	1	2	2	5

4.2.1.1 Female Diagnosis

The distribution of female diagnoses at Site 1 is illustrated in Figure 10. The pie chart indicates that advanced maternal age; 23% (n = 130), other (immunological, growths/fibroids, growths & myoma's, myoma, premature ovarian failure myomectomy, uterine factor, HIV positive, poor responder, genetic factor, congenital abnormality, cancer, ova vitrification, low AMH value and oophorectomy); 21% (n = 118) and normal (only male factor); 17% (n = 97) were the most common diagnoses among the patients. Tubal factor and endometriosis only contributed to 8% and 11% of the total female factor diagnosis, respectively.

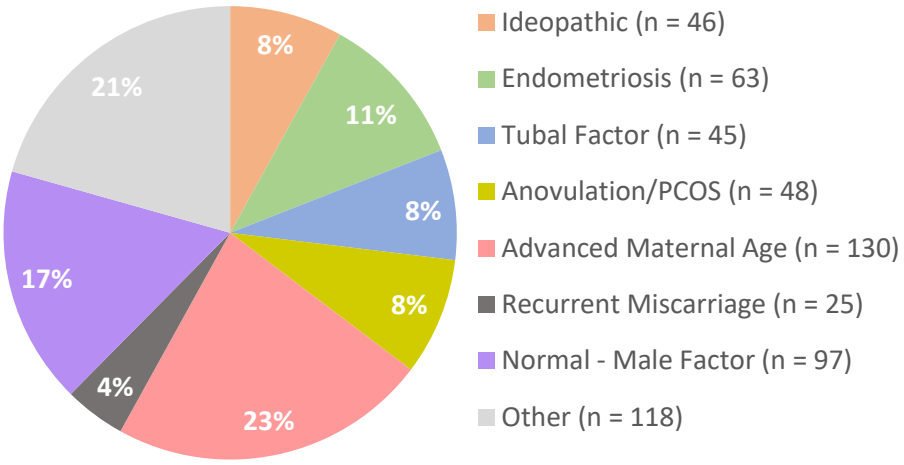


Figure 10: Pie chart showing the distribution of main female diagnoses at Site 1 (n = 572).

4.2.1.2 BMI

BMI distribution among patients attending Site 1 were categorized into two groups as shown in Figure 11. The first group was patients with a BMI < 30 kg/m^2 and 90% of the patients (n = 337) was in this group. The second group was patients with a BMI ≥ 30 kg/m^2 and 10% of the patients (n = 39) was in this group. The total of the cycles (n = 376) with an indicated BMI differs from the total number of cycles that occurred during the time of investigation (n = 572) due to missing data.

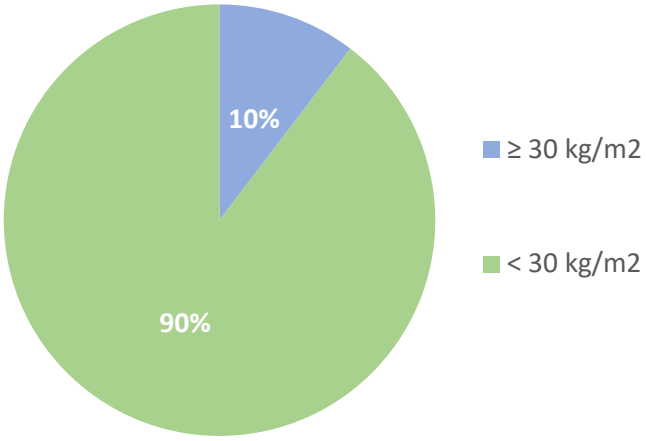


Figure 11: Pie graph showing the distribution of BMI, grouped, at Site 1 (n = 376).

4.2.1.3 Male Diagnosis

Table 11 presents the different **male diagnoses** and their distribution among the total number of cycles at Site 1 (n = 528). Of the various diagnoses the most predominant was G-Pattern Morphology (44.13%; n = 233) and Normozoospermia (12.31%; n = 65).

Table 11: Distribution of main male diagnoses at Site 1 (n = 528).

Male Diagnosis	Number of Patients (n)	Percentage of Patients (%)
N-Pattern Morphology (>14%)	65	12,31%
Oligozoospermia	43	8,14%
Oligo - Teratozoospermia (0 - 4%)	16	3,03%
Oligozoospermia (G-Pattern 5 - 14%)	12	2,27%
Oligo- Astheno - P-Pattern Morphology (0 - 4%)	13	2,46%
Oligo- Asthenozoospermia (G-Pattern 5 - 14%)	2	0,38%
Oligo- Asthenozoospermia (N-Pattern >14%)	0	0,00%
P-Pattern Morphology (0 - 4%)	33	6,25%
G-Pattern Morphology (5 - 14%)	233	44,13%
Immunological Factor	12	2,27%
Donor Sperm	18	3,41%
Other	11	2,08%
Astheno- Teratozoospermia/G-Pattern (0 - 4%/5 - 14%)	3	0,57%
Retrograde Ejaculation	1	0,19%
Testis Biopsy	20	3,79%
Donor And Own Sperm	5	0,95%
Azoospermia	32	6,06%
Previous Vasectomy	7	1,33%
HIV Positive	2	0,38%
Total	528	100,00%

4.2.1.4 Distribution of Cycles Types

At Site 1, the patient cycle types were grouped to illustrate how many patients made use of donor and surrogate cycles (Figure 12). The data indicated that of the total of patients that underwent ART treatment at Site 1; 79% (n = 464) used their own ova, 19% (n = 108) made use of donor ova and 2% (n = 14) made use of a surrogate.

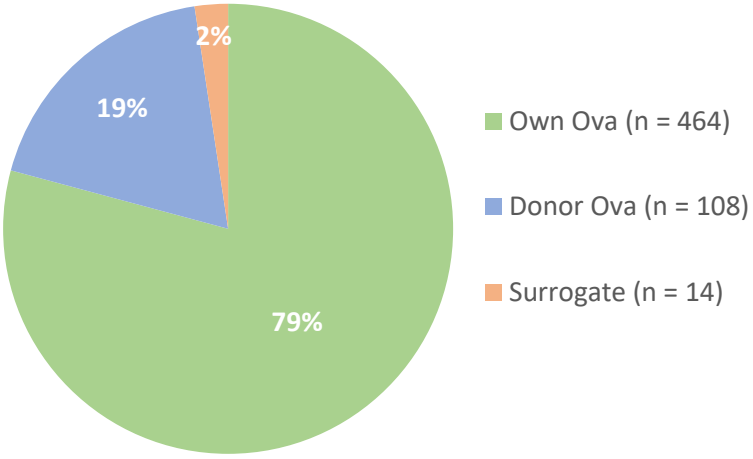


Figure 12: Pie chart showing the distribution of cycle types at Site 1 (n = 572).

4.2.1.5 Treatment Distribution

All the different types and combinations of treatment procedures at Site 1 are indicated in Table 12. The data showed that the most predominant treatment procedures were; PICSI (43.53%; n = 249), FET (18.70%; n = 107) and a combination of IVF and PICSI (13.63; n = 78).

Table 12: Distribution of treatment procedures at Site 1 (n = 572).

Treatment Type	Percentage (n)
IVF	1,74% (10/572)
ICSI	9,44% (54/572)
PICSI	43,53% (249/572)
IVF/ICSI	0,17% (1/572)
IVF/PICSI	13,63% (78/572)
PICSI/IMSI	1,57% (9/572)
FET	18,70% (107/572)
FROZEN OVA - PICSI	0,69% (4/572)
IMSI	0,17% (1/572)
PICSI - PGD/S	2,97% (17/572)
OOCYTE VITRIFICATION	5,06% (29/572)
ICSI/PICSI	0,69% (4/572)
AI/PICSI	0,17% (1/572)
ASPIRATED - NO PROCEDURE	1,04% (6/572)
CYCLE CANCELLED - NO ASPIRATION	0,17% (1/572)
IVF/FET	0,17% (1/572)

4.2.1.6 Cycle Description

Figure 13 presents descriptive data regarding the cycles, at Site 1, during the study period. The data indicated that a total of 460 (n = 572 cycles) patients were aspirated (for fresh cycles) if the procedure of ova vitrification is included. If the procedure of ova vitrification is excluded there were 436 (n = 572 cycles) aspirations eligible for embryo transfer. Of the 436 aspirated cycles eligible for embryo transfer (excluding ova vitrification cycles; n = 29), 371 patients received an embryo transfer (excluding FET cycles; n = 107), indicating an 85.09% transfer rate.

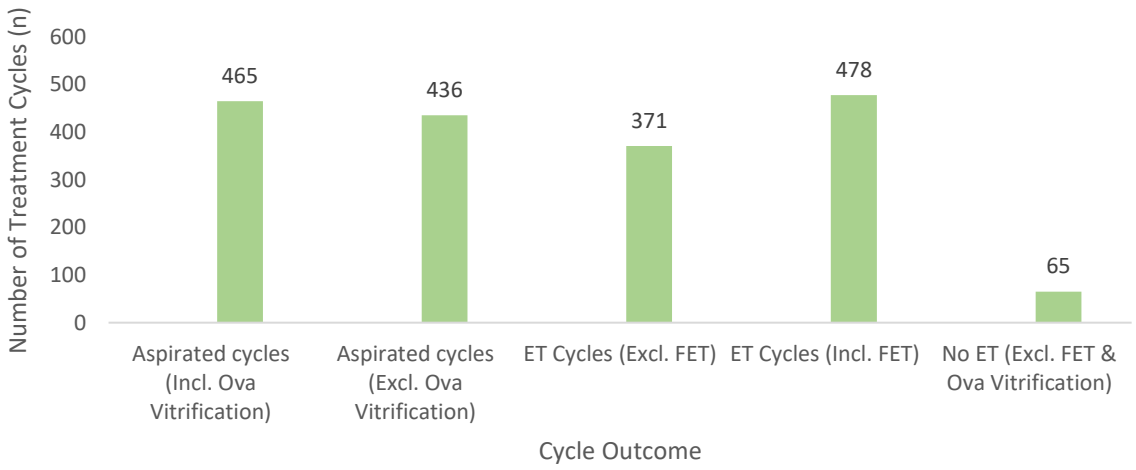


Figure 13: Distribution of treatment cycle outcomes at Site 1.

In 65 cycles eligible for an embryo transfer, no embryo transfer occurred. The various reasons for no transfer are shown in Figure 14. The most common reasons for a cycle with no embryos transferred was poor quality embryos (34%; n = 22) and freeze all cycles for PGD/PGS (26%; n = 17).

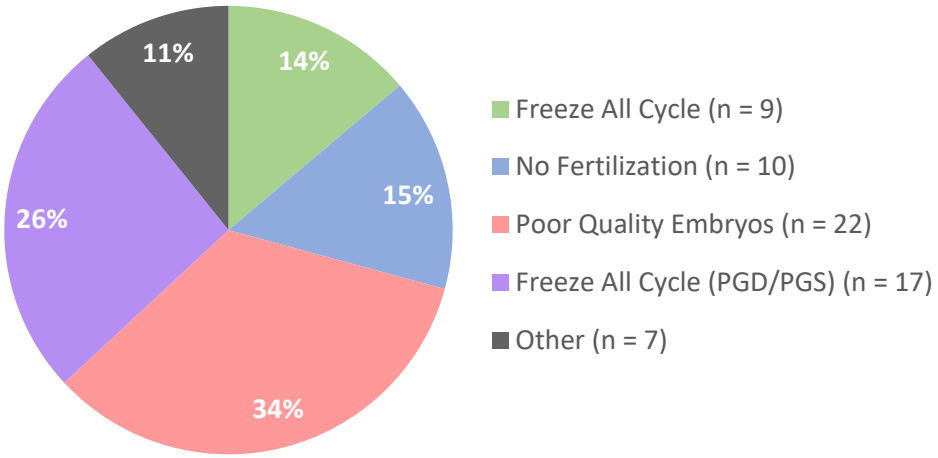


Figure 14: Distribution of cycles with no embryo transfer at Site 1 (n = 65).

4.2.1.7 Overall Pregnancy Outcome

The overall pregnancy outcome per transfer is indicated in Figure 15. At the time of analysis 61 patients out of 478 patients (that received an embryo transfer) did not have a known pregnancy result. In 417 cycles a pregnancy outcome was known. A total of 235 patients were not pregnant after ART treatment at Site 1. The number of miscarriages (< 7 weeks and 7-14 weeks) was 19 and a visible foetal sac/s was present in 113 of the patients. Eleven (11) babies were born during the study period.

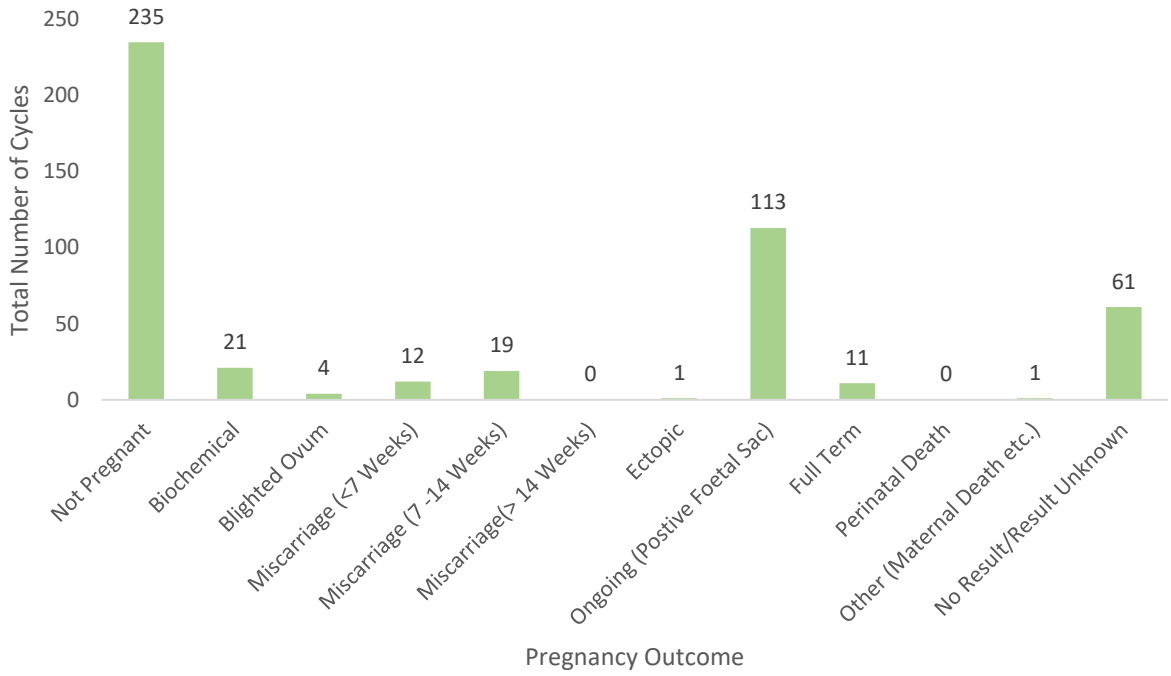


Figure 15: Distribution of pregnancy outcomes (cycle number) at Site 1 (n = 478).

4.2.1.8 Clinical Pregnancy Outcome/ET (of Cycles with a Known Pregnancy Outcome)

Table 13 shows an overview of the clinical pregnancy outcome at Site 1 of cycles with known pregnancy outcome (n = 417). The clinical pregnancy rate (CPR) data was subdivided into two categories, cycles with an oocyte [female] age ≤ 35 years and > 35 years, respectively. The group oocyte age ≤ 35 years had a CPR/ET of 36.01% (n = 103) compared to 27.36% (n = 52) in the group oocyte age > 35 years. The overall CPR /ET was 32.56% (n = 155)

Table 13: Clinical Pregnancy Outcome/Transfer at Site 1.

	n = ± SD
Average Number of Oocytes Aspirated	9,57 (± 7,08)
Average Number of Embryos Transferred	2,07 (± 1,03)
Average Oocyte [female] Age (years)	33,88 (± 5,89)
	Percentage (n)
Clinical Pregnancy Rate (Foetal Sac)/ET	37.17% (155/417)
Clinical Pregnancy Rate/ET - Oocyte Age ≤ 35 years	41.53% (103/248)
Clinical Pregnancy Rate/ET - Oocyte Age > 35 years	30.76% (52/169)
Clinical Pregnancy Rate/ET in Donor Cycles	48.31% (43/89)

4.2.2 Descriptive Data Results for Site 2 – The Public Fertility Clinic

Table 14 presents the descriptive statistics for the variables investigated that could potentially influence ART outcomes in the public sector fertility clinic. Table 14 provides an overall overview of the patient and cycle demographics at Site 2 during the period of investigation.

Table 14: Descriptive data of main factors influencing ART outcomes at Site 2.

Variable	N	Min	p25	p50	p75	Max
Patient Age (Years)	248	25	32	35	38	45
Donor Age (Years)	6	20	22	24.5	35	35
Oocyte Age (Years)	248	20	31	35	38	45
Anti-Mullerian Hormone [AMH] (ng/mL)	85	0.16	0.72	1.38	3.48	18.88
BMI (kg/m²)	232	17.80	23.59	26.97	31.075	47.78
Number of Oocytes Aspirated	241	0	2	3	5	30
Number MII Oocytes	224	0	2	3	4	30
Number of Oocytes Fertilized	225	0	1	2	3	28
Number of Good Quality Embryos on Day 2	198	0	1	2	3	14
Number of Good Quality Embryos on Day 3	173	0	1	1	2	10
Number of Good Quality Embryos on Day 4	40	0	0	1	2	4
Number of Good Quality Embryos on Day 5	19	0	0	1	3	9
Number of Good Quality Embryos on Day 6	1	0	0	0	0	0
Number of Good Quality Embryos on Day 7	0	-	-	-	-	-
Day of Embryo Transfer	185	1	3	3	3	6
Number of Embryos Transferred	248	0	0	2	2	4
Number of Good Quality Embryos Transferred	185	0	1	2	2	4

4.2.2.1 Female Diagnosis

The distribution of female diagnoses at Site 2 is illustrated in Figure 16. Four predominant diagnoses were evident as indicated in Figure 16. The most prevalent diagnoses were tubal factor infertility; 29% (n = 73), normal female/male only factor infertility; 18% (n = 45) and anovulation/PCOS; 14% (n = 34).

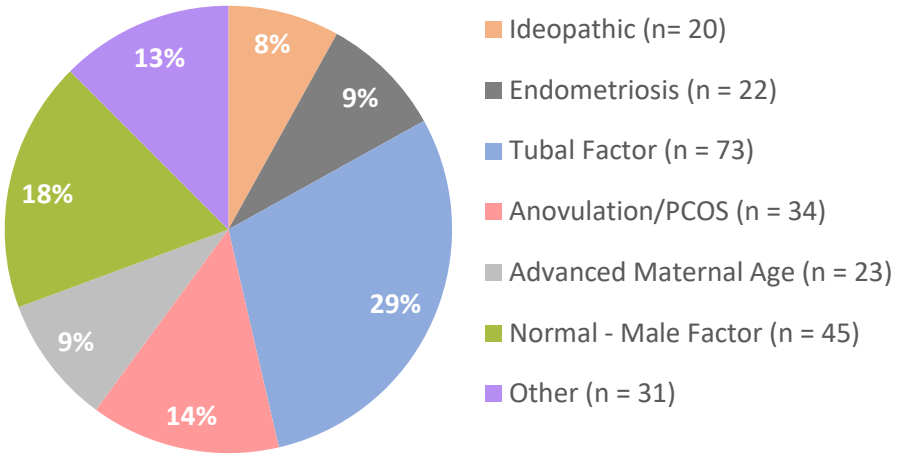


Figure 16: Pie chart showing the distribution of main female diagnoses at Site 2 (n = 248).

4.2.2.2. BMI

The BMI distribution among patients attending Site 2, is shown in Figure 17. The first group was patients with a BMI < 30 kg/m^2 and 68% of the patients (n = 144) was in this group. The second group was patients with a BMI ≥ 30 kg/m^2 and 32% (n = 67) was in this group. The total of the cycles (n = 211) with an indicated BMI differs from the total number of cycles that occurred during the time of investigation (n = 248) due to missing data.

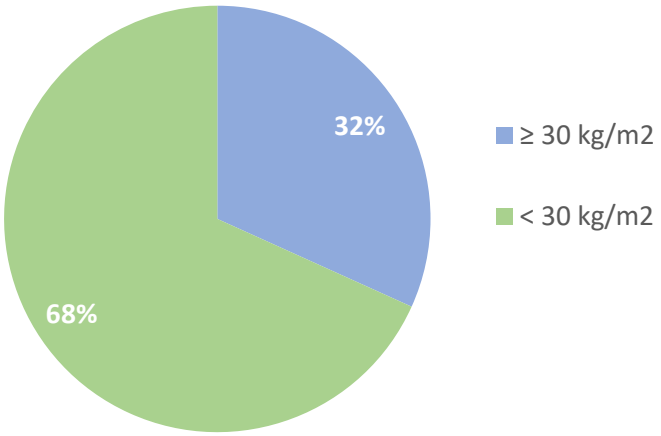


Figure 17: Pie graph showing the distribution of BMI, grouped, at Site 2 (n = 211).

4.2.2.3 Male Diagnosis

Table 15 demonstrates the male diagnosis distribution among all the cycles at Site 2 (n=248). The table indicates that the most frequent male diagnosis among male patients were; Normozoospermia (28.63%; n = 71), Teratozoospermia (20.56%; n = 51), Testis Biopsy (17.34%; n = 43) and G-Pattern Morphology (13.31%; n = 33).

Table 15: Distribution of main male diagnoses at Site 2 (n = 248).

Male Diagnosis	Number of Patients (n)	Percentage of Patients (%)
N-Pattern Morphology (>14%)	7	2.82%
Oligozoospermia	2	0,81%
Oligo - Teratozoospermia (0 - 4%)	20	8,06%
Oligozoospermia (G-Pattern 5 - 14%)	0	0,00%
Oligo- Astheno - Teratozoospermia (0 - 4%)	8	3,23%
Oligo- Asthenozoospermia (G-Pattern 5 - 14%)	1	0,40%
Oligo- Asthenozoospermia (N-Pattern >15%)	0	0,00%
P-Pattern Morphology (0 - 4%)	51	20,56%
G-Pattern Morphology (5 - 14%)	97	39.11%
Immunological Factor	2	0,81%
Donor Sperm	8	3,23%
Other	1	0,40%
Astheno- Teratozoospermia/G-Pattern (0 - 4%/5 - 14%)	2	0,81%
Retrograde Ejaculation	0	0,00%
Testis Biopsy	43	17,34%
Donor And Own Sperm	0	0,00%
Azoospermia	5	2,02%
Previous Vasectomy	1	0,40%
HIV Positive	0	0,00%
Total	248	100,00%

4.2.2.4 Distribution of Cycles Types

Figure 18 shows the distribution of cycle types at Site 2. Of the total number of cycles during the time of investigation (n = 248), the female patient made use of her own ova in 98% (n = 242) of the cycles, compared to 2% (n = 6) of patients making use of donor ova. There were no surrogate cycles during this time at Site 2.

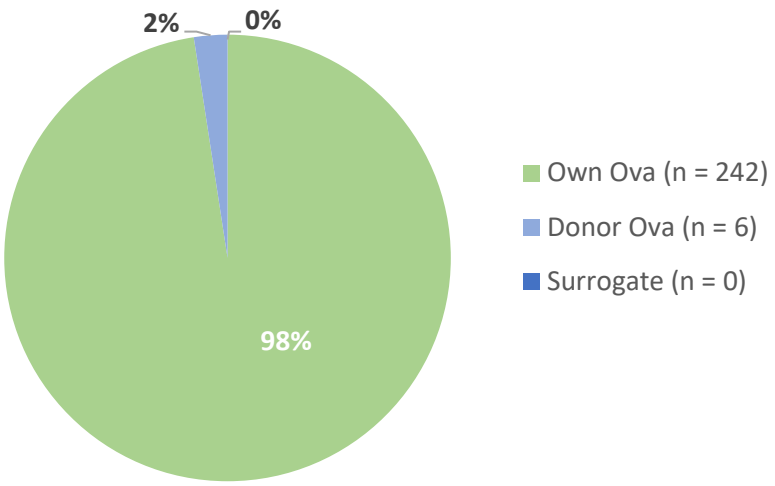


Figure 18: Pie chart showing the distribution of cycles types at Site 2 (n = 248).

4.2.2.5 Treatment Distribution

The treatment procedures applied at Site 2 during the time of investigation are indicated in Table 16. The most frequently used procedures were ICSI (58.87%; n = 146) and IVF (31.45%; n = 78). There was a total of 16 cancelled cycles (6.45%).

Table 16: Distribution of treatment procedures at Site 2 (n = 248).

Treatment Type	Percentage (n)
IVF	31,45% (78/248)
ICSI	58,87% (146/248)
PICSI	0,40% (1/248)
IVF/ICSI	0,00% (0/248)
IVF/PICSI	0,00% (0/248)
PICSI/IMSI	0,00% (0/248)
FET	2,82% (7/248)
FROZEN OVA - PICSI	0,00% (0/248)
IMSI	0,00% (0/248)
PICSI - PGD/S	0,00% (0/248)
OOCYTE VITRIFICATION	0,00% (0/248)
ICSI/PICSI	0,00% (0/248)
AI/PICSI	0,00% (0/248)
ASPIRATED - NO PROCEDURE	6,45% (16/248)
IVF/FET	0,00% (0/248)

4.2.2.6 Cycle Description

A total of 241 patients were aspirated at Site 2 during the investigation period. The total number of cycles were 248, but 7 of the cycles were FET. There were no ova vitrification during this period. Of the 241 aspirated cycles, 178 cycles received and embryo transfer, excluding Frozen Embryo Transfer [FET] (n = 7), resulting in a transfer rate of 73.85% (178/241) as indicated in Figure 19.

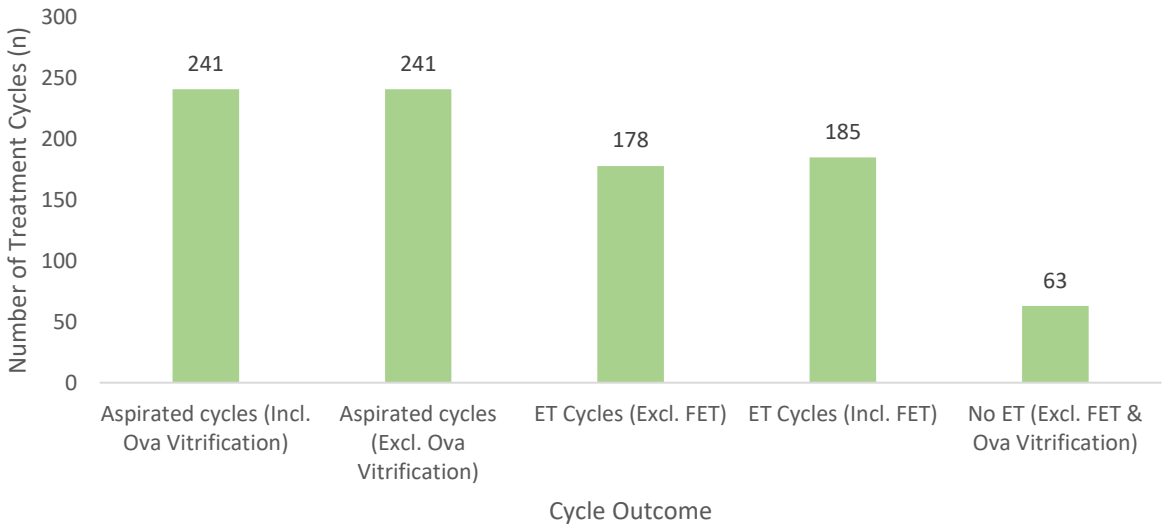


Figure 19: Distribution of treatment cycle outcomes at Site 2.

In 241 cycles eligible for an embryo transfer, 63 of the cycles did not receive an embryo transfer. The various reasons for no transfer are shown in Figure 20. The two main reasons for no transfer were no oocyte fertilization (32%; n = 20) and poor quality embryos (29%; n = 18).

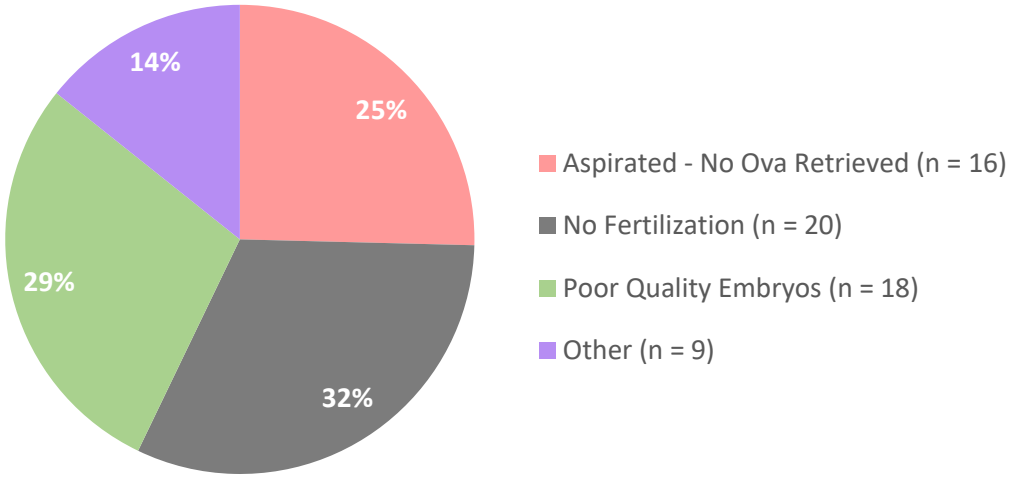


Figure 20: Distribution of cycles with no embryo transfer at Site 2 (n = 63).

4.2.2.7 Overall Pregnancy Outcome

The overall pregnancy outcome per transfer of the 185 cycles that received an embryo transfer (7 FET's included) are shown in Figure 21. At the time of analysis 148 of the cycles had a negative pregnancy outcome. A foetal heart/s was present in 5 patients and 14 babies have been born during the study period. The number of miscarriages (<7 weeks and > 7-14 weeks) were 12.

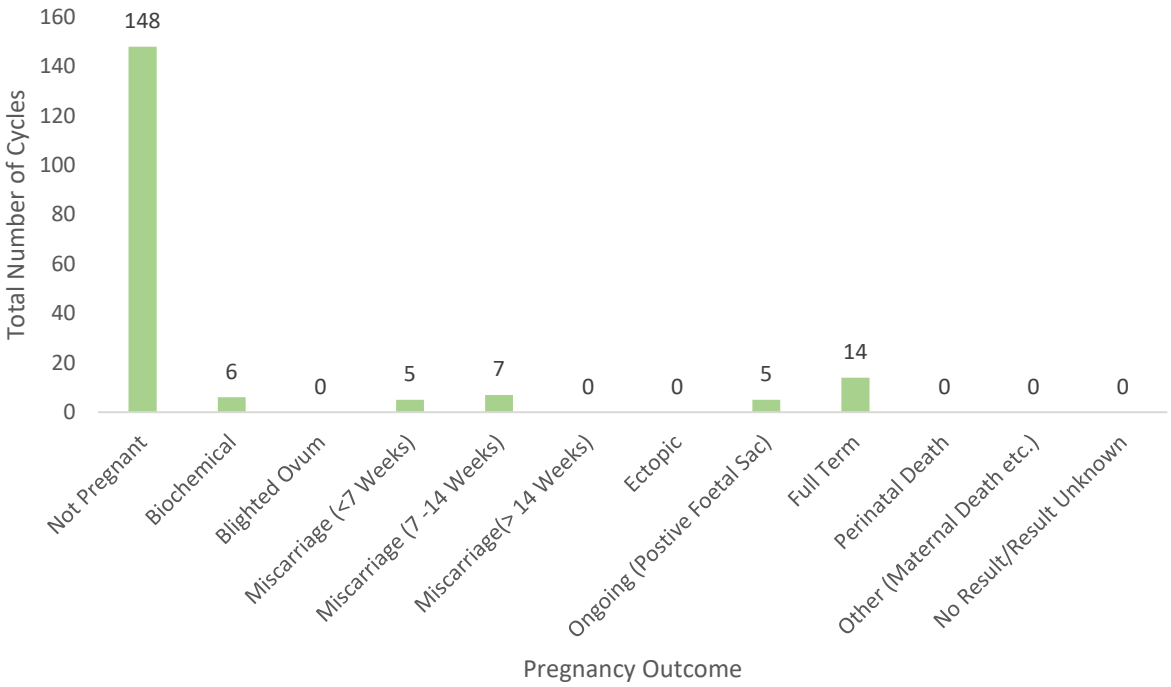


Figure 21: Distribution of pregnancy outcomes (cycle number) at Site 2 (n = 185).

4.2.2.8 Clinical Pregnancy Outcome/ET (of Cycles with a Known Pregnancy Outcome)

Table 17 shows an overview of the clinical pregnancy outcome at Site 2 of cycles with known pregnancy outcome (n=185). The clinical pregnancy rate (CPR)/ET for patients with an oocyte age of ≤ 35 years was 19.46% (n= 22) compared to 9.72**%** (n=7) in patients that had an oocyte age > 35 years. The overall CPR/ET, regardless of oocyte age, was 15.67% (n=29).

Table 17: Clinical Pregnancy Outcome/Transfer at Site 2.

	n = ± SD
Average Number of Oocytes Aspirated	4,14 (± 3,56)
Average Number of Embryos Transferred	2,07 (± 1,14)
Average Oocyte Age (years)	35 (± 4,50)
	Percentage (n)
Clinical Pregnancy (Foetal Sac)/ET	15,67% (29/185)
Clinical Pregnancy/ET - Oocyte Age ≤ 35 years	19,46% (22/113)
Clinical Pregnancy/ET - Oocyte Age > 35 years	9,72% (7/72)
Clinical Pregnancy/ET in Donor Cycles	20,00% (1/5)

4.2.3 Statistical Analysis for Site 1 and 2 – The Private and Public Fertility Clinic Pooled and Separate Data

4.2.3.1 Descriptive Data – Effect of Different Factors on Clinical Pregnancy Rate (CPR)

a) Age of Oocytes (Female Age)

The Lowess smooth graph (Figure 22) demonstrates the association between oocyte age and clinical pregnancy rate (CPR) in both Sites pooled. The results indicated that with an increase in oocyte age, CPR declines. There was a 20% difference across the oocyte age range.

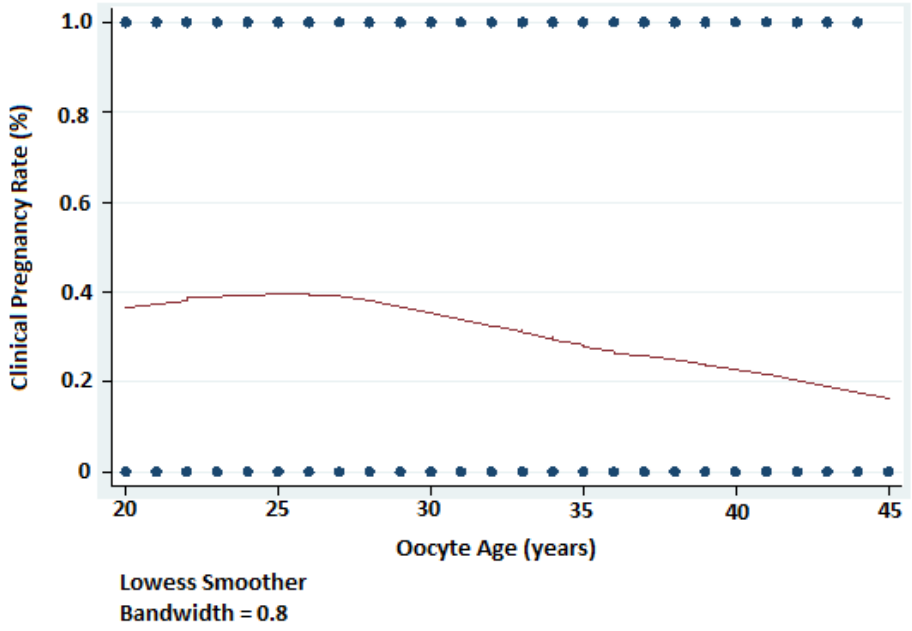


Figure 22: Lowess smooth graph showing the association between CPR and oocyte age for both Sites (pooled).

Lowess smooth graph (Figure 23) shows the association between CPR and oocyte age for each Site separately. Site 2 had very few cycles that had a relatively young oocyte age, < 26 years. Overall trends are the same at both Sites confirming results from pooled analysis as indicated in Figure 22. No interaction was observed between oocyte age and Site with respect to the outcome of CPR.

Figure 23: Lowess smooth graph showing the association between CPR and oocyte age for each Site separately.

b) Endometriosis

In a pooled analysis of both Sites, the results indicated no significant difference in CPR in cycles that had patients presenting with endometriosis (29.41%; n = 20) compared to those who did not have endometriosis (29.76% n = 164), p = 0.952.

The CPR, at Site 1, in cycles with patients presenting with endometriosis was 29.41% (n = 15) compared to 36.65% (n = 140) in patients that did not have endometriosis (p = 0.31). The CPR in patients with endometriosis in Site 2 was also 29.41% (n = 5) compared to 14.20% (n = 24) in patients that did not have endometriosis (p = 0.09).

As previously mentioned the CPR for cycles with female patients presenting with endometriosis was the same at both Sites; 29.41%. The differential comes in with the CPR in the cycles with patients not presenting with endometriosis. Site 2 CPR is 14.20% (n = 24) compared to 36.65% (n = 140) at Site 1. This indicated that the results obtained are strongly confounded by the patient population in both Sites.

c) TFI

The results of the pooled data (Site 1 and 2) with regard to the effect of tubal factor infertility on CPR indicated that there was no statistical significant difference in CPR when comparing tubal factor infertility patient cycles with non-tubal factor infertility patient cycles. Cycles with

patients presenting with tubal factor infertility had a CPR of 22.99% (n = 20) compared to cycles with patients that did not present with tubal factor infertility, 30.89% (n = 164). Thus, a lower CPR was observed in patients presenting with tubal factor, but this difference was not statistically significant (p = 0.138).

At Site 1 the CPR in cycles where patients presented with tubal factor infertility was 29.73% (n = 11) compared to 36.36% (n = 144) in cycles where patients did not present with tubal factor infertility (p = 0.42).

At Site 2 the CPR in cycles where patients presented with tubal factor infertility was 18.00% (n = 9) compared to 14.71% (n = 20) in cycles where patients did not present with tubal factor infertility (p = 0.58).

d) BMI

Figure 24 presents a non-parametric Lowess smooth graph of the association between CPR and BMI. The data used in this graph is pooled from Site and Site 2. The graph excludes one patient with a BMI < 16 from Site 1. The results indicated that there was a small decrease in CPR with an increase in BMI. Data was sparse above a BMI value > 40.

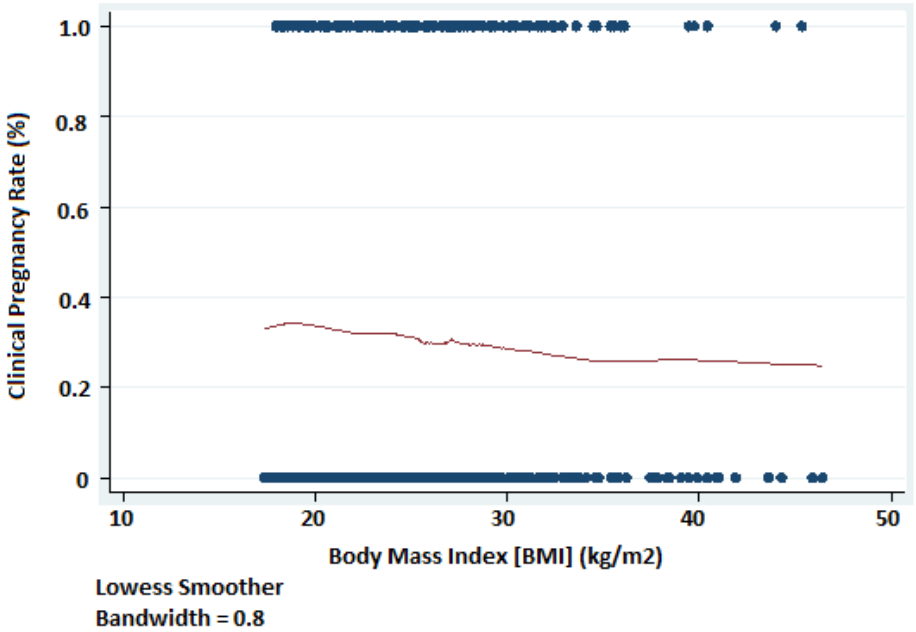


Figure 24: Lowess smooth graph showing the association between CPR and BMI for both Sites (pooled).

The Lowess smooth graph presented in Figure 25, illustrates the association between CPR and BMI for each Site separately. The number of participants with a BMI value > 40 was very small. The graph indicates that CPR decreases with an increased BMI at Site 1, with a rapid decline from a BMI value ≥ 30. At Site 2, CPR rapidly declines around a BMI value of 20 and starts to increase from a BMI of 30, this could be due to an interaction of other factors.

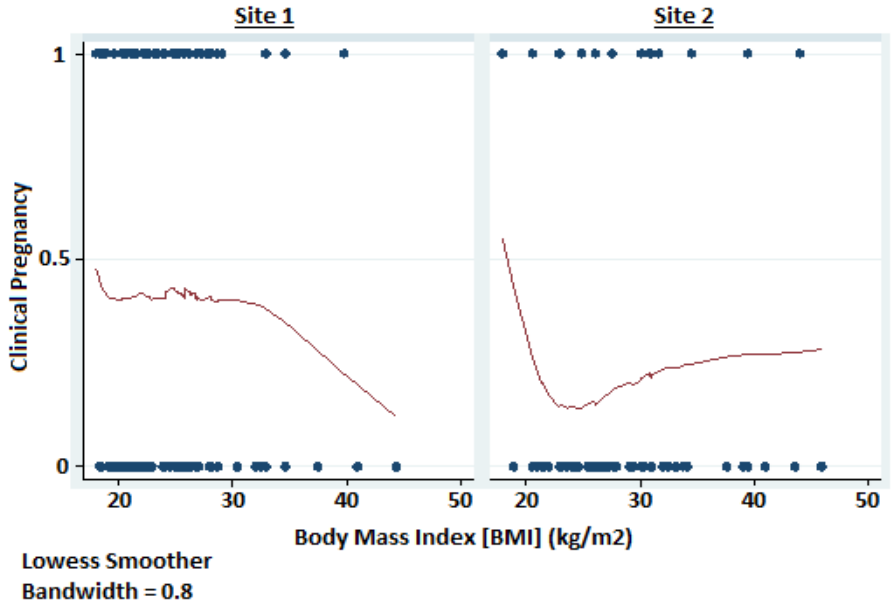


Figure 25: Lowess smooth graph showing the association between CPR and BMI for Site 1 and Site 2 separately.

Figure 26 illustrates the association between CPR and BMI at each Site, taking into account the effect of cycles presenting with and without endometriosis. The results demonstrated that cycles presenting with endometriosis had a lower CPR but the association was the same with regard to BMI at both Sites.

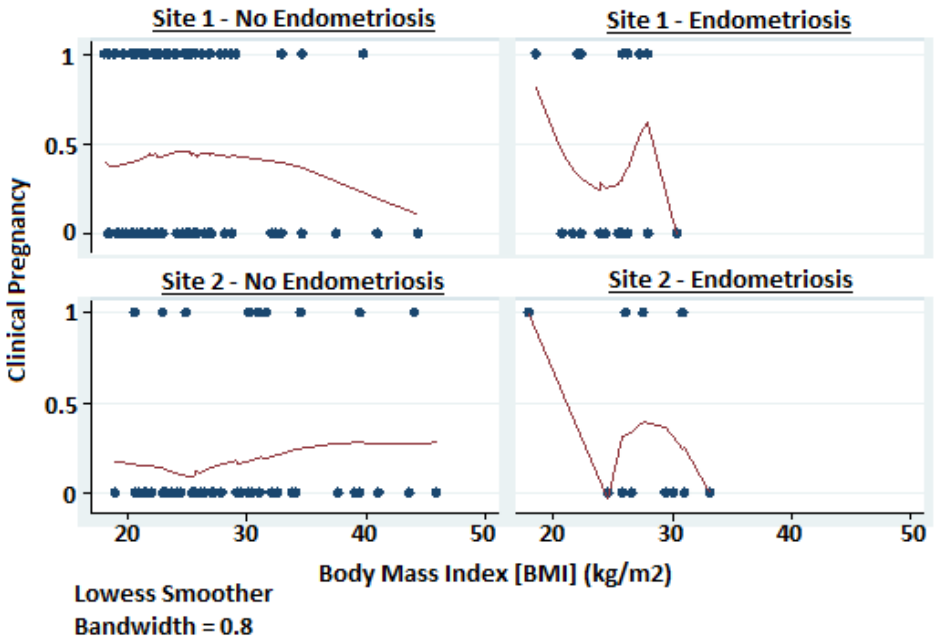


Figure 26: Lowess smooth graph showing the association between CPR and BMI and considering endometriosis at the two different Sites.

e) AMH

The data presented in Figure 27 demonstrates the association between AMH and CPR for both Sites, pooled. The data was restricted to an AMH value <8. The results indicated that there was no association with regard to CPR for AMH up to a value of 4, but for AMH > 4 an increase in CPR was observed. The number of cycles with a patient AMH value of > 4 was very small, although this factor was initially one of the factors investigated, various amounts of was data missing in both Sites, thus analysis was limited.

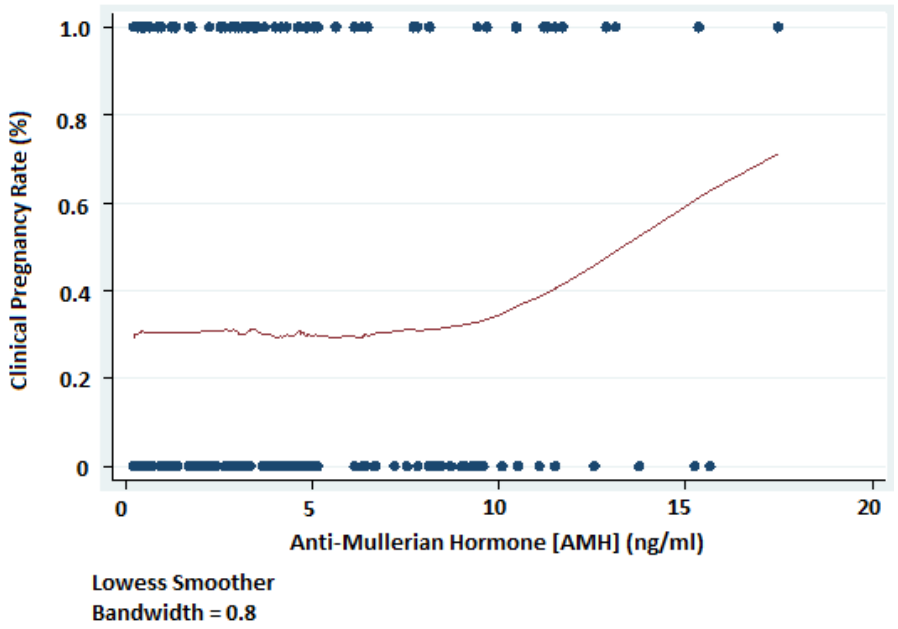


Figure 27: Lowess smooth graph showing the association between CPR and AMH for both Sites (pooled).

Figure 28 demonstrates the association between AMH and CPR for the two Sites separately. The trend shown in Figure 28 is similar to that of the pooled data (Figure 27), but at different levels. As previously mentioned large numbers of AMH data were missing and this limited its use in the statistical models evaluating AMH effect on clinical pregnancy outcome.

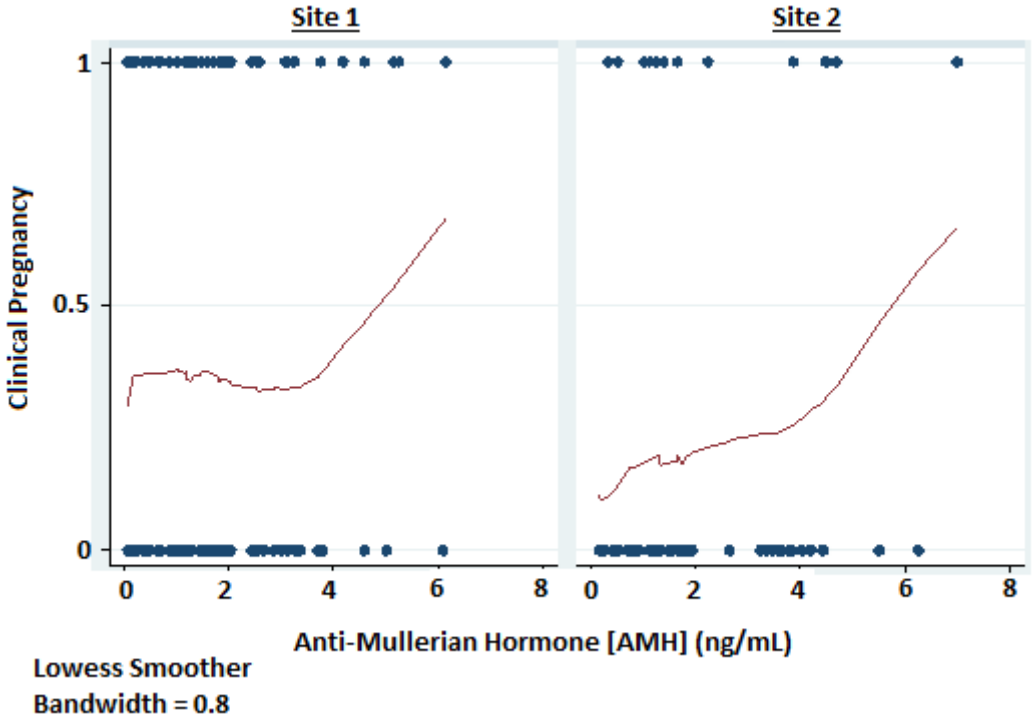


Figure 28: Lowess smooth graph showing the association between CPR and AMH at the two different Sites.

4.2.3.2 Further Statistical Analysis – Site 1 & 2 Combined

a) Binomial Regression Model (Adjusted for Site & Age of Oocytes)

A binomial regression model was performed for each of the determinant factors investigating their association with CPR. The model was adjusted for Site and the age of oocytes (female age). The data of both Sites were pooled to improve the accuracy of the outcomes.

i) BMI

Table 18 presents the outcome of the binomial regression model analyses applied to determine the association between BMI and CPR. As shown in the table, BMI is not associated with CPR in the basic adjusted model (p = 0.41).

Table 18: Association between BMI and CPR (adjusted for site & age of oocytes) for both Sites (pooled data).

Risk Ratio (rr)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
1.01	0.01	0.81	0.41	0.98	1.03

ii) AMH

The results from the adjusted binomial regression model presented in Table 19, demonstrates that AMH value is not associated with CPR (p = 0.17). AMH was not used in further models due to the significant numbers of missing data. More than 65% of the cycles had a missing AMH value and this could introduce severe bias in any result.

Table 19: Association between AMH and CPR (adjusted for site & age of oocytes) for both Sites (pooled data).

Risk Ratio (rr)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
1.04	0.03	1.37	0.17	0.98	1.11

iii) Endometriosis

Endometriosis is not associated with CPR according to the adjusted binomial regression model results as indicated in Table 20 (p = 0.72).

Table 20: Association between endometriosis and CPR (adjusted for site & age of oocytes) for both Sites (pooled data).

Risk Ratio (rr)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
0.93	0.18	- 0.36	0.72	0.63	1.37

iv) TFI

Table 21 presents the results of an association between tubal factor infertility and CPR. The results of the adjusted binomial regression model indicated that the two variables are not associated (p = 0.72).

Table 21: Association between tubal factor diagnosis and CPR (adjusted for site & age of oocytes) for both Sites (pooled data).

Risk Ratio (rr)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
0.93	0.18	- 0.35	0.72	0.62	1.38

b) Combined Binomial Regression Model (Adjusted for Site & Age of Oocytes)

A combined binomial regression model was then applied to the data and in this model BMI, endometriosis, tubal factor infertility, oocyte age and Site were all considered to determine the association of each factor with CPR. The results as presented in Table 22 indicated that BMI (p = 0.41), endometriosis (p = 0.99) and tubal factor infertility (p = 0.71) are not associated with CPR.

The age of oocytes (female age), is significantly (negatively) associated with CPR with a p-value of 0.02; rr = 0.98 (95%CI: 0.95 to 0.99). This result indicated that for every extra year added to the age of the oocytes (female partner), the probability of a clinical pregnancy is 2%

less than that of the base year (relative). Site is also significantly associated with CPR (p = 0.00). Site 2 has a significant negative association (lower CPR) with CPR.

Table 22: Combined regression model showing the association between BMI, endometriosis, tubal factor infertility, oocyte age and Site and CPR (pooled data).

	Risk Ratio (rr)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
BMI	1.01	0.01	0.82	0.41	0.98	1.03
Endometriosis	1.00	0.19	0.01	0.99	0.67	1.48
Tubal Factor	0.92	0.19	- 0.37	0.71	0.60	1.40
Oocyte Age	0.97	0.01	- 2.24	0.02	0.95	0.99
Site	0.40	0.07	- 4.61	0.00	0.27	0.59

c) Extended Combined Binomial Regression Model

The previous models indicated a strong impact of Site on the results. The interaction between Site and each of the other determining factors was then investigated in an extended, combined binomial regression model. The results of this model indicated a significant interaction between Site and endometriosis. This term was therefore included in the final model (Table 23).

There were no other interactions by Site, for example oocyte age. Therefore, the parameters estimated for oocyte age is the pooled estimate from both Sites.

The association between endometriosis and CPR were significantly different between the two Sites. The results from Site 1 indicated that endometriosis is not associated with CPR, rr = 0.88 (95%CI: 1.01 to 6.57), p =0.569, but Site 2 showed a positive associated with CPR, rr = 2.26 (95%CI: 0.99 to 5.18) p = 0.054. Both these estimates were adjusted for the other factors in the specific model.

The model also indicated that oocyte age (female age) is negatively associated with CPR. The results: rr = 0.98 (95%CI: 0.96 to 0.99), p = 0.021 indicate that the probability of having a clinical pregnancy decreases by a relative 2% with every added year in oocyte age. The association presented linear over age and non-linear effects are not significant, these tests are not included in the results.

BMI showed no association with CPR (p = 0.40). The same effect was observed for both Sites indicating no association.

Tubal factor infertility was also not associated with CPR (p = 0.796). No association was observed with Site and therefore this estimate of the risk ratio is a stable one across both Sites.

Table 23: Extended combined binomial regression model showing the association of Site with each of the determining factors.

	Risk Ratio (rr)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
BMI	1.01	0.01	0.84	0.40	0.98	1.03
Endometriosis	0.87	0.20	- 0.57	0.56	0.56	1.37
Site & Endometriosis	2.57	1.23	1,98	0.04	1.00	6.57
Tubal Factor	0.94	0.20	- 0.26	0.79	0.62	1.43
Oocyte Age	0.97	0.00	-2.31	0.02	0.95	0.99
Site	0.35	0.07	- 4.74	0.00	0.23	0.54

d) Binomial Regression Models (with Odds Ratio to Quantify Risk)

The previous relative risk models did not converge and therefore a binomial regression model with **odds ratios** was used to quantify the risk.

Previous analysis showed no interaction with regard to Site, therefore the data of both Sites was pooled to provide a more accurate analysis of the risk factors.

i) Binomial Regression Model with Odds Ratios with all Interactions

In these models the number of MII oocytes aspirated was included since they were very different for the two Sites (See Tables 10 and 14 on page 55 and 63, respectively).

The first model used in this analysis included all the interactions. The reason for this was to test if any association with CPR is different between the two different Sites. The results of this model, presented in Table 24, indicated that there were no factors that had any interaction with Site.

Table 24: Binomial regression model with odds ratios with all interactions.

	Odds Ratio (or)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
Site & BMI	0.97	0.04	- 0.53	0.59	0.89	1.06
Site & Endometriosis	3.29	2.39	1.64	0.10	0.79	13.69
Site & Tubal Factor	2.20	1.49	1.16	0.24	0.58	8.31
Site & Oocyte Age	0.96	0.05	- 0.62	0.53	0.86	1.07
Site & Number of MII Oocytes	0.94	0.05	- 0.92	0.35	0.83	1.06

ii) Binomial Regression Model with Odds Ratios with only certain Interactions

Certain factors were then excluded from the odds ratio model to determine whether the result would differ from the above mentioned but, again, the results showed that none of the factors

had a significant interaction with Site (Table 25). The factors excluded were; BMI and number of MII oocytes.

Table 25: Binomial regression model with odds ratios with only certain interactions.

	Odds Ratio (or)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
Site & Endometriosis	3.31	2.41	1.65	0.10	0.79	13.79
Site & Tubal Factor	2.19	1.48	1.16	0.24	0.58	8.82
Site & Oocyte Age	0.96	0.05	- 0.57	0.56	0.87	1.07

iii) Binomial Regression Model with Odds Ratios with Diagnostic Factors Interactions.

Only the diagnostic factors, BMI and tubal factor infertility, were included in this model to determine whether the result would differ from the above mentioned models. The results showed neither, BMI nor tubal factor infertility had a significant interaction with Site (Table 26).

Table 26: Binomial regression model with odds ratios with diagnostic factors interactions.

	Odds Ratio (or)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
Site & Endometriosis	3.16	2.28	1.60	0.11	0.76	13.03
Site & Tubal Factor	2.24	1.51	1.19	0.23	0.59	8.41

e) Final Binomial Regression Model with Odds Ratios with all Interactions omitted (since they were not significant)

Results are presented in Table 27. The model used in this analyses was the main effects model, where an adjustment was made for Site only. Pooled data was used to ensure an

increased stability of the estimates. The effect of Site, adjusts for the level of response in CPR, but does not adjust for the type of association.

In this model, only Site (p = 0.001) and number of MII oocytes (p = 0.001) available are significant factors associated with CPR.

Site 2 had a significantly lower CPR, or = 0.39 (95%CI: 0.22 to 0.66), p = 0.001, but the risk profile with respect to other factors was the same as for Site 1. The number of MII oocytes available is significantly associated with CPR, or = 1.08 (95%CI: 1.03 to 1.12) p = 0.001. This outcome (number of MII oocytes) is true for both Sites. The number of MII oocytes was used as a continuous variable and CPR increases by 8% for every additional mature oocyte available.

Through the adjustment of the number of MII oocytes in the model, the interaction of endometriosis and Site ceases to exist. None of the other factors were significant except Site. Increasing the availability of the number of MII oocytes at Site 2 will offset the risk profile of the Site.

Table 27: Final binomial regression model with odds ratios with all interactions omitted.

	Odds Ratio (or)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
BMI	1.03	0.02	1.43	0.15	0.98	1.07
Endometriosis	1.22	0.40	0.60	0.54	0.64	2.32
Tubal Factor	0.95	0.31	- 0.15	0.87	0.49	1.82
Oocyte Age	0.98	0.02	- 0.50	0.61	0.94	1.03
Site	0.38	0.10	- 3.46	0.001	0.22	0.66
Number MII Oocytes	1.07	0.02	3.45	0.001	1.03	1.12

To reflect the association between CPR and number of MII oocytes, a Lowess smooth graph shows clearly that the linear association between the number of MII oocytes and CPR is visible at both Sites. (Figure 29). At Site 2 the CPR is lower compared to Site 1 but a positive association is still visible. Thus, if the difference due to Site is taken into account, the association between the number of MII oocytes and CPR is the same across both Sites.

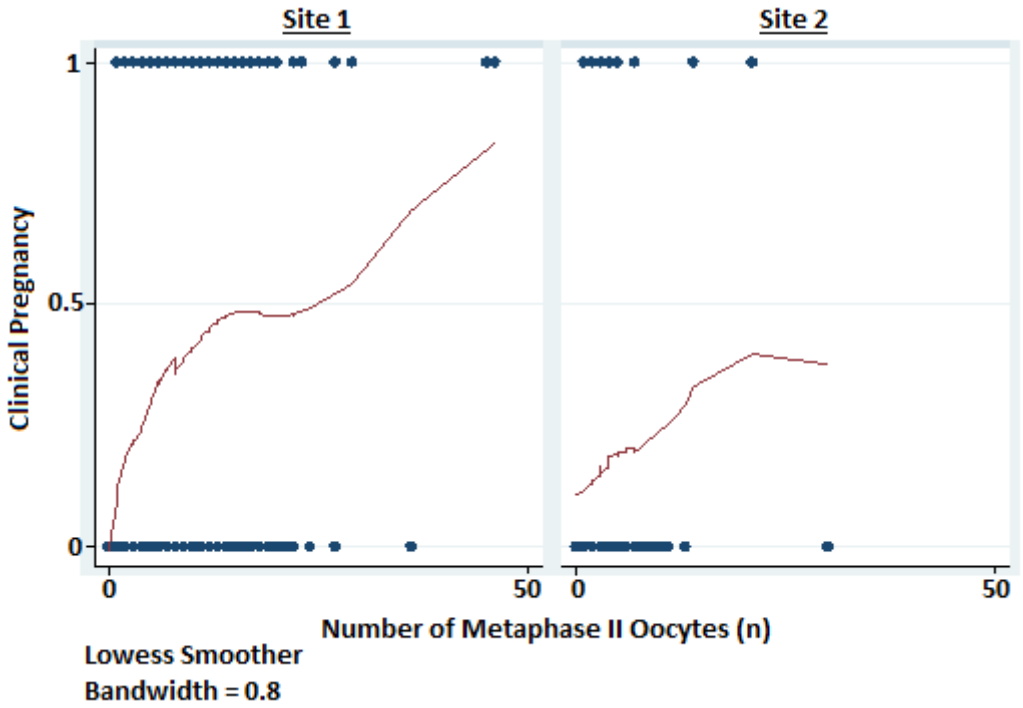


Figure 29: Lowess smooth graph showing the association between CPR and the number of MII Oocytes for each Site separately.

4.2.3.3 Binomial Regression Models with for Site 1 & Site 2 Separately

The results of the Site-specific models confirm the results obtained from the pooled analysis but due to the increase in the number of cycles when pooled, the precision and accuracy of the result are improved.

a) Site 1

The results from a Binomial Regression risk ratio model (determining which of the factors: BMI, endometriosis, tubal factor infertility and oocyte age are associated with CPR at Site 1), are presented in Table 28. Results indicated that oocyte age (female age) was the only factor that had a statistically significant negative association with CPR, rr = 0.97 (95%CI: 0.95 to 0.99), p = 0.03.

Table 28: Binomial regression risk ratio model for Site 1.

	Risk Ratio (rr)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
BMI	1.01	0.01	0.78	0.43	0.98	1.03
Endometriosis	0.86	0.19	- 0.62	0.53	0.55	1.35
Tubal Factor	0.82	0.21	- 0.72	0.47	0.49	1.38
Oocyte Age	0.97	0.01	- 2.13	0.03	0.95	0.99

Table 29 represents a binomial regression model with odds ratios used to quantify the risk for Site 1. Results indicated that when the number of MII oocytes are added to the model, the association between CPR and oocyte age is no longer statistically significant (p = 0.91) and only the number of MII oocytes has a significant association (positive) with CPR, rr = 1.08 (95%CI: 1.03 to 1.14), p = 0.001.

Table 29: Binomial regression odds ratio model with number of MII oocytes available added to the model for Site 1.

	Odds Ratio (or)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
BMI	1.04	0.02	1.59	0.11	0.99	1.09
Endometriosis	0.915	0.35	- 0.23	0.81	0.43	1.94
Tubal Factor	0.67	0.31	- 0.84	0.39	0.26	1.69
Oocyte Age	0.99	0.02	- 0.11	0.91	0.95	1.04
Number MII Oocytes	1.08	0.02	3.45	0.001	1.03	1.14

b) Site 2

Table 30 provides an overview of a binomial risk ratio regression model, determining which of the factors: BMI, endometriosis, tubal factor infertility and oocyte age are associated with CPR

at Site 2. The results of the model indicated that endometriosis was the only factor that had a statistically significant positive association with CPR, rr = 2.59 (95%CI: 1.08 to 6.25), p = 0.03.

Table 30: Binomial regression risk ratio model for Site 2.

	Risk Ratio (rr)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
BMI	1.01	0.03	0.33	0.74	0.95	1.07
Endometriosis	2.59	1.16	2.13	0.03	1.08	6.25
Tubal Factor	1.36	0.55	0.76	0.44	0.61	3.03
Oocyte Age	0.96	0.03	- 0.82	0.41	0.89	1.05

Table 31 represents a binomial regression model with odds ratios used to quantify the risk at Site 2. The results indicated that when the number of MII oocytes are added to the model, the association between CPR and endometriosis is no longer statistically significant (p = 0.07). None of the factors indicated a statistically significant association with CPR.

Table 31: Binomial regression odds ratio model with number of MII oocytes available added to the model for Site 2.

	Odds Ratio (or)	Standard Error	z - value	P > |z|	[95% Confidence Interval]	
BMI	1.01	0.03	o.48	0.63	0.94	1.09
Endometriosis	3.01	1.85	1.79	0.07	0.89	10.10
Tubal Factor	1.47	0.72	0.80	0.42	0.56	3.84
Oocyte Age	0.96	0.04	- 0.74	0.46	0.87	1.06
Number MII Oocytes	1.02	0.05	0.47	0.63	0.91	1.15

As mentioned before - the number of MII oocytes available is such a dominant factor that all other risk factors are surpassed and by increasing the availability of the number of MII oocytes at Site 2, the offset of the risk profile of the Site will occur.

CHAPTER 5 - DISCUSSION AND CONCLUSION

Discussion

By definition, infertility is, "*a disease, defined by the failure to achieve a successful pregnancy after 12 months or more of appropriate, timed unprotected intercourse or therapeutic donor insemination*" (Practice Committee of the American Society of Reproductive Medicine, 2013). Infertility treatment, more specifically ART, is accepted widely and globally, and most countries have a number of facilities that can readily provide fertility care, although in developing countries this is not always the case (Ombelet, 2014). ART, in most developing countries, is completely unavailable, scarcely available, or so expensive, that the majority of infertile couples simply cannot afford it (Murage *et al.,* 2011). Multiple strategies should be considered in the process of making infertility treatment more accessible in developing countries. Some of these strategies include simplifying diagnostic procedures, ovarian stimulation protocols, assisted reproduction procedures and laboratory procedures and equipment to ultimately reduce the overall cost (Ombelet, 2007).

The success of ART treatment can be influenced by many factors. These factors include; the equipment, laboratory techniques, patient profile and selection, quality control, skill and experience of clinicians and embryologists and funding available.

Aim and Research Questions

The current study was done at two fertility clinics, one in the public and one in the private sector and aimed to answer two research questions:

a) *How does the **laboratory equipment**, specifically the two types of CO_2 incubators used at the private laboratory, affect embryo quality and development and ultimately the CPR?*
b) *Which **patient factors** (specifically female - diagnosis, number of oocytes, age, BMI and AMH) have a significant and independent impact on CPR at the public and private fertility clinics, respectively?*

Laboratory Equipment – *Comparison of Two CO_2 Incubators: A Retrospective Study*

The first research question was investigated through a retrospective analysis of data at a private fertility clinic during 2013 to 2014.

Exclusion Criteria for the Retrospective Study

To minimize the possible influence of confounding factors on the outcomes of the current retrospective study, a strict exclusion criteria was applied. The use of a strict exclusion criteria resulted in a significant number of cycles being excluded from the study, but increased the reliability of the outcomes as significant factors, which have already been shown to influence success rates, for example advanced maternal age (Dew *et al.,* 1998; Committee of American Society for Reproductive Medicine, 2006), were eliminated. The strict exclusion criteria also resulted in a skewed distribution of numbers between the two incubators. Various reasons contributed to the higher number of cycles cultured within the Forma Scientific CO_2 Incubator. Firstly, in 2013 and 2014 at the private clinic, there were more Forma Scientific CO_2 Incubators (n = 6) than MINC™ Benchtop Incubators (n = 2) in use. Secondly, the possibility existed that more cycles in the Forma could be accommodated compared to the MINC. Although these circumstances were not ideal for the purposes of the retrospective study, statistical analysis indicated that the results were of significant value.

Importance of the CO_2 Incubator

Not only does a CO_2 incubator contribute to the high cost of the overall ART service, but it is also the most important piece of equipment to be considered when it comes to the effective culturing of gametes and/or embryos (Higdon *et al.,* 2008). Optimal culture conditions are essential for successful embryo development, implantation and ultimately, pregnancy outcome (Meseguer *et al.,* 2012) and the choice and use of specific incubators can be directly proportional to the pregnancy outcome of a patient cycle. Multiple studies to date have recommended benchtop incubators, as these incubators have shown increased recovery rates of temperature and gas, which is beneficial for optimal embryo development (Cooke *et al.,* 2004; Fujiwara *et al.,* 2007; Hill *et al.,* 2013). However, no study has been able to provide a distinctive answer to indicate which incubator has superior outcomes when comparing embryo development and ultimately, clinical pregnancy (Swain, 2014). Cooke *et al.,* (2004) conducted a study comparing a K – MINC™ 1000 incubator and a Forma 3110 incubator. The results indicated that there was a significant difference in the percentage change at opening in the MINC™ compared to the Forma (temperature: 1.3% vs. 31%; humidity: 43% vs. 44%; CO_2: 33% vs. 58%; $p < 0.01$). The recovery time to 90% also showed a significant difference

in the MINC™ compared to the Forma (temperature: 1 min. vs. 180 min.; humidity: 12 min. vs. 180 min.; CO_2: 8 min. vs. 120 min.; $p < 0.01$). Similarly, Hill *et al.* (2013) concluded from a retrospective data analysis a higher internal environment recovery rate with Planer BT-37 benchtop incubators compared to ThermoForma® large incubators. The results of the previously mentioned studies that found superior outcomes with the use of a benchtop culture incubator, contributed to our expectation and hypothesis that the MINC™ benchtop incubator would yield better quality embryos and result in a higher CPR compared to the conventional large Forma CO_2 incubator.

Outcomes of the Current Study

The results of the current study indicated clearly that the patient profiles in the two incubators, the Forma Scientific CO_2 Incubator (Forma – 20% O_2) and the MINC™ Benchtop Incubator (MINC – 5% O_2) were not significantly different. Factors such as female diagnosis, male diagnosis, age of ova (female age), number of ova aspirated per cycle and percentage normal sperm morphology showed no statistically significant difference between the two incubators and therefore indicated that patients were very well paired in the two incubator types. The above-mentioned factors can also have an effect on the outcomes (fertilization, embryo quality and pregnancy), but could be excluded since they were not significantly different in the two incubator types.

The outcomes of interest for this study were; fertilization, embryo quality and clinical pregnancy. The results of the current retrospective analysis indicated that the two incubators investigated, the Forma and the MINC, had very similar outcomes in terms of fertilization rate, embryo quality and ultimately clinical pregnancy rate. Outcomes were almost identical or slightly better in the MINC compared to the Forma.

Incubators and Fertilization Rate

The results of the current study showed a non-significant difference in fertilization rate of oocytes in the two incubators (Forma; 79.41% vs MINC: 79.06%).

Several studies have reported similar results to the current study when investigating the effect of different CO_2 incubators on fertilization rate. Steinkampf *et al.* (2014) reported no significant difference in fertilization rate when comparing a Forma Model 3110 (using 6% CO_2 and 20% O_2) and a Planer Model BT37GP benchtop incubator (with premixed 6% CO_2, 5% O_2 and 89% N_2), 68% vs. 67%. Kovačič *et al. (*2008) and Sobrinho *et al.* (2011) also concluded that the fertilization rate was not significantly different between oocytes cultured in low oxygen (5%)

compared to atmospheric oxygen (20%). A recent study by Nastri *et al.* (2016) reported the results of a systematic review and met-analysis including 21 published studies. The aim was to compare low oxygen to atmospheric oxygen tension for embryo culture. The results showed low quality evidence that there was no difference in the fertilization rate between the two groups (rr = 1.0; 95% CI 1.0 – 1.0).

Higdon *et al.* (2008) reported interesting results from an extensive study where the authors determined the effect of incubator management on ART outcome. When looking specifically at the IVF fertilization rate, the results indicated that dual gas incubators (5% O_2; 95% in air - 20% O_2) had an increased fertilization rate compared to triple gas incubators (7% CO_2; 5% O_2 and 88% N_2), IVF: 68% vs 62%; p = 0.05. For ICSI it was not significant: 74% vs 72%; p = 0.4]. In contrast, Lee *et al.* (2010) also found a significant increase in fertilization rate in the K-MINC 1000 incubator compared to the Forma 3110 incubator (72% vs 67%; $p < 0.05$).

It seems therefore that at the level of fertilization, incubator type and O_2 concentration do not play a conclusive, significant role. The outcome of the current study did not support the stated hypothesis.

Incubators and Embryo Quality

The statistical evaluation of embryo quality (Veeck & Zaninovic, 2013) - determined on embryo culture days 2, 3 and 5 – indicated that there was no significant difference in the average number of good quality embryos cultured in the two incubators. After re-modelling and analysing data as percentage of good quality embryos per number of oocytes aspirated, day 3 GQE became significant. After being adjusted for the age of the oocytes (female age), use of donor oocytes and the number of MII oocytes available, it was shown that the MINC had a 15% (relative) higher probability of having good quality embryos on day 3 compared to the Forma.

Few studies consider individual fertilization results for specific culture days when determining the effect of different incubators on embryo quality. The most frequent outcomes measured are fertilization rate, cleavage rate (culture day 2 – 4) and blastulation rate (culture day ≥ 5), but the results of the current study agree with several published reports.

Several studies have reported no significant difference in embryo quality on various culturing days when comparing bench-top incubators and standard large-box incubators. Paternot *et al.* (2013), showed no overall impact on embryo quality after culturing the embryos in a bench-top incubator or at lower O_2 concentration. Similarly, a study by Steinkampf *et al.* (2014) also reported no significant difference in the number of good quality embryos on culture day 3 when

comparing a Forma Model 3110 (using 6% CO_2/ 20% O_2) and a Planer model BT37GP benchtop incubator (with premixed 6% CO_2, 5% O_2 and 89% N_2). Lee *et al.* (2010) also found no significant difference with regard to embryo quality on culture day when comparing the K-MINC 1000 incubator to the Forma 3110 incubator (72% vs 67%; $p > 0.05$). Podsiadly *et al.* (2016) similarly conducted a study comparing the Genea Embryo Review Incubator (GERI) time-lapse monitoring system to a standard bench top incubator and found that both systems provided similar outcomes in terms of embryo quality.

Studies reporting significant differences in embryo quality with different incubators include that of Kovačič *et al. (*2008, 2010) and Guarneri *et al.* (2014). The 2008 study by Kovačič *et a.,* focused on the specific effect of oxygen concentration on various culture days and found that reduced oxygen, 5% versus 20%, resulted in a significantly higher proportion of good quality embryos, specifically on culture day 3 (whereas the other culture days showed no significant difference, not including blastulation rate). Another study by Kovačič *et al.* (2010) indicated that low oxygen resulted in a higher proportion of good quality embryos on culture day 2. Guarneri *et al.* (2014) conducted a similar study where the results indicated that the combined use of atmospheric and controlled oxygen or an exclusive use of low oxygen tension can result in good quality embryos.

Our hypothesis was that embryo quality should be better in the MINC incubator where the internal environment, recovery period with regards to pH, temperature and gas concentrations was shown to be superior in previous reports (Cooke *et al.,* 2002; Fujiwara *et al.,* 2007; Lee *et al.,* 2010).

The outcome of a higher proportion of day 3 GQE in the MINC compared to the Forma, in the current study, is therefore in agreement with previous published results (Fujiwara *et al.,* 2007). It must however be noted that this was only true after being adjusted for the age of the oocytes (female age), use of donor oocytes and the number of MII oocytes available. It was however unexpected that day 5 embryo quality did not show a significant difference between the two incubators since the O_2 concentration is thought to be most important at the blastocyst stage (Waldenstrom *et al.,* 2009). Blastocyst evaluation is however very subjective and since five different embryologists are involved in embryo grading at the study laboratory, subjective evaluation could have had an effect on the outcome. In general therefore, the study outcome for embryo quality did not support the hypothesis stated except for day 3 embryo quality.

Incubators and Clinical Pregnancy

For the purpose of this study, a clinical pregnancy is defined as the visibility of *any product of conception* seven weeks after an embryo transfer (World Health Organization, (2016). CPR is therefore expressed as foetal sac/embryo transfer at seven weeks gestation for this study.

The results of the current study indicated no statistical significant difference in CPR between the MINC and the Forma patients (47.17% and 45.43%; respectively).

The information available from published studies with regard to pregnancy outcome of embryos cultured in different incubators and different O_2 concentrations is diverse. Nanassy *et al.* (2010) indicated through retrospective analyses of two culture conditions (5% vs 20% O_2) that there was no statistical significant difference in CPR between the two culture systems (58.56% vs 64.36%). A study published by Swain (2014) also concluded that there is no statistical differences in terms of pregnancy outcome when comparing a conventional front-load incubator and a small top-load incubator.

A study conducted in Texas in 2009, indicated however that a reduction in O_2 concentration from 21% to 5%, when culturing embryos, resulted in an increased live birth rate (42.6% vs. 54.4%; 95% CI 1.9 – 27.0; p = 0.04) (Meintjies *et al.,* 2009). Another study that indicated similar results was a systematic review by Nastri *et al.* (2016). The authors reported that a lower oxygen concentration compared to standard atmospheric oxygen tension resulted in a ± 5% improvement in live birth, ongoing pregnancy and clinical pregnancy.

Introducing Time–lapse Incubators

In other recent studies, bench - top incubators equipped with time-lapse monitoring technology have been compared to incubators without the time-lapse technology (Meseguer *et al.,* 2012; Goldberg *et al.,* 2015; Meseguer, 2016; Podsiadly *et al.,* 2016). Embryo evaluation, using time – lapse images, have been promoted as being superior to the classical embryo morphology evaluation methods. The results obtained in these studies have also been contradictory, with some studies claiming increased embryo quality and pregnancy outcomes and others not. One study comparing a time-lapse monitoring system (EmbryoScope™) and a Heracell™ 150 large-box incubator found an increase in blastocyst formation rate and ongoing pregnancy rate, but none of these findings were of statistical significance (Cruz *et al.,* 2011). In 2012, a large retrospective study was done by Meseguer *et al.* comparing results of the time-lapse monitoring system with standard large-box incubators. The results showed that the relative probability of clinical pregnancy was significantly increased when embryos were cultured in the time-lapse monitoring system compared to standard large-box incubators.

Rubio *et al.* (2014) found similar results when comparing a standard large–box incubator with the EmbryoScope™ time-lapse incubator and reported a significant increase in ongoing pregnancy and implantation rates. Similarly, Goldberg *et al.* (2015) found an increase in blastocyst formation and clinical pregnancy with embryos cultured in the time-lapse monitoring system, EmbryoScope™, compared to the standard incubator (Forma) lapse monitoring system with a standard incubator. Most of these studies have been criticized since they actually used two different kinds of incubators and better outcomes could probably be attributed to the improved culture conditions in the time-lapse (bench-top) incubators.
Although the results of current study regarding CPR outcome did not show a significant increase in the MINC incubator group as was expected, and did therefore not support the hypothesis, it was similar in outcome to several of the other studies published.

Regardless of the results of previous studies comparing incubator ART outcomes, the process of selecting an incubator for ART culture purposes should focus on the availability and utilization of low O_2 capability (Swain, 2014). This opinion has been widely published and is now accepted as one of the most important conditions of an ART incubator. Other factors such as cost, space and practicality must also be taken into consideration when selecting an ART culture incubator as they too play an extremely important role (Hill *et al.,* 2013). Smaller incubators are more convenient and economical to use and deliver very good results, but they remain more expensive compared to the standard culture incubators (Hill *et al.,* 2013). Most of the smaller incubators can also accommodate a smaller number of patients compared to the larger incubators. However, small bench-top incubators have an excellent internal environment recovery period, especially with regard to temperature (Walker *et al.,* 2013), pH and gas concentrations (Swain *et al.,* 2016). This characteristic has been widely published (Lee *et al.* 2010) and can influence embryo development and pregnancy.

Limitations of the Study

One of the biggest limiting factors of this retrospective study was its retrospective nature and the uneven distribution of patients between the two incubators. Uneven patient distribution could not be avoided since the time period chosen was the only period when both incubator types were used simultaneously. A prospective study will be ideal, but large box incubators are not being used as frequently as in the past, and a concurrent comparison will be difficult.

Conclusive Remarks and Recommendations

In conclusion, although the outcomes investigated (FR, percentage GQE and CPR), for the studied patient cohort, were increased in the MINC incubator compared to the Forma, none

were statistically significant. As the only statistically significant difference between the two incubators under investigation observed was the average number of good quality embryos on day 3 ($p = 0.209$), the hypothesis of the study is therefore rejected as no statistical significant difference was observed in the overall outcome in terms of CPR.

However, since certain clinics still make use of Forma incubators for embryo culture, the outcome of the study gives some assurance that it can still be used effectively. Future studies could concentrate on incubator alternatives, especially in terms of cost analysis, for example the INVO-cell device, which could have major implications for the low-cost ART ventures.

Effect of Female Patient Profile on Art Success – A Prospective Study

The second research question was investigated by means of a prospective analysis of data at a private and public clinic during 2015 and 2016.

Introduction

The patient profile plays an extremely important role in the outcome of an ART cycle (Lintsen *et al.,* 2007). In the current prospective study, data from two fertility clinics (2015 - 2016), one private and one public, were analysed to identify possible independent factors specifically influencing CPR. The two clinics are relatively similar with regard to standard operating procedures such as semen preparation, oocyte aspiration, embryo culture/evaluation and embryo transfer. The same embryo culture media is used at both laboratories. The public laboratory however, offers an affordable ART service with a different ovarian stimulation protocol and only made use of a conventional large Forma CO_2 incubator during the study period. Since data capturing is done regularly at both laboratories, for quality control purposes, it was evident that patient profiles differ within the two clinics. The most notable differences were; the **average number of oocytes aspirated** (the private clinic's average aspirated number showed a two-fold increase compared to the public clinic), the **average number of GQE available** were higher at the private clinic, the **day of transfer** was predominantly day 3 at the public clinic and day 5 at the private clinic, **tubal factor diagnosis** was approximately four times more prevalent in the public clinic, the public clinic had three times more patients with a **high BMI** (≥ 30) and **donor cycles** (10 times more patients made use of an oocyte donor at the private clinic).

These pronounced differences were the foundation for the formulation of the research question as stated and the factors investigated with regard to their effect on CPR were; age of oocytes (female age), endometriosis, TFI, BMI, AMH and the number of MII oocytes available.

In the majority of the prospective study's statistical analysis, the data of the two clinics were pooled. Statistical analysis of pooled data presents more accurate results in terms of the effect of the factors investigated and their statistical significance on clinical pregnancy outcome, due to larger cycle numbers. The statistical analysis also revealed that the pooled results did not differ in terms of outcome compared to the results obtained from data that remained separate for the two clinics.

For the purpose of this study, a clinical pregnancy is defined as the visibility of *any product of conception* seven weeks after an embryo transfer (World Health Organization, 2016) and CPR is expressed as foetal heart/ET at seven weeks gestation.

Different statistical models were considered to analyse the vast amount of data and to test the hypothesis.

General and Final Statistical Outcome for the Private Clinic – Site 1

The statistical results obtained for the **private clinic** data using the first binomial regression analyses model where BMI, endometriosis, tubal factor infertility and female age were considered, showed that only female age played a significant role in CPR outcome. As suspected, younger patients had a higher CPR. In a second binomial regression analyses model, where the number of MII oocytes available was also considered, the effect of female age disappeared and only the number of MII oocytes available was significant for CPR. The female diagnoses, endometriosis and TFI had no significant influence on CPR. AMH levels and BMI also showed no significant relation with CPR.

General and Final Statistical Outcome for the Public Clinic – Site 2

For the **public clinic,** various results were observed. In the first binomial regression analyses model where BMI, endometriosis, TFI and female age were considered, only endometriosis had a significant effect on CPR. Patients with endometriosis had an increased pregnancy rate. In the second binomial regression model where the number of MII oocytes was also taken into consideration, the significance of endometriosis disappeared and no other factor, including number of oocytes, had a significant effect on CPR.

Oocyte Age and CPR

Background

Oocyte age (female age) at the time of an ART cycle, whether donor or own ova, plays a pivotal role in the overall pregnancy outcome (Wang *et al.,* 2008). Oocyte quality depends on multiple biological processes and many of these processes can only be assessed at molecular level, but the fact remains that female age is primarily related to the biological capability to reproduce (Gioacchini *et al.,* 2013). Various articles have been published indicating that women in their late thirties show a significant reduction in fecundity and an increased probability of infertility (Steiner & Jukic, 2016). The decline in fertility as a female patient's age

increases is primarily the result of an age-related decrease in oocyte quality rather than changes in endometrial receptivity (Bentov & Casper, 2013).

Statistical Results and Outcomes of Models Tested

The prospective descriptive results of the current study indicated that the average oocyte age in both clinics was similar and that an increasing oocyte age results in a decrease in CPR, similar to what has been published widely in the literature. In both clinics the CPR of women > 35 years were 10% less than those ≤ 35 years. An extended combined binomial regression model (risk ratio) of the pooled data indicated a negative association between oocyte age and CPR and showed that the probability of having a clinical pregnancy decreased by 2% of every year added to oocyte age.

When the two clinics' data were separated and the first binomial regression model was used to analyse the data, only the private clinic had a statistically significant negative association between oocyte age and CPR. For the public clinic, the association between oocyte age and CPR was not significant. However, when number of MII oocytes was added in the second binomial regression model (with odds ratio to quantify risk), oocyte age had no significant association with CPR in the pooled data as well as for the two clinics separately. In a third binomial regression model (with odds ratio to quantify risk) and where all interactions with the site were omitted, oocyte age had no significant association with CPR for pooled data and for the two clinics separately.

It is therefore clear that oocyte age has an effect on CPR, but when other confounding factors are also considered in the regression models, its significant association ceases to exist.

Comparison of Results with the Relevant Literature

The results obtained from the private clinic correlates with various published studies. A recent study by González-Foruria *et al.* (2016) indicated that among three female age groups analysed (≤ 35 years, 36 – 39 years and ≥ 40 years), pregnancy rates per cycle decreased significantly in the older patient cycles (11.4% vs. 11.6% vs. 5.9%). Gleicher *et al.* (2014) stated that the live birth rate for women aged 44 and 45 years (using their own oocytes) was 1.4% and 2.7%, respectively.

A possible reason for the difference observed in the two clinics in the current study, in terms of the effect of oocyte age on CPR, could be the availability and use of donors. The high success rate of donor oocyte ART cycles, specifically for recipients with advanced maternal

age, indicate that this factor influences the overall pregnancy rates significantly. The public clinic rarely makes use of donor cycles, due to the high cost for patients. Therefore, in the public clinic, older patients use their own oocytes (with 42 years being the maximum age to start an ART cycle at the public clinic) and oocyte age distribution was less varied compared to that of the private clinic. In the private clinic, a larger proportion of patients use donor oocytes from very young donors. This occurrence could therefore also contribute to the lower average oocyte age observed at the private clinic, even though the maximum patient (recipient) age at the private clinic was 54 years compared to 45 years at the public clinic.

Conclusion/Remarks

From the results of this study it can be concluded that age of ova (female age) is a predominant factor that influences CPR. When however, the number of MII oocytes is included in the analyses model, the outcome changes, and the number of MII oocytes remains as the only significant factor influencing CPR when all other factors are considered – and this was only observed in the private clinic. The number of mature ova aspirated therefore has a more significant effect on CPR than age of ova.

For the public clinic, neither oocyte age nor number of MII oocytes was significantly associated with CPR when all the other factors were also considered. This result is difficult to explain, but could be attributed to the relative low numbers of oocytes obtained in the program as well as the higher incidence of elevated BMI and tubal factor infertility.

Since oocyte age plays a significant role in CPR, the statistical models were adjusted for oocyte age when determining the association between the factors such as BMI, AMH, endometriosis and tubal factor infertility and CPR.

Endometriosis and CPR

Background

An estimated of 5 to 10% of the female population is affected by **endometriosis**, with a higher prevalence of ± 20 to 30% among women with infertility (Giudice, 2010; Prescott *et al.,* 2016). Various articles and studies have been published identifying endometriosis with infertility but no direct relationship has been proven.

Statistical Results and Outcomes of Models Tested

The descriptive results from the prospective data obtained at the private clinic indicated that 11% of patients presented with endometriosis. The public clinic showed a slightly lower prevalence of 9%.

A descriptive pooled analysis of both clinics indicated no significant association between endometriosis and CPR. Analysis of data separated for each clinic indicated the same results. An interesting observation was the difference in CPR with patients not presenting with endometriosis between the two clinics. In the private clinic a CPR of 36.65% was reported and the public clinic a CPR of 14.20% for patients without endometriosis. This observation could indicate the difference in the patient population between the two clinics or it could just be due to the large difference in the number of patients observed in this specific category.

Analyses with first binomial regression model and a second combined binomial regression model, with pooled data, confirmed no association between endometriosis and CPR. A third and extended combined binomial regression model showed that the association between endometriosis and CPR were significantly different between the two clinics. The private clinic data showed no association between endometriosis and CPR and this result remained the same in the model with the addition of the number of MII oocytes. The public clinic data analyses however, indicated a significant positive association between endometriosis and CPR, but when the number of MII oocytes was included in the model, the association between endometriosis and CPR were no longer of statistical significance. This observation has not been documented in previous studies, therefore it is possible that the other factors considered in the model contributed to the unexpected outcome.

Comparison of Results with the Relevant Literature

In 1998, a study by Bergendal *et al.* indicated that patients presenting with endometriosis had a significantly decreased fertilization rate but the pregnancy rates did not differ significantly from patients not presenting with endometriosis. Other studies have confirmed this observation with regard to the association between endometriosis and pregnancy rates (Matalliotakis *et al.,* 2007; Singh *et al.,* 2014).

The data obtained from this study, and also the findings from other studies confirm that the effect of endometriosis on pregnancy outcome is still unsure. A study needs to be conducted with a large number of patients and where the influence of other factors that can potentially influence the results, are also considered. The effect of endometriosis and tubal factor

infertility has been compared in a few studies (Omland *et al.,* 2001; Omland *et al.,* 2005; Mathieu d'Argent *et al.,* 2010). To determine however, the overall effect of endometriosis on infertility, a study needs to be conducted that matches patients with endometriosis (and all other areas of diagnosis) with patients that do not present with endometriosis. This type of study might provide a clearer association between pregnancy outcome and endometriosis.

Conclusion/Remarks

In the current study the diagnosis of endometriosis was not independently associated with CPR when all the other contributing factors were also considered. This was true for both clinics.

TFI and CPR

Background

Tubal pathology, contributing to 30 – 35% of infertility among women, has been identified as one of the most common causes of infertility (Kawwass *et al.,* 2013). **TFI** can range from moderate to severe, pre- and postoperatively. Since such a wide range of severity exists within this diagnosis, it is difficult to conclude a distinctive association between TFI and pregnancy outcomes. A study by Zou *et al.* (2014) demonstrated that the pregnancy prognosis differed among the different classifications of TFI. The results of one classification system within this study indicated that patients with extreme and severe TFI had a 0% pregnancy rate compared to 42.9% pregnancy rate in patients with moderate TFI. These findings emphasize the difficulty in drawing inferences between TFI and pregnancy outcome. Various factors contribute to the TFI diagnosis and although patients may have the same diagnosis, the magnitude thereof may differ, thus resulting in different outcomes.

Another important factor contributing to the prognosis of a patient with a TFI diagnosis is whether the hydrosalpinx has been removed or not. Numerous studies have shown that the presence of a hydrosalpinx is associated with an impaired ART outcome (Strandell & Lindhard, 2002; Dreyer *et al.,* 2016). A recent study by Dreyer *et al.* (2016) indicated a statistically significant increased ongoing pregnancy rate after hysteroscopic proximal occlusion removal compared to laparoscopic salpingectomy. This type of study provides more detailed information on the effect of treatment for hydrosalpinges on pregnancy outcome, but more in-depth studies need to be done determining overall TFI to determine its association with CPR. Future studies should aim at dividing patients presenting with TFI according to the severity thereof and further subdivided according to whether or not they have had any form of treatment

for hydrosalpinges, including; drainage, neosalpingostomy, salpingectomy or proximal tubal occlusion (Dun & Nezhat, 2012).

Statistical Results and Outcomes of Models Tested

The patient profile regarding TFI was very different for the public compared to the private clinic, 29% and 8% of the total number of patients, respectively. In the current study, the patient group with TFI always had a decreased CPR compared to the non-TFI group (for the pooled data as well the separate clinic data) but TFI was not associated with CPR in any of the models tested.

Comparison of Results with the Relevant Literature

TFI is often very prevalent in public clinic settings – in a Brazilian study published by Pantoja & Fernandez (2015), TFI was present in 73.3% of patients. In the current study, the number was much less (29%) but still significantly higher than that of the private clinic (8%).

The pregnancy outcomes of patients presenting with TFI is difficult to assess since it was shown by Zou *et al.* (2014) that outcomes differ in the mild, moderate and severe groups, with pregnancies ranging from 44% - 19% The presence of a hydrosalpinx also influences outcomes (Strandell & Lindhard, 2002; Dreyer *et al.,* 2016) and removal thereof has a significant positive effect on CPR (Dreyer *et al.,* 2016). In most of the cases at the public clinic, the hydrosalpinx was removed, but in some cases, it was unclear. In the private clinic, as part of the clinical protocol, all hydrosalpinges are removed before any ART treatment is considered. The presence of a hydrosalpinx was not a specific component of the investigation and all tubal factor patients were included to determine whether an association existed with CPR. TFI was not associated with CPR in any of the models tested in the current study, not for the public or private clinic.

Conclusion/Remarks

This result was unexpected especially for the public clinic, where almost a third of patients present with TFI and where the CPR in the TFI group is decreased compared to the non-TFI group. The difference in CPR was however not very big (4%) indicating that other confounding factors (female age, number of oocytes) also played a role in CPR outcome. Since the study also did not differentiate between the severity of the tubal factor and whether a hydrosalpinx was present or not, this could explain the unexpected result. The hypothesis for TFI on CPR is therefore rejected.

AMH and CPR

Background

Although AMH values in female patients are of significant importance for the recruitment and selection of follicle development (Cui *et al.,* 2016), its function as a biochemical marker for predicting ovarian reserve has been highly debated over the past few years. A study by Tobler *et al.* (2015) demonstrated that 60% of the clinics (n = 796) that participated in the study, used AMH levels as a first line test for evaluating ovarian reserve. When comparing AMH and AFC as measures for ovarian reserve, it has been shown that both have a good predictive value but objectivity, convenience and potential standardization of AMH level throughout the menstrual cycle, increase the likelihood of AMH becoming the standardised test (La Marca *et al.,* 2007; Fleming *et al.,* 2015).

A recent study that conducted a multivariate analysis of over 500 autologous cycles indicated that cycles with extremely low AMH levels compared with matched age-related normal AMH cycles, showed a twofold decrease in LBR per cycle (Seifer *et al.,* 2016). Nelson *et al.* (2016) demonstrated through a validated prediction model that AMH, in combination with clinical characteristics, can serve as an accurate predictive tool indicating live birth likelihood. The same outcome was observed by Tal *et al.* (2014) indicating that increased AMH levels were associated with higher clinical pregnancy rates. Tal *et al.* (2015) later published another study that was contradictory to their previous study. This study indicated that AMH had a weak association with implantation and clinical pregnancy rates in ART. A recent study also showed that infertile patients had similar AMH levels compared to controls with no history of infertility (Hvidman et al., 2016).

Statistical Results and Outcomes of Models Tested

Although pooled and separate data for the two clinics indicated an increase in CPR with an increase in AMH value, the association was not statistically significant. AMH was one of the main factors to be investigated in the prospective part of the study, but this category had a significant number of missing data, at both clinics. Due to the small number of AMH values available from the total patient population at clinics, further statistical analysis on AMH and CPR was not done and was also not included in any of the statistical regression models.

Comparison of Results with the Relevant Literature

In a study by Seifer *et al.* (2015) it was reported that patients with a low AMH (≤ 0.16 ng/mL) had a twofold lower live birth rate (9.5%/cycle) and a fivefold greater cancellation rate when

compared to age matched patients with normal AMH cycles. Fiçicioğlu *et al.* (2006) however, reported no association between early follicular serum AMH and pregnancy success. In general the results of the current study agrees with these findings.

Conclusion/Remarks

AMH is not routinely done for all patients due to the fact that it is not yet considered a standardised test with regard to a fertility treatment work-up (Practice Committee of the American Society for Reproductive Medicine, 2015). AMH testing is also mainly done in older patients and less frequently done in younger patients. One should also consider that AMH testing is relatively expensive, limiting its routine use in the public sector and AFC as an alternative marker should be considered. Future studies should aim at determining the association between AMH levels and specific pregnancy outcomes, specifically miscarriage rate since a recent study indicated that low AMH levels and subsequently diminished ovarian reserve might result in recurrent miscarriage (Atasever *et al.,* 2016).

Lifestyle Behaviour and CPR

The effects of lifestyle behaviour on the success of ART treatment have been documented in many publications. In a prospective study by Domar *et al,* (2012), the researchers reported that infertility patients often continue with adverse lifestyle behaviour in spite of being advised against it. Various lifestyle factors have been linked to decreased probability of successful ART outcomes including; obesity (Lintsen *et al.,* 2005), smoking (Lintsen *et al.,* 2005; Dechanet *et al.,* 2011) and excessive alcohol consumption (Rossi *et al.,* 2011).

BMI and CPR

Background

Numerous studies have indicated the adverse effect of obesity (BMI ≥ 30kg/m^2) on the female reproductive system. These effects include; anovulation, endocrine disorders and decreased oocyte quality (Wang *et al.,* 2016), but have shown to be inconsistent, when determining to what extent BMI influences infertility. A possible additional mechanism explaining the association of CPR and BMI could be the reduction in endometrial quality (Bosdou *et al.,* 2016; Rhee *et al.,* 2016). In contrast, being underweight may also reduce fertility through a shortened luteal phase, increased FSH levels and secondary amenorrhea (McKinnon *et al.,* 2016).

Comparison of Results with the Relevant Literature

Although the majority of literature indicates a significant effect of BMI on CPR, some studies indicated contradictory results and outcomes.

It was evident in the current study that CPR decreased with an increase in BMI. This was true for the pooled, as well as the separate clinic data, but the association of BMI with CPR was not statistically significant. Additional data including the effect of BMI in patients with and without endometriosis in the two separate clinics revealed that endometriosis reduced CPR but did not change the association between BMI and CPR. Studies showing no effect of BMI on CPR have been published and agree with the findings of the current study. A study by Matalliotakis *et al.* (2008) indicated no statistical significant association between BMI, CPR and overall ART outcome, although a lower stimulation response in women with an increased BMI was observed. Another study conducted in 2015 indicated similar results, stating that there was no evidence found that weight status influenced infertility treatment outcome (Schliep *et al.,* 2015). A large study by Provost *et al.* (2016) however, indicated that the success of ART cycles decreased, statistically significantly, with an increased BMI. Various other studies have found similar results (Law *et al.,* 2007; Luke *et al.,* 2011; Pinborg *et al.,* 2011; Comstock *et al.,* 2015). In a retrospective cohort study by Luke *et al.* (2009), reporting on 50172 cycles, it was found that obese women had a lower chance of pregnancy compared to females with a normal BMI.

Conclusion/Remarks

The non-significant result of the influence of BMI on CPR was unexpected especially for the public clinic where more than a third of patients were classified as obese. The CPR in the obese group was decreased and one would have expected a significant effect. The study hypothesis was however rejected for BMI and the unexpected result can possibly be explained by the fact that other determining factors contributed to the outcome.

The results from the current study indicated a marked difference in the distribution of patients with a BMI ≥ 30 kg/m² compared to patients with a BMI <30 kg/m² in the two clinics. In the private clinic, it was 10% vs. 90% respectively and for the public clinic 32% vs. 68%. Patients with a high BMI was therefore significantly more prevalent in the public clinic and patient demographics differed between clinics. It can be argued that private clinic patients are more affluent and have different eating habits and also have better access to organized exercise compared to the public clinic patient. There could also be ethnic differences although this was not specifically addressed in the study. This argument was underlined in an article by Bosdou

et al. (2016) showing that economic differences and sociocultural factors among different communities influence physical activity and dietary intake. The author recommended that future diagnosis, counselling and treatment of infertility should take region-specific risk factors into account.

The BMI of many of the patients was not documented, due to incomplete patient medical records. This was therefore a limitation of the study and could have had an effect on the analytical outcome. Clinics, private and public should be informed to include information on BMI in patient medical records to ensure that future studies can make use of the information. Another limitation for this part of the study was that the BMI recorded was that of the patients undergoing the treatment, and not that of the surrogate or ova donor – this could have had an effect on outcome, specifically in the private clinic where many donor cycles are done. In the public clinic, very few donor cycles are done and the limitation is therefore not applicable there. In future studies on BMI effect on CPR, information of the BMI of the donor should be recorded to get an accurate analyses outcome.

Ovarian Stimulation Protocols and CPR

The effect of ovarian stimulation on CPR was not one of the aims of the study and was not specifically investigated, but since it differed in the two clinics, it is important to include in the discussion.

Standard vs. Mild Ovarian Stimulation

The success of ART is critically dependent on optimizing ovarian stimulation protocols that endeavour to provide good quality oocytes and embryos (Kligman & Rosenwaks, 2001). Although the topic of mild ovarian stimulation compared to standard ovarian stimulation in terms of ART success has been highly debated, the practicality of the type of ovarian stimulation used depends largely on the socio-economic background of the clinic and its patients. The use of standard ovarian stimulation has been shown to be very successful with regard to ART outcome, but its high cost (Heng, 2007) can make it impractical in clinics where funds are limited.

A randomized controlled trial by Feliciani *et al.* (2009) showed that mild ovarian stimulation had similar ongoing pregnancy rates compared to long agonist ovarian stimulation. Another positive outcome of mild ovarian stimulation has been the possibility that the use thereof may increase the relative proportion of genetically normal oocytes and also the number of mature oocytes (Baart *et al.,* 2007; Gianaroli *et al.,* 2010; Blumenfeld, 2015), adding to the notion of

quality rather than quantity, but more studies are needed to support this claim. Standard, controlled ovarian stimulation has also been associated with a higher number of immature oocytes available from the total oocyte yield, but has not been associated with impaired pregnancy outcomes (Kok *et al.,* 2006). In 2007, Heijnen *et al.* conducted a randomized, non-inferiority effectiveness trial to determine whether mild IVF treatment had the same probability in resulting in a live birth pregnancy compared to standard IVF treatment. The results indicated that the cumulative live birth pregnancy rate for the mild treatment group was 43.4% compared to 44.7% for the standard treatment group. Standard treatment resulted in 1.3% more full term live births compared to mild treatment (95% CI: - 9.8%). These findings indicate mild IVF treatment can result in similar cumulative live birth outcome, compared to standard IVF treatment (Heijnen et al., 2007).

On the other hand, Gleicher *et al.* (2012) indicated that low intensity IVF reduced pregnancy success without demonstrating relative cost advantages. Supporting this, a study by Groen *et al.* (2013), demonstrated that modified natural cycle IVF was not cost effective since controlled ovarian hyperstimulation IVF resulted in a higher cumulative live birth rate. A meta-analysis study published locally by Matsaseng *et al.* (2013) also found significantly in favour for conventional stimulation compared to mild stimulation in terms of live birth/ET outcome. Although acceptable results have been demonstrated with regard to mild ovarian stimulation protocols (Ubaldi *et al.,* 2007; Datta 2016), the question remains whether the use of mild ovarian stimulation can be justified in terms of successful ART outcome?

Ovarian Stimulation and Oocyte Number and Quality

Follicular development and oocyte maturation are two closely related factors and controlled ovarian stimulation for ART affects the cells that are responsible for these two processes (Bosch *et al.,* 2016). Various studies have shown that ART success is, to a large extent, dependant on the maturity and quality of the oocytes and multiple studies link the type of controlled ovarian stimulation used in a cycle, directly to this outcome (Sunkara *et al.,* 2011; Ji *et al.,* 2013). It was also shown in a study by Heng (2007) that the mature oocyte yield is directly proportional to the stimulation protocol followed during an ART cycle.

Ovarian Stimulation, Oocyte Number and CPR

Results from a study published by Sunkara *et al.* (2011) showed a non-linear association between the number of oocytes and live birth with 15 oocytes giving the highest live birth rate. The results of the current study also indicated that the number of MII oocytes available had a significant effect on CPR - more MII oocytes resulted in an increased CPR. The significant

association between the number of MII oocytes and CPR was demonstrated in the final binomial regression model, using odds ratios and including interactions of all other determinant factors. Final analysis of the pooled data indicated that only the clinic and the number of MII oocytes available had a statistically significant association with CPR, taking all other factors; BMI, TFI, endometriosis and oocyte age also into account. The analysis showed that CPR increases by 8% for every additional MII oocyte available. Although the association between the number of MII oocytes available and CPR remained consistent, the association was not statistically significant for the public clinic. It is possible to speculate that this result is because of the relative small oocyte yield in the public clinic, which is an indication of the effect of the mild ovarian stimulation protocol followed. This was not the case in the private clinic. A statistically significant association between the number of MII oocytes available and CPR was found. The private clinic uses standard ovarian stimulation protocols, a higher mature oocyte yield is possible, and higher number of MII oocytes resulted in an increased CPR.

Stoop *et al.* (2012) made the interesting observation that the oocyte utilization rate between the ages of 23 to 37 years was more dependent on age than on ovarian response. For the ≥ 38 year old group ovarian reserve was more important. Lemmen *et al.* (2016) reported similar results. In a possible follow-up study, it might therefore be important to separate data into two age groups to determine a more accurate association between the number of MII oocytes and CPR.

Limitations of the Study

Although it was not the aim of this study to compare the outcomes in public and private ART clinics, it is noteworthy that due to lack of funds, the public facility does differ from the private one in certain aspects. These differences were not investigated, but could have contributed to the significant difference in CPR.

- The public ART laboratory is situated in the emergency obstetrics theatre complex of Tygerberg Hospital, and is not equipped with the suggested "*clean room*" specifications for ART laboratories. There is no HEPA air filter system and also no positive pressure applied in the laboratory. Inline HEPA/VOC filters are also not used. Therefore, air quality, shown to be an important factor in ART results (Heitman *et al.,* 2015) was not optimal during the study period.
- Ovarian stimulation is also different, as mentioned before.
- The oocyte aspiration procedure at the public clinic is performed with only local anaesthesia, and it's uncertain whether this could have an effect on overall ART outcome.

- For the duration of the study, a conventional large CO_2 Forma incubator was used at the public clinic. Although the results of the retrospective study showed no significant effect on CPR for the different types of incubators, its role in CPR cannot be ignored.
- A number of patients had to be excluded from the study – especially at the private clinic - since they did not receive consent forms.
- A significant number of data was missing from patient files – and made statistical analyses of certain factors extremely difficult.
- Due to the policy at the private clinic, students are not allowed to interact with patients directly – and the student conducting the research had to rely on the fertility sister, embryologists and clinicians for patient information.

All other factors were the same in both laboratories: experience and skill of the embryologists and clinicians, culture medium and culture procedures, embryo evaluation and embryo transfer method.

Conclusive Remarks

It is clear from the results of the current study that ART outcome is multifactorial and that identifying one or two independent factors determining CPR at a specific fertility clinic, is difficult to achieve.

It is also obvious from the data that the demographics and patient profiles in clinics differ and might have an influence on outcome. In the current study, it was especially true for TFI, BMI, oocyte donor availability, number of oocytes available and CPR. Social and cultural factors that exist within a social context, influence clinical practice by different healthcare services (Adisasmita *et al.,* 2008). An example of differences between private and public clinics is that in most cases public clinics have an age restriction with regard to the patients they accept for ART treatment and in most cases an extensive waiting period exists. Private clinics however, have more “freedom” in managing and treating their patients (Castilla, 2009). Although these factors may not be directly associated with the outcomes observed in this study, they do provide a better understanding thereof.

Similar trends - for the association of factors investigated with CPR - were found for the pooled data and the two clinics separately. Increased female age was associated with decreased CPR and so was TFI and increased BMI. A higher number of MII oocytes resulted in an increased CPR. The results of the final regression analysis of the current study indicated that for the private clinic, only the number of MII oocytes available, influenced CPR significantly –

more MII oocytes resulted in an increased CPR. In the public clinic, no significant association for any of the investigated factors was found.

One can therefore speculate that if the number of MII oocytes available could be increased at the public clinic, CPR might also increase. To achieve this, the stimulation protocol will most probably have to be adapted. The implication for such a strategy is of course, increased cost. In reality, all the factors (success and cost) have to be considered to the best benefit to the patient. Economic challenges to infertility treatment access was not a factor investigated in the current study, but the issue must be considered as it influences most of the factors that could have a relation to pregnancy outcome. Infertility has still not been widely accepted as a disease and therefore the treatment, including ovarian stimulation and laboratory output, is costly (Davis & Sokol, 2016).

The complexity of fertility treatments also contribute to their inaccessibility in many countries. Paulson *et al.* (2016) reviewed four methods to determine whether they would be effective in making infertility treatment access more available. The methods were; mild ovarian stimulation protocols, in vitro maturation, modified natural cycles and intravaginal culturing of embryos. Although unconventional, all these methods have demonstrated live birth outcomes and need further investigation (Davis & Sokol 2016; Paulson *et al*., 2016). Future studies should aim at exploring these methods, to provide a better understanding of the factors that influence pregnancy outcomes.

Recommendations

Finally, after reviewing the outcomes of the study, certain recommendations can be made:

For the Public Clinic:

- Acquisition of a benchtop CO_2 incubator with 5% O_2 is recommended.
    - Fortunately, funds became available recently and the public clinic started using a Miri® (ESCO Medical, South Africa) benchtop incubator since May 2016. The effect of this new incubator on outcomes will be monitored and possibly be part of a new study.
- Modified stimulation protocols should be considered to achieve a higher number of MII oocytes without compromising oocyte quality and an increase cycle cost.
    - Currently, a modified stimulation protocol (Segawa *et al.,* 2007; Okimura *et al.,* 2008) has been implemented since April 2016 aiming at improving both quality and number of oocytes aspirated and is part of future studies aiming to maximize success but minimize cost for public clinic patients.

- Implementation of at least inline filters for the CO_2 incubators should be encouraged.
    - The recently introduced Miri® incubator is equipped with filters and UV sterilization of the gas and should result in better results.

For the Private Clinic:

- For future research studies, a better coordination between the students and personnel should be implemented.
- Protocols and all possible limiting factors should be thoroughly discussed and agreed upon before the study starts.
- Patient medical records must be complete for all patients – Information such as BMI, for example, should be available for all patients and fertility sisters and doctors that mostly correspond with patients, should ensure that the patient provides all the information necessary for a complete medical record.

APPENDICES

Appendix I – Semen Preparation

(Adapted from the *WHO laboratory manual for the examination and processing of human semen*, 5th edition, 2010)

(Tygerberg Fertility Clinic SOP 5 and Aevitas Fertility Clinic SOP)

Swim-Up Method

- Mix semen sample well (ensure sample has liquefied)
- Dilute sample in a 1:2 ratio (semen: sperm washing medium [SAGE® Advantage HEPES buffered sperm preparation medium - SpermPrep®]) in a test tube
- Vortex sample, centrifuge at 450 x g for 10 minutes
- Remove supernatant, leave pellet undisturbed
- Resuspend pellet in 2 mL SpermPrep®
- Centrifuge at 450 g for 10 minutes
- After second centrifugation, remove supernatant and carefully overlay pellet with 0.5 mL SpermPrep®
- Leave to stand at 45° angle in an 37°C incubator for one hour
- Aspirate the top layer of the sample, leaving pellet undisturbed.

Density Gradient Centrifugation

- This technique makes use of 3 different density gradient stock solutions (stock solution used is SilSelect® FertiPro);
    - 90% : 0.90mL of stock solution plus 1.0mL SpermPrep®
    - 70% : 0.70mL of stock solution plus 0.3mL SpermPrep®
    - 45% : 0.45mL of stock solution plus 0.55mL SpermPrep®
- The solutions are carefully layered on top of each other, starting with the 90% solution at the bottom of a conical tube and allowed to equilibrate at 37°C
- Mix semen sample well (ensure sample has liquefied)
- Dilute sample in a 1:2 ratio (semen: sperm washing medium [SAGE® Advantage HEPES buffered sperm preparation medium - SpermPrep®]) in a test tube
- Centrifuge at 350 to 450 x g for 10 minutes
- Resuspend pellet in 1 mL SpermPrep® and carefully overlay on gradient
- Centrifuge sample at 35 x g between 15 – 18 minutes
- Remove top gradient layers, leaving only the pellet
- Resuspend pellet in 0.3 – 0.5 mL medium and aspirate into a clean conical tube

- The resuspended sperm sample is diluted with 2mL medium and centrifuged at 300 – 450 x g for 10 minutes
- The last step is repeated and the resultant pellet is resuspended in 0.3 – 0.5mL medium.

Appendix II – Standard Ovarian Stimulation Protocol

(*The Practice Committee of the American Society for Reproductive Medicine*, 2013)

(Aevitas Fertility Clinic SOP)

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.

Appendix III - Oocyte Retrieval Method – Public Clinic

(Matsaseng & Kruger, 2014)

Approximately, 15 to 30 minutes before an oocyte retrieval procedure is performed the patient is consciously sedated using only local Pethidene® (100mg) intramuscularly and a cervical block with 1% Lignocaine®. The clinician inserts a needle (guided by means of a transvaginal ultrasound) through the vaginal wall and into an ovarian follicle. The other end of the needle is attached to a suction device that expels follicular fluid into a laboratory tube.

Once the follicle is entered, suction is gently applied to aspirate follicular fluid and with it, hopefully, cellular material including the oocyte. The follicular fluid is handed to an embryologist in the IVF laboratory to identify and quantify the ova. Next, other follicles are aspirated. Once the ovarian follicles have been aspirated on one ovary, the needle is withdrawn, and the procedure repeated on the other ovary. In the mild stimulation programme more than 10 oocytes is seldom recovered. After completion, the needle is withdrawn, and haemostasis is achieved.

The procedure lasts between 5 to 10 minutes after which the patient recovers for approximately 30 minutes and once they are fully awake and in the company of a family member, they are discharged.

Appendix IV - Oocyte Retrieval Method – Private Clinic

(Aevitas Fertility Clinic SOP)

The oocyte retrieval procedure is performed under conscious sedation (Dormicum® IV) and using transvaginal ultrasound guidance. The clinician inserts a needle through the vaginal wall and into an ovarian follicle, taking care not to injure organs located between the vaginal wall and the ovary. The other end of the needle is attached to a suction device that expels follicular fluid.

Once the follicle is entered, suction is gently applied to aspirate follicular fluid and with they, hopefully, cellular material including the oocyte. The follicular fluid is handed to an embryologist in the IVF laboratory to identify and quantify the ova. Next, other follicles are aspirated. Once the ovarian follicles have been aspirated on one ovary, the needle is withdrawn, and the procedure repeated on the other ovary. It is not unusual to recover 20 oocytes as women are generally hyper-stimulated in advance of this procedure. After completion, the needle is withdrawn, and haemostasis is achieved.

The procedure usually approximately lasts 15 to 30 minutes.

Appendix V - In Vitro Fertilization

(Tygerberg Fertility Clinic SOP 9 and Aevitas Fertility Clinic SOP)

Make sure that all forms and documents are prepared
Check patient's file and record to eliminate all possible uncertainties/queries

Aspiration

Medium preparation – previous day
See section on medium preparation

Ovum Pick up

- Check suction pump (100-120 mmHg)
- Prepare glass polished pipettes for pick up
- Place pick-up tubes in heated block
- Hand theatre medium tube to sister when needed
- 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
- Determine maturity [see appendix at end of section]
    - GV, MI, MII
    - Spread complexes to visualize the first polar body (if possible)
    - Note maturity and all other features on the ova form
- Put the complexes (with as little as possible blood and medium) in the pick-up tubes (MI and MII separate)
- When finished, rinse all complexes in small Petri dish with gassed fertilization medium – check number obtained
- Transfer to 4 well NUNC dish – maturities separate and not more than 5 complexes per well
- Incubate in the CO_2 incubator until insemination
- Complete all forms

Insemination

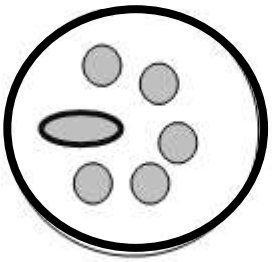
- Inseminate complexes with the correct number/volume of prepared sperm
    - Morphology ≤ 4 % - up to 2 x 10^6 sperm/ovum
    - Morphology 4, ≤ 14 % – 500 000 sperm/ovum
    - Morphology 14 % – 100 000 sperm/ovum
        - *(work out the correct volume)*

- Do insemination ± 40 hours post HCG administration if at all possible
- Incubate overnight at 37 ºC, 6% CO_2

Embryo evaluation

Dish preparation for embryo culture

- Prepare a culture dish with cleavage medium drops (Quinns – Cooper Surgical) for the following day
- Prepare culture drops under oil (Quinns – Cooper Surgical)
    - Work as fast as possible to prevent evaporation
    - Work on a cold surface
    - Make drops equal to the number of oocytes (but add one extra for rinsing)
    - Maximum number of drops is 10 – make 2 dishes if more than 10



For 6 embryos

Fertilization check

Day 1 (2 PN)

- Clean oocytes with denuding pipettes (Cook Australia)
    - If oocytes are not clearly visible – dislodge first with two sterile, hypodermic needles
- Rinse well in clean 4 well NUNC dish
- Check for pronuclei (PN) and polar body (PB) number on the inverted microscope with heated stage
    - Note any abnormal number of PN [> or < than 2], or any other anomalies
- Transfer to preincubated cleavage medium drops covered with mineral oil in a small Petri dish
- Incubate overnight at 37 ºC, 6% CO_2

Embryo quality/morphology check

Day 2 (2- 4 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation*
- Select embryos for transfer if a day 2 transfer
    - *See section on embryo transfer*

Day 3 (6-8 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation*
- Transfer embryos to preincubated blastocyst medium drops covered with mineral oil
- Select embryos for transfer if a day 3 transfer
    - *See section on embryo transfer*

Day 5/6 (Blastocyst transfer)

- Check for cell stage/blastocyst morphology on the inverted microscope with heated stage
    - *See section on blastocyst morphology evaluation*
- Transfer embryos/blastocysts to preincubated blastocyst medium drops covered with mineral oil
- Select embryos/blastocysts for transfer if a day 5 transfer
    - *See section on embryo transfer*

Cryopreservation

Select blastocysts for vitrification after the transfer – See Appendix XIII.

Appendix VI - Intra-cytoplasmic Sperm Injection

(Tygerberg Fertility Clinic SOP 10 and Aevitas Fertility Clinic SOP)

Make sure that all forms and documents are prepared
Check patient's file and record to eliminate all possible uncertainties/queries

Aspiration

Medium preparation – previous day

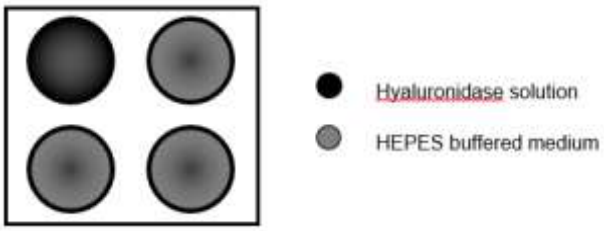
Ovum Pick up

- Check suction pump (100-120 mmHg)
- Prepare glass polished pipettes for pick up
- Place pick-up tubes in heated block
- Hand theatre medium tube to sister when needed
- 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 finished, 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

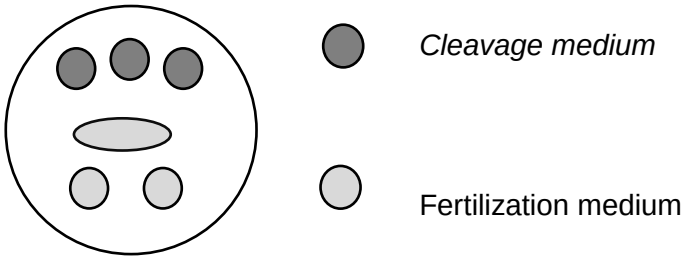
- Prepare pipettes for the process: fire polished glass Pasteur pipettes, hand drawn glass pipettes and the Cook stripper (Marcus Medical) pipette
- Prepare a 4 well NUNC dish for denuding: **[USE ONE DISH FOR EVERY 8 OOCYTES]**
    - In well 2, 3 and 4 place ± 0.8 – 0.9 ml warmed HEPES buffered flushing medium (Quinns, Cooper Surgical)
    - In well 1 place 0.6 ml HEPES buffered flushing medium and add 0.3 ml hyaluronidase [80 UI/ml] (Quinns, 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



- *Place the denuded oocytes into a pre-prepared holding or "rugby ball" dish, into the elliptical fertilization wash medium drop.*

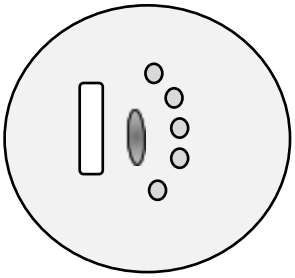
- *[this dish is prepared the previous day and cultured at 6% CO_2/37ºC – drops are covered with oil – Quinns - Cooper Surgical)*



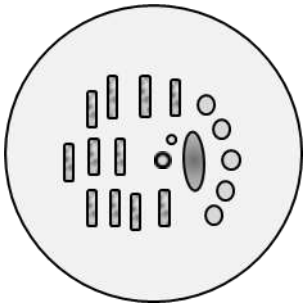
- Determine the maturity of the oocytes and transfer to the clean fertilization drops – all MII oocytes to the left drop and all MI and GV to the right drop
- Culture until injection
- Complete all forms
- Set up the inverted microscope for ICSI [heated stage, holding and injection pipettes, manipulators]

Injection

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



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



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

Sperm Immobilization

- Add sperm cells to the sperm preparation medium
- Select motile, morphological normal spermatozoa form the drop with the ICSI injection pipette and immobilize the sperm cell
    - Collect enough sperm cells for the injection procedure.

Sperm injection

- Place MII oocytes to be injected in the oocyte drops (2 per drop)
- Select an immobilized sperm cell and carefully inject oocyte
- Continue until all oocytes are injected
- Transfer back into the 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

EMBRYO EVALUATION IS EXACTLY AS FOR IVF

Embryo evaluation

Dish preparation for embryo culture

- Prepare a culture dish with cleavage medium drops (Quinns – Cooper Surgical) for the next day
- Make drops under oil (Quinns – Cooper Surgical)
    - Work as fast as possible to prevent evaporation
    - Work on a cold surface
    - Make drops equal to the number of oocytes (but add one extra for rinsing)
    - Maximum number of drops is 10 – make 2 dishes if more than 10

Fertilization check

Day 1 (2 PN)

- Check for pro nuclei (PN) and polar body (PB) number on the inverted microscope with heated stage
    - Note any abnormal number of PN [> or < than 2], or any other anomalies
- Transfer to preincubated cleavage medium drops covered with mineral oil in a small Petri dish
- Incubate overnight at 37 ºC, 6% CO_2

Embryo quality/morphology check

Day 2 (2- 4 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation*
- Select embryos for transfer if a day 2 transfer
    - *See section on embryo transfer*

Day 3 (6-8 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation*
- Transfer embryos to preincubated blastocyst medium drops covered with mineral oil
- Select embryos for transfer if a day 3 transfer
    - *See section on embryo transfer*

Day 5/6 (Blastocyst transfer)

- Check for cell stage/blastocyst morphology on the inverted microscope with heated stage
    - *See section on blastocyst morphology evaluation*
- Transfer embryos/blastocysts to preincubated blastocyst medium drops covered with mineral oil
- Select embryos/blastocysts for transfer if a day 5 transfer
    - *See section on embryo transfer*

Cryopreservation

Select blastocysts for vitrification after transfers – See Appendix XIII.

Appendix VII – Physiological Intra-Cytoplasmic Sperm Injection

(Adapted Origio PICSI® Protocol)

(Tygerberg Fertility Clinic SOP 11 and Aevitas Fertility Clinic SOP)

Preparation for use:

Hydrate the hyaluronan microdots by placing single 10-µL droplets of Human Tubal Fluid (HTF) containing at least 5 mg/mL serum protein, or other suitable sperm diluent, at the end of each locating line covering the area where the microdot is situated (Figure 1). Alternatively, the sperm suspension can be added directly to the dry microdot. Drops of polyvinylpyrrolidone (PVP) or other fluids useful for manipulating sperm may also be placed elsewhere on the dish at this time.

Carefully flood the dish with tissue culture oil to prepare it for use. Hydrating the microdot before applying the sperm gives the hyaluronan time to swell.

Swelling and sperm binding begin normally in 5 minutes or less. However 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.

Using the PICSI® Sperm Selection

Device:

- Add the sperm to the pre-hydrated microdot in a volume equal to or greater than that used to pre-hydrate the dot (approximately 10 µL).
- 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.

- 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 100,000hyaluronan-binding sperm per mL (approximately 1,000-2,000 total sperm in10-20 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 they are 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.
- Continue collecting until 20-50sperm are captured.
- Expel the captured sperm into a PVP drop to process them for ICSI (inactivating the tail, re-evaluating motility and morphology.)
- From the PVP droplet, select and load single, processed sperm for injection into the oocytes according to your standard injection protocol.

Temperature:

- Sperm bind best to hyaluronan hydrogel at temperatures below 30°C.
- At temperatures above30°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, 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. At 33°C or even at 37°C, many bound sperm will remain available for selection.

Instructions for Use

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 in to 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. 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 VIII – Intracytoplasmic Morphological Sperm Injection

(Tygerberg Fertility Clinic SOP 12 and Aevitas Fertility Clinic SOP)

Make sure that all forms and documents are prepared
Check patient's file and record to eliminate all possible uncertainties/queries

Aspiration

Medium preparation – previous day

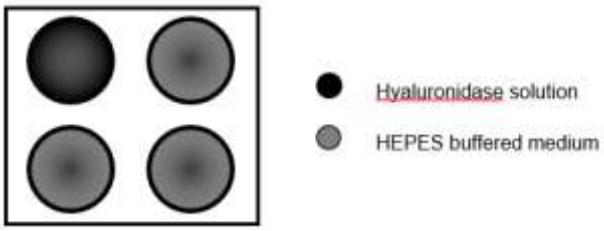
Ovum Pick up

- Check suction pump (100-120 mmHg)
- Prepare glass polished pipettes for pick up
- Place pick-up tubes in heated block
- Hand theatre medium tube to sister when needed
- 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 finished, 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

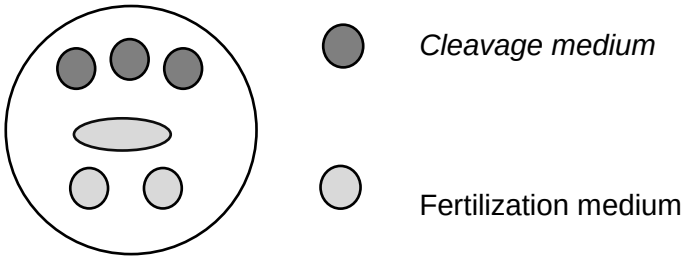
- Prepare pipettes for the process: fire polished glass Pasteur pipettes, hand drawn glass pipettes and the Cook stripper (Marcus Medical) pipette
- Prepare a 4 well NUNC dish for denuding: **[USE ONE DISH FOR EVERY 8 OOCYTES]**
    - In well 2, 3 and 4 place ± 0.8 – 0.9 ml warmed HEPES buffered flushing medium (Quinns, Cooper Surgical)
    - In well 1 place 0.6 ml HEPES buffered flushing medium and add 0.3 ml hyaluronidase [80 UI/ml] (Quinns, 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



- *Place the denuded oocytes into a pre-prepared holding or "rugby ball" dish, into the elliptical fertilization wash medium drop.*

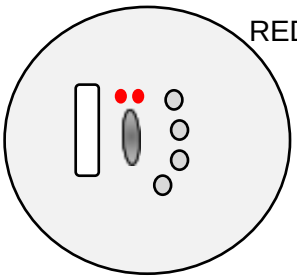
- *[this dish is prepared the previous day and cultured at 6% CO_2/37ºC – drops are covered with oil – Quinns- Cooper Surgical)*



- 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

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



RED DOTS ARE SMALL SPERM PREP DROPS

- 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 form the drop with the ICSI injection pipette using the 20x objective
- Place the selected sperm into the left SP drop and focus on the edge of the drop.
- Change the heated stage *to the setting where the dish overlaps with the opening on the stage,* the dish needs to come in contact with the objective.
- Put the 100x objective in place and *suspend* a small drop of oil onto the objective
- Place the glass bottom dish containing your sperm onto the oil covered objective. The left SP drop should be in the centre of the objective.
- Using the *more robust* magnification to focus on your SP edge. Bring your needle down and make an indentation in the edge. The sperm will swim into the indentation.
- Select the morphologically normal sperm without any vacuoles and move them to the SP drop to the right.
- After selecting enough sperm change your heated stage again and proceed with normal ICSI protocol.
- immobilize the sperm cell
    - Collect enough sperm cells for the injection procedure.

Sperm injection

- Place MII oocytes to be injected in the oocyte drops (2 per drop)
- Select an immobilized sperm cell and carefully inject oocyte
- Continue until all oocytes are injected

- Transfer back into the 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

EMBRYO EVALUATION IS EXACTLY AS FOR IVF

Embryo evaluation

Dish preparation for embryo culture

- Prepare a culture dish with cleavage medium drops (Quinns – Cooper Surgical) for the next day
- Make drops under oil (Quinns – Cooper Surgical)
    - Work as fast as possible to prevent evaporation
    - Work on a cold surface
    - Make drops equal to the number of oocytes (but add one extra for rinsing)
    - Maximum number of drops is 10 – make 2 dishes if more than 10

Fertilization check

Day 1 (2 PN)

- Check for pro nuclei (PN) and polar body (PB) number on the inverted microscope with heated stage
    - Note any abnormal number of PN [> or < than 2], or any other anomalies
- Transfer to preincubated cleavage medium drops covered with mineral oil in a small Petri dish
- Incubate overnight at 37 ºC, 6% CO_2

Embryo quality/morphology check

Day 2 (2- 4 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation*
- Select embryos for transfer if a day 2 transfer
    - *See section on embryo transfer*

Day 3 (6-8 cell)

- Check for cell stage and embryo morphology on the inverted microscope with heated stage
    - *See section on embryo morphology evaluation*
- Transfer embryos to preincubated blastocyst medium drops covered with mineral oil
- Select embryos for transfer if a day 3 transfer
    - *See section on embryo transfer*

Day 5/6 (Blastocyst transfer)

- Check for cell stage/blastocyst morphology on the inverted microscope with heated stage
    - *See section on blastocyst morphology evaluation*
- Transfer embryos/blastocysts to preincubated blastocyst medium drops covered with mineral oil
- Select embryos/blastocysts for transfer if a day 5 transfer
    - *See section on embryo transfer*

Cryopreservation

Select blastocysts for vitrification after transfers – See Appendix XIII.

Appendix IX - Embryo Grading Day 2 and Day 3

(Modified from *Veeck*, 2003)

(Tygerberg Fertility Clinic SOP 17 and Aevitas Fertility Clinic SOP)

Grade 1: Embryo with few blastomeres of any size, severe or complete fragmentation.

Grade 2: Embryo with blastomeres of equal and unequal size, significant cytoplasmic fragmentation.

Grade 3: Embryo with blastomeres of distinctly unequal size, few or no cytoplasmic fragments.

Grade 4: Embryo with blastomeres of equal size; minor cytoplasmic fragments.

Grade 5: Embryo, with blastomeres of equal size; no cytoplasmic fragments.

Appendix X - Grading Criteria for Good Quality Embryos

(Modified from *Veeck*, 2003)

EMBRYO GRADING (GQED 2 - 7; PQED 2 - 7)		
	Cell	**Grading**
	2	4 (- / +)
Day 2	3	5 (- / +)
	4	
	> 4	
	6	4 (- / +)
Day 3	7	5 (- / +)
	8	
	> 8	
	10 cell	4 (- / +)
Day 4	Early Compact (EC / VK)	5 (- / +)
	Compact (C / K)	
	> Compact (C / K)	
Day 5	Early Blastocyst (EB / VB)	
	1A; 1B	NO C Grading
	> 1A; 1B	
Day 6	> 3AA; 3AB; 3BA; 3BB	NO C Grading
	> 3AA; 3AB; 3BA; 3BB	
Day 7	3AA; 3AB; 3BA; 3BB	NO C Grading
	> 3AA; 3AB; 3BA; 3BB	

Appendix XI - Grading Criteria for Human Blastocysts

(*Veeck*, 2003)

(Tygerberg Fertility Clinic SOP 17 and Aevitas Fertility Clinic SOP)

See Figure 30

Degree of expansion and hatching status:

1. Early blastocyst; the blastocoel filling more than half the volume of the conceptus, but no expansion in overall size as compared to earlier stages.
2. Blastocyst; the blastocoel filling more than half of the volume of the 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 through the zona.
5. Fully hatched blastocyst; (non-preimplantation genetic diagnosis). Free blastocyst fully removed from zona pellucida.
6. Hatching or hatched blastocyst; (preimplantation genetic diagnosis).

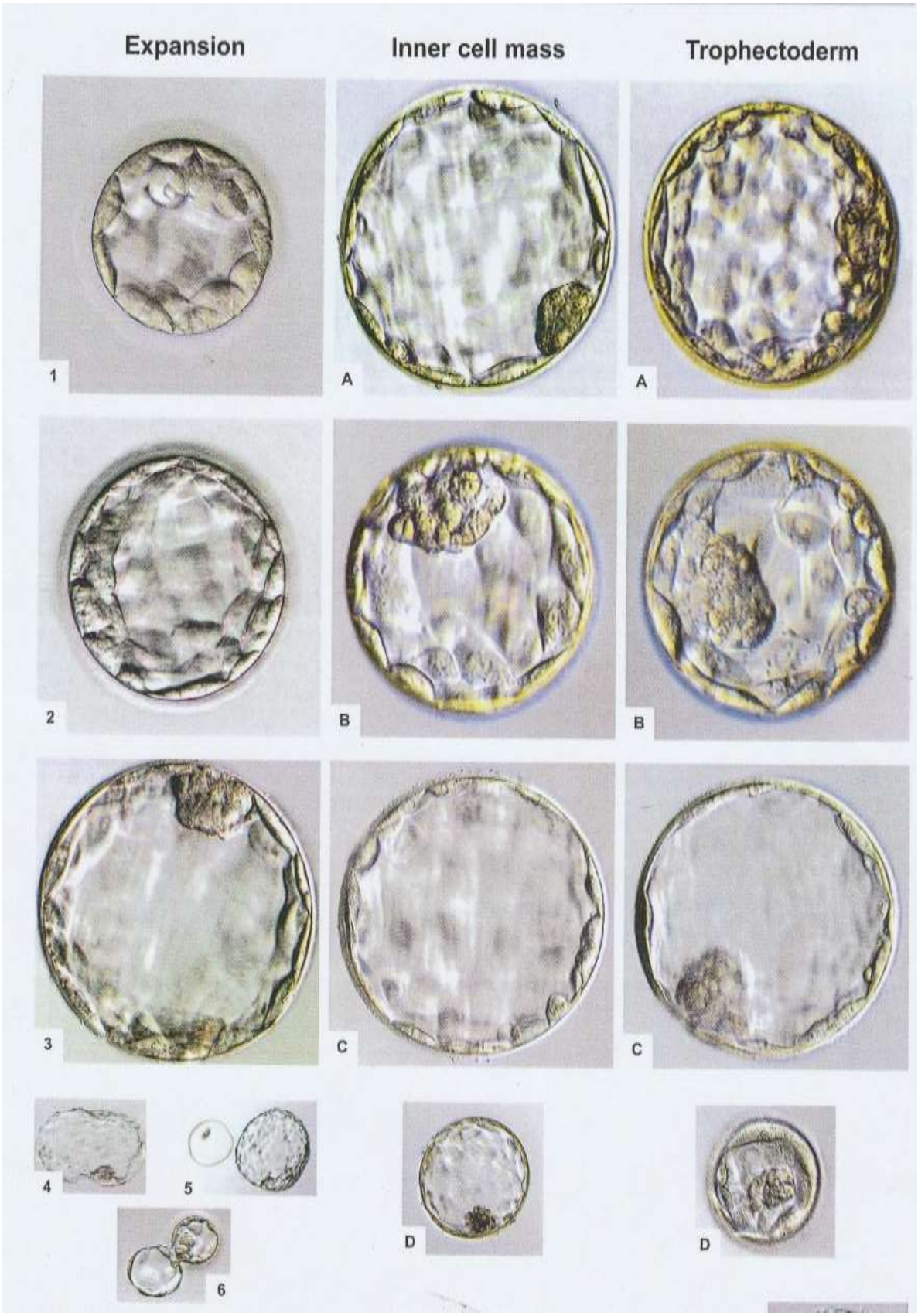
Inner Cell Mass (ICM) grading:

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.

Figure 30. Grading criteria for human blastocysts (*Veeck*, 2003)



Appendix XII - Cryopreservation and Thawing of Oocytes

(Kitazato® BioPharma Protocol)

Oocyte - Vitrification

Vitrification Kit

The Kitazato vitrification kit consists of:

Equilibration medium

Vitrification medium

Note: Oocytes of all maturational stages can be vitrified (germinal vesicle; metaphase I; metaphase II)

Preparation

1. 1x centre-well organ culture dish (Falcon 35 3037)
2. Attenuated glass pipettes/or Stripper pipette with stripper tips
3. Liquid nitrogen in large polystyrene holder
4. Labelled Cryotop (patient's name, identity number & date of freezing)
   - If more than one Cryotop is to be used you can either use different coloured Cryotop for each oocyte set, or simply label each Cryotop on the very top with a number corresponding to the set of oocytes that are placed onto it.
5. Gassed fertilization medium for the first drop of the equilibration process.
6. 1ml of the equilibration medium (Solution 1) and 1ml of the vitrification medium (Solution 2) into Eppendorf tubes and bring to ROOM TEMPERATURE
7. When removing the cumulus, keep the stripper-tip for loading of the oocytes on the Cryotop later.

METHOD

1. Equilibration (ROOM TEMPERATURE)

   - In the lid of the 1ml centre-well organ culture dish make the drops.
   - Place oocytes into fertilization medium drop for 1minute (a).
   - After 1 minute drag fertilization drop into the first equilibration drop. Keep in the first drop for 3 minutes.

- During the first minute keep oocytes in the ‘tunnel’ between the drops (b). In the second and third minutes gradually move the oocytes further into the equilibration drop (c & d).

- After 3 minutes drag the first equilibration drop into the second equilibration drop. Keep oocytes in the second drop for 3 minutes. (Method as above).
- After 3 minutes transfer the oocytes from the second equilibration drop to the last, large equilibration drop. Keep in this drop for at least 9 minutes. (DO NOT drag the other drops into the last drop). (Oocytes may be held in this last droplet for longer than 9 minutes if necessary).
    - If you have a number of oocytes to vitrify, make a few parallel rows of media and take the oocytes through the equilibration steps to the last equilibration drop. Once in the last drop, you can leave the other oocytes in the last drop while you take the first set of oocytes into the vitrification and loading stages.

2. Vitrification (ROOM TEMPERATURE)

- Place the 1ml of vitrification medium (solution 2) into the centre-well organ culture dish.
- After the 9 minutes in the last equilibration drop, transfer the oocytes into the 1ml vitrification medium (put on the bottom of the dish) for no more than 1 minute (this includes the time for loading onto the Cryotop).
- The oocytes will float and move around. Keep catching them and transferring them to the bottom of the dish.

3. Loading Cryotop

NB! The most important thing during the vitrification procedure is to load as little medium onto the Cryotop as possible. Load the oocytes in as little medium and then suck off excess medium around the oocytes, being careful not to suck the oocytes up again.

- Load a MAXIMUM of 4 oocytes per Cryotop, preferably less.
- Place the top of the Cryotop into the liquid nitrogen.
- Using an attenuated glass pipette (by hand or by mouth) or a stripper-tip pipette, transfer all oocytes onto the tip of the Cryotop (ensure that the Cryotop is turned so that the writing on the Cryotop is facing up – this ensures that the scientist that is thawing the oocytes knows which side the oocytes are on).

- Suck off as much excess medium as possible. Work quickly.
- Plunge the tip of the Cryotop directly in the liquid nitrogen.
- Insert the tip into the top of the Cryotop.

- When transferring the Cryotops to the tank, remove the tube from the goblet and transfer into the polystyrene holder that the Cryotops are in. Transfer all the Cryotops into the tube under liquid nitrogen and then transfer the tube back to the goblet. This ensures that the Cryotops are never exposed to air. Only put the cotton wool into the tube once the tube has been transferred back to the tank. This will prevent air bubbles forming in the tube.

Oocyte Thawing

Thawing Kit

The Kitazato thawing kit consists of:

Thawing medium – 1.0 Mol Sucrose
Diluent medium – 0.5 Mol Sucrose
Washing medium 1 – No sucrose. Just a culture medium
Washing medium 2 – Same as washing medium 1

Preparation

1. 1x centre-well organ culture dish (Falcon 35 3037) (warmed in incubator to 37ºC).
2. Attenuated glass pipettes/ or Stripper pipette with stripper tips.
3. Liquid nitrogen in large polystyrene holder.
4. 1ml of Thawing medium (solution 1) in Eppendorf tube and bring to 37ºC.
5. 0.4ml Diluent medium (solution 2) into Eppendorf tube and bring to ROOM TEMPERATURE.
6. Combine 0.2ml Diluent medium and 0.2ml Washing Medium (we'll call it solution 2b - 0.25M sucrose) together in Eppendorf and bring to ROOM TEMPERATURE.
7. 0.5ml Washing medium 1 or 2 (solution 3) into Eppendorf and bring to ROOM TEMPERATURE.
8. 0.5ml gassed fertilization / cleavage medium (37ºC) for the final drops of the thawing process. (The fertilization / cleavage medium is not essential as the washing medium will suffice, but if you are going to transfer the oocytes to fertilization / cleavage medium it will be good to wash the oocytes in cleavage medium).

Method

1. Preparation
   - Place liquid nitrogen into a large polystyrene holder.
   - Transfer the entire tube holding the patient's Cryotops from the tank into the polystyrene holder.
   - Remove the required Cryotops from the tube and replace the tube into the tank leaving the Cryotops for thawing in the polystyrene holder.

2. Solution 1 (37ºC)

   - Work on a room temperature stage.
   - Place the 1ml of solution 1 (37ºC) into the warmed centre-well organ culture dish.
   - Work quickly so as not to reduce the temperature of the 37ºC medium too much.
   - Ensure that the microscope is adjusted prior to removing the Cryotop as speed is important.
   - Under liquid nitrogen remove the top of the Cryotop.
   - Working very quickly, place the tip of the Cryotop into solution 1. Under microscopic vision gently wave the tip until the oocytes dislodge. (If necessary, use a pipette to blow / remove the oocytes from the Cryotop). Remove the Cryotop as quickly as possible so as not to reduce the temperature of the medium too much.
   - Leave oocytes in solution 1 for 1 minute.

3. Solution 2 & 2b (ROOM TEMPERATURE)

   - In the lid of the 1ml centre-well organ culture dish make the drops.
   - Transfer oocytes from the centre-well dish into Solution 2 for 3 minutes.
   - Transfer the oocytes into Solution 2b for 3 minutes.
   - Transfer the oocytes into the first washing drop and move them through the washing droplets to the last drop of washing medium.
   - Drag the last washing drop into the first drop of fertilization / cleavage medium (so as not to shock the oocytes). Do the same into the second drop.

- Pick up the oocytes and transfer them into the last fertilization / cleavage drop.
- Transfer the oocytes to the gassed fertilization / cleavage medium and place into the incubator for 1 or 2 hours prior to ICSI.

Appendix XIII - Cryopreservation and Thawing of Embryos/Blastocysts

(VitriFreeze™ and VitriThaw™ FertiPro Protocol)

Ensure all media are well mixed before use.

Preliminary steps:

In a 4-well dish fill the first well with 300 µl of Pre-incubation medium, the second with VitriFreeze 1 and the third with VitriFreeze 2 solution. Next open as many packs of HSV devices as will be required for the vitrification step, taking into account that 1 HSV device can hold up to 2 embryos.
Conveniently place the separate parts of the HSV device on the workbench for easy access later in the procedure.

Freezing preparation:

Transfer the embryos from the blastocyst cell culture medium in to each of the VitriFreeze solutions using the following scheme:

Stage	*Pre-incubation*	*Vitri Freeze 1*	*Vitri Freeze 2*	*Temperature*
Early Blastocyst / Morulae	*2'*	*2'*	*30"*	*Room temperature*
Blastocyst – expanded blastocyst	*2'*	*3'*	*30"*	*37°C*
Blastocyst – expanded blastocyst + artificial shrinkage*	*2'*	*2'*	*30"*	*Room Temperature*

* Before starting the vitrification procedure, in order to reduce the negative effect of the blastocoel, expanded blastocysts should be collapsed by reducing artificially with a glass pipette the volume of the blastocoel (Vanderzwalmen *et al*, 2002; Son *et al*., 2003 Hiraoka 2004).

Vitrification

1. Using an attenuated pipette or an equally suitable device, deposit maximum 2 blastocysts in a volume of approximately 0.3µl of VitriFreeze 2, in the gutter of the tip of the vitrification straw.
2. Place the vitrification straw in the outer sheath and seal it as indicated in the instructions for use of the HSV device.
3. Plunge the sealed device into the liquid nitrogen.

Thawing

1. Remove the vitrification straw from the outer sheath as indicated in the instructions for use of the HSV device.
2. Immediately plunge the vitrification straw into pre-heated VitriThaw Thawing medium 1 (37°C) and leave in thawing 1 for 3 minutes.
3. Transfer into VitriThaw Thawing medium 2 (37°C) and leave in this medium for 2 minutes.
4. Transfer into VitriThaw Thawing medium 3 (37°C) and leave in this medium for 2 minutes.
5. Finally transfer into VitriThaw Thawing medium 4 (37°C) and wash for at least 1 minute.
6. Transfer into blastocyst culture medium for continued cell culture.

Appendix XIV - Embryo Transfer Method

(Matsaseng & Kruger, 2014)

(Tygerberg Fertility Clinic SOP 18 and Aevitas Fertility Clinic SOP)

Routinely, an embryo transfer occurs 2 to 5 days after the oocyte retrieval. The procedure starts by placing a speculum in the vagina to visualize the cervix, which is cleaned with gauze wetted with HEPES buffered (flushing) culture medium. 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 atraumatically and embryos transferred easily and exactly at the right place.

A soft transfer catheter, which contains the embryos to be transferred, is inserted through the cervical canal and advanced to the uterine cavity. After insertion of the catheter, the media containing the embryos are deposited into the uterine cavity. It is important that, after the deposit of the embryos, the embryologist checks the catheter immediately to ensure that the embryos don't remain inside it.

The transfer is guided with an ultrasound (abdominal ultrasound) to ensure correct placement in the uterine cavity. Anaesthesia is not required when performing an embryo transfer.

Appendix XV - Consent Form – Prospective Study

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

"Investigation into Possible Factors Influencing Clinical Pregnancy Rate in a public and private sector ART Clinic"

REFERENCE NUMBER: S15/03/050A (Health Research Ethical Committee Approval Number)
PRINCIPAL INVESTIGATOR: Nicole Ashley Nel
ADDRESS: Aevitas Fertility Clinic, Vincent Pallotti Hospital, Pinelands
Tygerberg Hospital, Tygerberg, Cape Town
CONTACT NUMBER: +27 82 799 9444

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you will be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part. This study has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

Many people have difficulty to fall pregnant and have a baby of their own. There are many solutions for this problem – one being the use of Assisted Reproductive Techniques (ART's). There are many factors and reasons why a couple may have difficulty to fall pregnant. When Fertility doctors need to find out what these reasons are they do a thorough workup/investigation of the couple. The investigations include both the male and female partner's medical history, certain lifestyle habits (smoking, alcohol use, Body Mass Index) and medical tests so that contributing factors to the couple's infertility can be identified. The contributing factors could be from the male

partner, female partner or both. The success of ART treatment may also be influenced by many factors such as female age, the number of eggs and the quality of the embryos. The aim of this study will be to investigate which factors may have a significant effect on a successful clinical pregnancy [a positive fetal heartbeat at 7 weeks] outcome at two different ART laboratories – one a private [Drs Aevitas Clinic at Vincent Pallotti Hospital] and the other a public sector [Tygerberg Fertility Clinic, Tygerberg Hospital] Fertility Clinic.

To do this study we need to use the routine data collected in each clinic from your files. The data and information will be collected in an **anonymous** manner and you will not be identified and none of your personal information will be used.

Why have you been invited to participate?

You have been identified as a suitable candidate for the study since you agreed to undergo either IVF or ICSI treatment at the respective Fertility Clinics and fall into the group of patients who falls within the inclusion criteria of the study.

What will your responsibilities be?

All we ask is that you read through the information and sign the form provided to give consent for the use of your medical records – you will stay anonymous.

Will you benefit from taking part in this research?

You may benefit because your consent to take part in the study and the results of the study may help to improve the outcome of successful pregnancy, also for other sub-fertile couples.

Are there in risks involved in your taking part in this research?

There are no risks.

Who will have access to your medical records?

Your identity and any additional information about your treatment will be confidential and protected. If the research is used in any kind of publication, your identity will remain anonymous. Your information will be available only to the principle and co-investigators involved in the research project.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

There will not be any form of injury as a direct or indirect result of you giving consent to use the information needed for the study.

Will you be paid to take part in this study and are there any costs involved?

No, you will not be paid and there are no costs involved.

Is there anything else that you should know or do?

-You can contact myself, the researcher, or the supervisor of the project Dr. Marie-Lena de Beer (+27 83 708 8964) if you have any further queries or encounter any problems.

-You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.

-You will receive a copy of this information and consent form for your own records.

Declaration by participants

By signing below, we (Initials & Surname) …….. and

…….. agree to take part in a research study entitled: ***"Investigation into Possible Factors Influencing Clinical Pregnancy Rate in a public and private sector ART Clinic"***

We declare that:

- We have read or had read to us this information and consent form and it is written in a language with which we are fluent and comfortable.
- We have had a chance to ask questions and all our questions have been adequately answered.
- We understand that taking part in this study is **voluntary** and we have not been pressurised to take part.
- We may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- We may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in our best interests, or if we do not follow the study plan, as agreed to.

Signed at (*place*) ..... on (*date*) ……

…….. ……

Signature of participant [partner one] Signature of witness

……..

Signature of participant [partner two]

Declaration by investigator

I *(Initials & Surname)* …… declare that:

- I explained the information in this document to ……..

and ……..……

- I encouraged them to ask questions and took adequate time to answer them
- I am satisfied that they adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below*

Signed at (*place*) ..... on (*date*) ……

....

Signature of investigator Signature of witness

Declaration by interpreter

I *(name)* …… declare that:

- I assisted the investigator (*name*) ……..to explain the information

in this document to (*name of participants*) ……

and ……..……using the language medium of Afrikaans/Xhosa.

- We encouraged them to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participants fully understand the content of this informed consent document and has had all their questions satisfactorily answered.

Signed at (*place*) ..... on (*date*) ……

....

Signature of interpreter Signature of witness

Appendix XVI - Retrospective Data Results

1. Distribution of patients between different incubators

```
. gen inc2=inc

. tab inc , missing
```

INC	Freq.	Percent	Cum.
1	69	17.78	17.78
2	72	18.56	36.34
3	45	11.60	47.94
4	64	16.49	64.43
5	77	19.85	84.28
8	14	3.61	87.89
9	14	3.61	91.49
10	17	4.38	95.88
11	13	3.35	99.23
12	1	0.26	99.48
14	1	0.26	99.74
16	1	0.26	100.00
Total	388	100.00	

2. Distribution of patients grouped between two incubators under investigation

```
. recode inc2 (1/5=1) (8/11=2) (12/16=.)
(inc2: 319 changes made)

. tab inc2 , missing
```

inc2	Freq.	Percent	Cum.	
1	327	84.28	84.28	
2	58	14.95	99.23	
.	3	0.77	100.00	not considered for analysis
Total	388	100.00		

3. Age of Ova distribution between incubators

```
. tab ageova inc2, col
```

Key
frequency
column percentage

AgeOva	inc2 1	inc2 2	Total
19	2	0	2
	0.61	0.00	0.52
20	2	1	3
	0.61	1.72	0.78
21	7	0	7
	2.14	0.00	1.82
22	18	1	19
	5.50	1.72	4.94
23	10	3	13
	3.06	5.17	3.38
24	12	3	15

```
           |      3.67       5.17 |      3.90
-----------+--------------------+----------
        25 |        17          1 |        18
           |      5.20       1.72 |      4.68
-----------+--------------------+----------
        26 |        19          1 |        20
           |      5.81       1.72 |      5.19
-----------+--------------------+----------
        27 |        14          5 |        19
           |      4.28       8.62 |      4.94
-----------+--------------------+----------
        28 |        14          2 |        16
           |      4.28       3.45 |      4.16
-----------+--------------------+----------
        29 |        17          5 |        22
           |      5.20       8.62 |      5.71
-----------+--------------------+----------
        30 |        26          1 |        27
           |      7.95       1.72 |      7.01
-----------+--------------------+----------
        31 |        18          6 |        24
           |      5.50      10.34 |      6.23
-----------+--------------------+----------
        32 |        26          3 |        29
           |      7.95       5.17 |      7.53
-----------+--------------------+----------
        33 |        24          5 |        29
           |      7.34       8.62 |      7.53
-----------+--------------------+----------
        34 |        22          3 |        25
           |      6.73       5.17 |      6.49
-----------+--------------------+----------
        35 |        15          8 |        23
           |      4.59      13.79 |      5.97
-----------+--------------------+----------
        36 |        33          3 |        36
           |     10.09       5.17 |      9.35
-----------+--------------------+----------
        37 |        31          7 |        38
           |      9.48      12.07 |      9.87
-----------+--------------------+----------
     Total |       327         58 |       385
           |    100.00     100.00 |    100.00
```

```
. ttest ageova, by(inc2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     327    30.24771    .2698549    4.879824    29.71683    30.77858
   Mincs |      58    30.96552    .6089499    4.637625    29.74612    32.18492
---------+--------------------------------------------------------------------
combined |     385    30.35584    .2469271     4.84506    29.87035    30.84134
---------+--------------------------------------------------------------------
    diff |           -.7178108    .6902328               -2.074931    .6393091
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =  -1.0400
Ho: diff = 0                                     degrees of freedom =      383

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1495         Pr(|T| > |t|) = 0.2990          Pr(T > t) = 0.8505

No difference
```

4. Male diagnoses (coded) between incubators

```
. tabulate mdiag inc2, exact

           |         inc2
     Mdiag |     Forma      Mincs |     Total
-----------+--------------------+----------
         1 |       126         20 |       146
         2 |        18          2 |        20
```

```
          3 |          8          1 |          9
          4 |          4          0 |          4
          5 |         14          3 |         17
          6 |          2          2 |          4
          8 |         25          5 |         30
          9 |         46         13 |         59
         10 |          3          0 |          3
         11 |          8          0 |          8
         12 |         20          3 |         23
         13 |          1          0 |          1
         15 |         20          4 |         24
         16 |          1          0 |          1
         20 |          0          1 |          1
------------+----------------------+----------
      Total |        296         54 |        350

           Fisher's exact =                 0.519

No difference in mdiag profile
. tab mdiag inc2 ,col

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

           |         inc2
     Mdiag |     Forma      Mincs |     Total
-----------+----------------------+----------
         1 |       126         20 |       146
           |     42.57      37.04 |     41.71
-----------+----------------------+----------
         2 |        18          2 |        20
           |      6.08       3.70 |      5.71
-----------+----------------------+----------
         3 |         8          1 |         9
           |      2.70       1.85 |      2.57
-----------+----------------------+----------
         4 |         4          0 |         4
           |      1.35       0.00 |      1.14
-----------+----------------------+----------
         5 |        14          3 |        17
           |      4.73       5.56 |      4.86
-----------+----------------------+----------
         6 |         2          2 |         4
           |      0.68       3.70 |      1.14
-----------+----------------------+----------
         8 |        25          5 |        30
           |      8.45       9.26 |      8.57
-----------+----------------------+----------
         9 |        46         13 |        59
           |     15.54      24.07 |     16.86
-----------+----------------------+----------
        10 |         3          0 |         3
           |      1.01       0.00 |      0.86
-----------+----------------------+----------
        11 |         8          0 |         8
           |      2.70       0.00 |      2.29
-----------+----------------------+----------
        12 |        20          3 |        23
           |      6.76       5.56 |      6.57
-----------+----------------------+----------
        13 |         1          0 |         1
           |      0.34       0.00 |      0.29
-----------+----------------------+----------
        15 |        20          4 |        24
           |      6.76       7.41 |      6.86
-----------+----------------------+----------
        16 |         1          0 |         1
           |      0.34       0.00 |      0.29
-----------+----------------------+----------
        20 |         0          1 |         1
           |      0.00       1.85 |      0.29
-----------+----------------------+----------
      Total |       296         54 |       350
```

```
           |    100.00     100.00 |    100.00
```

5. Female diagnoses (coded) between incubators

```
. tabulate fdiag inc2, exact
```

Fdiag	inc2 Forma	Mincs	Total
1	187	32	219
2	18	2	20
13	3	1	4
14	21	1	22
15	22	9	31
16	41	11	52
17	8	0	8
18	1	0	1
19	2	0	2
28	1	0	1
Total	304	56	360

```
           Fisher's exact =                 0.283
```

```
tab fdiag inc2 ,col
```

```
+--------------------+
| Key                |
|--------------------|
|     frequency      |
| column percentage  |
+--------------------+
```

Fdiag	inc2 Forma	Mincs	Total
1	187	32	219
	61.51	57.14	60.83
2	18	2	20
	5.92	3.57	5.56
13	3	1	4
	0.99	1.79	1.11
14	21	1	22
	6.91	1.79	6.11
15	22	9	31
	7.24	16.07	8.61
16	41	11	52
	13.49	19.64	14.44
17	8	0	8
	2.63	0.00	2.22
18	1	0	1
	0.33	0.00	0.28
19	2	0	2
	0.66	0.00	0.56
28	1	0	1
	0.33	0.00	0.28
Total	304	56	360
	100.00	100.00	100.00

6. Procedures (coded) between incubators

```
. . tab proc inc2 ,col

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

           |         inc2
      Proc |     Forma      Mincs |     Total
-----------+----------------------+----------
         1 |        34          6 |        40
           |     10.40      10.34 |     10.39
-----------+----------------------+----------
         2 |       116         13 |       129
           |     35.47      22.41 |     33.51
-----------+----------------------+----------
         3 |        24          5 |        29
           |      7.34       8.62 |      7.53
-----------+----------------------+----------
         4 |        45          7 |        52
           |     13.76      12.07 |     13.51
-----------+----------------------+----------
         5 |        11          3 |        14
           |      3.36       5.17 |      3.64
-----------+----------------------+----------
         6 |        94         24 |       118
           |     28.75      41.38 |     30.65
-----------+----------------------+----------
         8 |         2          0 |         2
           |      0.61       0.00 |      0.52
-----------+----------------------+----------
        12 |         1          0 |         1
           |      0.31       0.00 |      0.26
-----------+----------------------+----------
     Total |       327         58 |       385
           |    100.00     100.00 |    100.00


. tabulate proc inc2, exact

           |         inc2
      Proc |     Forma      Mincs |     Total
-----------+----------------------+----------
         1 |        34          6 |        40
         2 |       116         13 |       129
         3 |        24          5 |        29
         4 |        45          7 |        52
         5 |        11          3 |        14
         6 |        94         24 |       118
         8 |         2          0 |         2
        12 |         1          0 |         1
-----------+----------------------+----------
     Total |       327         58 |       385

           Fisher's exact =                 0.435
```

7. Sperm morphology frequency

```
. tab morph inc2 ,col

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

           |         inc2
     Morph |     Forma      Mincs |     Total
-----------+----------------------+----------
```

1	9	2	11
	3.88	4.88	4.03
2	13	1	14
	5.60	2.44	5.13
3	16	3	19
	6.90	7.32	6.96
4	14	2	16
	6.03	4.88	5.86
5	10	2	12
	4.31	4.88	4.40
6	20	3	23
	8.62	7.32	8.42
7	23	2	25
	9.91	4.88	9.16
8	23	2	25
	9.91	4.88	9.16
9	19	4	23
	8.19	9.76	8.42
10	9	5	14
	3.88	12.20	5.13
11	23	3	26
	9.91	7.32	9.52
12	16	2	18
	6.90	4.88	6.59
13	8	3	11
	3.45	7.32	4.03
14	7	3	10
	3.02	7.32	3.66
15	4	1	5
	1.72	2.44	1.83
16	6	0	6
	2.59	0.00	2.20
17	3	0	3
	1.29	0.00	1.10
18	2	1	3
	0.86	2.44	1.10
19	2	1	3
	0.86	2.44	1.10
20	2	1	3
	0.86	2.44	1.10
22	2	0	2
	0.86	0.00	0.73
25	1	0	1
	0.43	0.00	0.37
Total	232	41	273
	100.00	100.00	100.00

```
.
end of do-file


. ttest morph, by(inc2)

Two-sample t test with equal variances
```

```
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     232    8.413793    .3020763     4.60109    7.818616     9.00897
   Mincs |      41    9.146341    .7404357    4.741102    7.649865    10.64282
---------+--------------------------------------------------------------------
combined |     273     8.52381    .2796734    4.620962     7.97321    9.074409
---------+--------------------------------------------------------------------
    diff |          -.7325484    .7830283               -2.27414    .8090435
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =  -0.9355
Ho: diff = 0                                     degrees of freedom =      271

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1752         Pr(|T| > |t|) = 0.3503          Pr(T > t) = 0.8248
No difference
```

8. Frequency of donor ova

```
. . tabulate donova inc2, exact col

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

           |         inc2
    DonOva |     Forma      Mincs |     Total
-----------+----------------------+----------
         0 |       228         46 |       274
           |     69.94      79.31 |     71.35
-----------+----------------------+----------
         1 |        98         12 |       110
           |     30.06      20.69 |     28.65
-----------+----------------------+----------
     Total |       326         58 |       384
           |    100.00     100.00 |    100.00

          Fisher's exact =                 0.159
   1-sided Fisher's exact =                 0.095
```

No difference despite 10% difference in proportion with donor ova. Study not powered to show this difference.

9. Number of ova distribution

```
. tab NrOva inc2 ,col

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

           |         inc2
     NrOva |     Forma      Mincs |     Total
-----------+----------------------+----------
         3 |         8          1 |         9
           |      2.45       1.72 |      2.34
-----------+----------------------+----------
         4 |         6          4 |        10
           |      1.84       6.90 |      2.60
-----------+----------------------+----------
         5 |        16          4 |        20
           |      4.91       6.90 |      5.21
-----------+----------------------+----------
         6 |        22          4 |        26
           |      6.75       6.90 |      6.77
-----------+----------------------+----------
         7 |        29          4 |        33
```

	8.90	6.90	8.59
8	19	2	21
	5.83	3.45	5.47
9	17	3	20
	5.21	5.17	5.21
10	18	6	24
	5.52	10.34	6.25
11	20	8	28
	6.13	13.79	7.29
12	17	2	19
	5.21	3.45	4.95
13	25	2	27
	7.67	3.45	7.03
14	17	1	18
	5.21	1.72	4.69
15	14	1	15
	4.29	1.72	3.91
16	13	1	14
	3.99	1.72	3.65
17	13	2	15
	3.99	3.45	3.91
18	12	1	13
	3.68	1.72	3.39
19	4	0	4
	1.23	0.00	1.04
20	11	1	12
	3.37	1.72	3.13
21	3	4	7
	0.92	6.90	1.82
22	5	1	6
	1.53	1.72	1.56
23	9	0	9
	2.76	0.00	2.34
24	3	2	5
	0.92	3.45	1.30
25	3	1	4
	0.92	1.72	1.04
26	2	0	2
	0.61	0.00	0.52
28	6	0	6
	1.84	0.00	1.56
29	2	1	3
	0.61	1.72	0.78
30	2	0	2
	0.61	0.00	0.52
31	2	0	2
	0.61	0.00	0.52
32	2	1	3
	0.61	1.72	0.78
34	2	0	2
	0.61	0.00	0.52

```
        38 |          1          0 |          1
           |       0.31       0.00 |       0.26
-----------+----------------------+----------
        40 |          1          0 |          1
           |       0.31       0.00 |       0.26
-----------+----------------------+----------
        41 |          0          1 |          1
           |       0.00       1.72 |       0.26
-----------+----------------------+----------
        50 |          1          0 |          1
           |       0.31       0.00 |       0.26
-----------+----------------------+----------
        56 |          1          0 |          1
           |       0.31       0.00 |       0.26
-----------+----------------------+----------
     Total |        326         58 |        384
           |     100.00     100.00 |     100.00


. ttest NrOva , by(inc2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     326    13.27914    .4199777    7.582895    12.45292    14.10536
   Mincs |      58    12.58621    1.020075    7.768661    10.54354    14.62887
---------+--------------------------------------------------------------------
combined |     384    13.17448     .388092    7.605019    12.41142    13.93754
---------+--------------------------------------------------------------------
    diff |            .6929342    1.084623               -1.439645    2.825514
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =   0.6389
Ho: diff = 0                                     degrees of freedom =      382

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.7384         Pr(|T| > |t|) = 0.5233          Pr(T > t) = 0.2616
No difference
```

10. Frequency of MI oocytes

```
. tab mi inc2 ,col

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

           |         inc2
        MI |     Forma      Mincs |     Total
-----------+----------------------+----------
         0 |       103         18 |       121
           |     31.50      31.03 |     31.43
-----------+----------------------+----------
         1 |        65         10 |        75
           |     19.88      17.24 |     19.48
-----------+----------------------+----------
         2 |        63         11 |        74
           |     19.27      18.97 |     19.22
-----------+----------------------+----------
         3 |        27          9 |        36
           |      8.26      15.52 |      9.35
-----------+----------------------+----------
         4 |        19          5 |        24
           |      5.81       8.62 |      6.23
-----------+----------------------+----------
         5 |        23          1 |        24
           |      7.03       1.72 |      6.23
-----------+----------------------+----------
         6 |         8          2 |        10
           |      2.45       3.45 |      2.60
-----------+----------------------+----------
         7 |         8          2 |        10
```

```
           |      2.45        3.45 |      2.60
-----------+---------------------+----------
         8 |         4           0 |         4
           |      1.22        0.00 |      1.04
-----------+---------------------+----------
         9 |         2           0 |         2
           |      0.61        0.00 |      0.52
-----------+---------------------+----------
        10 |         2           0 |         2
           |      0.61        0.00 |      0.52
-----------+---------------------+----------
        13 |         2           0 |         2
           |      0.61        0.00 |      0.52
-----------+---------------------+----------
        17 |         1           0 |         1
           |      0.31        0.00 |      0.26
-----------+---------------------+----------
     Total |       327          58 |       385
           |    100.00      100.00 |    100.00
```

```
. tabulate mi inc2, exact
```

```
           |         inc2
        MI |     Forma      Mincs |     Total
-----------+---------------------+----------
         0 |       103         18 |       121
         1 |        65         10 |        75
         2 |        63         11 |        74
         3 |        27          9 |        36
         4 |        19          5 |        24
         5 |        23          1 |        24
         6 |         8          2 |        10
         7 |         8          2 |        10
         8 |         4          0 |         4
         9 |         2          0 |         2
        10 |         2          0 |         2
        13 |         2          0 |         2
        17 |         1          0 |         1
-----------+---------------------+----------
     Total |       327         58 |       385

           Fisher's exact =                 0.760
```

11. Frequency MII oocytes

```
. tab mii inc2 ,col
```

```
+-------------------+
| Key               |
|-------------------|
|     frequency     |
| column percentage |
+-------------------+
```

```
           |         inc2
       MII |     Forma      Mincs |     Total
-----------+---------------------+----------
         2 |         3           1 |         4
           |      0.92        1.72 |      1.04
-----------+---------------------+----------
         3 |         9           3 |        12
           |      2.75        5.17 |      3.12
-----------+---------------------+----------
         4 |        21           3 |        24
           |      6.42        5.17 |      6.23
-----------+---------------------+----------
         5 |        23           4 |        27
           |      7.03        6.90 |      7.01
-----------+---------------------+----------
         6 |        36           8 |        44
           |     11.01       13.79 |     11.43
-----------+---------------------+----------
         7 |        22           8 |        30
```

	6.73	13.79	7.79
8	19	3	22
	5.81	5.17	5.71
9	24	2	26
	7.34	3.45	6.75
10	22	6	28
	6.73	10.34	7.27
11	21	2	23
	6.42	3.45	5.97
12	20	3	23
	6.12	5.17	5.97
13	16	2	18
	4.89	3.45	4.68
14	16	2	18
	4.89	3.45	4.68
15	11	2	13
	3.36	3.45	3.38
16	6	1	7
	1.83	1.72	1.82
17	9	0	9
	2.75	0.00	2.34
18	14	2	16
	4.28	3.45	4.16
19	5	0	5
	1.53	0.00	1.30
20	5	2	7
	1.53	3.45	1.82
21	3	0	3
	0.92	0.00	0.78
22	4	0	4
	1.22	0.00	1.04
23	4	0	4
	1.22	0.00	1.04
24	2	1	3
	0.61	1.72	0.78
25	3	0	3
	0.92	0.00	0.78
26	3	0	3
	0.92	0.00	0.78
27	0	1	1
	0.00	1.72	0.26
29	2	1	3
	0.61	1.72	0.78
32	1	0	1
	0.31	0.00	0.26
33	1	0	1
	0.31	0.00	0.26
37	1	0	1
	0.31	0.00	0.26
38	0	1	1
	0.00	1.72	0.26

```
        45 |          1           0 |          1
           |       0.31        0.00 |       0.26
-----------+----------------------+-----------
     Total |        327          58 |        385
           |     100.00      100.00 |     100.00
```

12. Proportion fertilized MI & MII

```
. ttest fertmii , by(inc2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     327    8.611621     .272772    4.932575    8.075005    9.148236
   Mincs |      58    8.258621    .7678351    5.847658    6.721057    9.796184
---------+--------------------------------------------------------------------
combined |     385    8.558442    .2586038    5.074173    8.049985    9.066898
---------+--------------------------------------------------------------------
    diff |            .3530001    .7236675               -1.069858    1.775859
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =   0.4878
Ho: diff = 0                                     degrees of freedom =      383

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.6870         Pr(|T| > |t|) = 0.6260          Pr(T > t) = 0.3130

.
end of do-file

.
. . ttest fertmi , by(inc2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     327    .0183486    .0074331    .1344143    .0037257    .0329716
   Mincs |      58    .0172414    .0172414    .1313064   -.0172839    .0517666
---------+--------------------------------------------------------------------
combined |     385    .0181818    .0068182    .1337824    .0047762    .0315875
---------+--------------------------------------------------------------------
    diff |            .0011072    .0190856               -.0364184    .0386329
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =   0.0580
Ho: diff = 0                                     degrees of freedom =      383

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5231         Pr(|T| > |t|) = 0.9538          Pr(T > t) = 0.4769

end of do-file


There were 17 pt where fertmii>mii. This cannot be. Thus assumed fertmii =mi and proportion
fertilised=100%

. . ttest p_fertmi , by(inc2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     224    .0113095    .0047403    .0709468    .0019679    .0206511
   Mincs |      40       .0125       .0125    .0790569   -.0127836    .0377836
---------+--------------------------------------------------------------------
combined |     264    .0114899    .0044359    .0720756    .0027554    .0202244
---------+--------------------------------------------------------------------
    diff |           -.0011905    .0123953               -.0255975    .0232166
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =  -0.0960
Ho: diff = 0                                     degrees of freedom =      262

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.4618         Pr(|T| > |t|) = 0.9236          Pr(T > t) = 0.5382
```

Very few pt with any fertilisation of mi

```
. . ttest p_fertmii , by(inc2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     327    .7941573    .0099234    .1794457    .7746354    .8136792
   Mincs |      58    .7906504    .0220263    .1677476    .7465434    .8347574
---------+--------------------------------------------------------------------
combined |     385     .793629    .0090476    .1775264      .77584     .811418
---------+--------------------------------------------------------------------
    diff |            .0035069    .0253257               -.0462878    .0533016
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =   0.1385
Ho: diff = 0                                     degrees of freedom =      383

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.5550         Pr(|T| > |t|) = 0.8899          Pr(T > t) = 0.4450
```

No difference in mii fertilisation rate 79% in both incubators

13. Frequency of good quality embryos on cycle day 2

```
tab GQDAY2 inc2 ,col

+--------------------+
| Key                |
|--------------------|
|     frequency      |
| column percentage  |
+--------------------+
```

GQDAY2	inc2 Forma	inc2 Mincs	Total
3	48	14	62
	15.05	24.56	16.49
4	51	11	62
	15.99	19.30	16.49
5	49	5	54
	15.36	8.77	14.36
6	33	3	36
	10.34	5.26	9.57
7	30	3	33
	9.40	5.26	8.78
8	22	4	26
	6.90	7.02	6.91
9	19	4	23
	5.96	7.02	6.12
10	22	5	27
	6.90	8.77	7.18
11	9	3	12
	2.82	5.26	3.19
12	12	0	12
	3.76	0.00	3.19
13	5	0	5
	1.57	0.00	1.33
14	3	2	5

```
           |      0.94        3.51 |      1.33
-----------+---------------------+----------
        15 |         4           0 |         4
           |      1.25        0.00 |      1.06
-----------+---------------------+----------
        16 |         1           1 |         2
           |      0.31        1.75 |      0.53
-----------+---------------------+----------
        17 |         1           1 |         2
           |      0.31        1.75 |      0.53
-----------+---------------------+----------
        18 |         4           1 |         5
           |      1.25        1.75 |      1.33
-----------+---------------------+----------
        19 |         1           0 |         1
           |      0.31        0.00 |      0.27
-----------+---------------------+----------
        20 |         1           0 |         1
           |      0.31        0.00 |      0.27
-----------+---------------------+----------
        21 |         2           0 |         2
           |      0.63        0.00 |      0.53
-----------+---------------------+----------
        24 |         1           0 |         1
           |      0.31        0.00 |      0.27
-----------+---------------------+----------
        26 |         1           0 |         1
           |      0.31        0.00 |      0.27
-----------+---------------------+----------
     Total |       319          57 |       376
           |    100.00      100.00 |    100.00
```

```
. ttest GQDAY2 , by(inc2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     319    6.940439     .217478    3.884281    6.512561    7.368316
   Mincs |      57    6.666667    .5168425    3.902075    5.631307    7.702027
---------+--------------------------------------------------------------------
combined |     376    6.898936    .2002509    3.883009    6.505181    7.292692
---------+--------------------------------------------------------------------
    diff |            .2737722    .5589464               -.8252992    1.372844
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =   0.4898
Ho: diff = 0                                     degrees of freedom =      374

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.6877         Pr(|T| > |t|) = 0.6246          Pr(T > t) = 0.3123
```

Number of good quality embryo on day2 the same

14. Frequency of good quality embryos on cycle day 3

```
. tab GQDAY3 inc2 ,col

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

           |         inc2
    GQDAY3 |     Forma      Mincs |     Total
-----------+---------------------+----------
         0 |         9           0 |         9
           |      2.83        0.00 |      2.41
-----------+---------------------+----------
         1 |        18           4 |        22
           |      5.66        7.14 |      5.88
-----------+---------------------+----------
         2 |        39           6 |        45
```

	12.26	10.71	12.03
3	67	10	77
	21.07	17.86	20.59
4	41	10	51
	12.89	17.86	13.64
5	44	8	52
	13.84	14.29	13.90
6	29	4	33
	9.12	7.14	8.82
7	20	2	22
	6.29	3.57	5.88
8	19	1	20
	5.97	1.79	5.35
9	11	4	15
	3.46	7.14	4.01
10	6	2	8
	1.89	3.57	2.14
11	4	1	5
	1.26	1.79	1.34
12	2	0	2
	0.63	0.00	0.53
13	1	0	1
	0.31	0.00	0.27
14	3	3	6
	0.94	5.36	1.60
15	1	1	2
	0.31	1.79	0.53
17	1	0	1
	0.31	0.00	0.27
19	2	0	2
	0.63	0.00	0.53
23	1	0	1
	0.31	0.00	0.27
Total	318	56	374
	100.00	100.00	100.00

```
. ttest GQDAY3 , by(inc2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     318    4.786164    .1802023    3.213467     4.43162    5.140707
   Mincs |      56    5.285714    .4689822    3.509542    4.345853    6.225576
---------+--------------------------------------------------------------------
combined |     374    4.860963    .1685424    3.259455     4.52955    5.192375
---------+--------------------------------------------------------------------
    diff |           -.4995508    .4722849               -1.428234    .4291322
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =  -1.0577
Ho: diff = 0                                     degrees of freedom =      372

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1454         Pr(|T| > |t|) = 0.2909          Pr(T > t) = 0.8546

. graph box GQDAY3 , over(inc2)
```

15. Frequency of good quality embryos on cycle day 5

```
. tab GQDAY5 inc2 ,col

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

           |         inc2
    GQDAY5 |     Forma      Mincs |     Total
-----------+----------------------+----------
         0 |        30          6 |        36
           |     12.35      15.00 |     12.72
-----------+----------------------+----------
         1 |        40         10 |        50
           |     16.46      25.00 |     17.67
-----------+----------------------+----------
         2 |        41          6 |        47
           |     16.87      15.00 |     16.61
-----------+----------------------+----------
         3 |        37          3 |        40
           |     15.23       7.50 |     14.13
-----------+----------------------+----------
         4 |        38          4 |        42
           |     15.64      10.00 |     14.84
-----------+----------------------+----------
         5 |        19          1 |        20
           |      7.82       2.50 |      7.07
-----------+----------------------+----------
         6 |        16          2 |        18
           |      6.58       5.00 |      6.36
-----------+----------------------+----------
         7 |         8          6 |        14
           |      3.29      15.00 |      4.95
-----------+----------------------+----------
         8 |         7          1 |         8
           |      2.88       2.50 |      2.83
-----------+----------------------+----------
         9 |         3          0 |         3
           |      1.23       0.00 |      1.06
-----------+----------------------+----------
        11 |         1          0 |         1
           |      0.41       0.00 |      0.35
-----------+----------------------+----------
        12 |         1          0 |         1
           |      0.41       0.00 |      0.35
-----------+----------------------+----------
        13 |         2          0 |         2
           |      0.82       0.00 |      0.71
-----------+----------------------+----------
        15 |         0          1 |         1
           |      0.00       2.50 |      0.35
-----------+----------------------+----------
     Total |       243         40 |       283
           |    100.00     100.00 |    100.00


. graph box GQDAY5 , over(inc2)


. ttest GQDAY5 , by(inc2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
   Forma |     243    3.144033    .1586405     2.47296    2.831541    3.456525
   Mincs |      40       3.225    .4993425    3.158119    2.214984    4.235016
---------+--------------------------------------------------------------------
combined |     283    3.155477    .1530401    2.574534    2.854231    3.456723
---------+--------------------------------------------------------------------
    diff |           -.0809671    .4400519               -.9471837    .7852496
```

```
------------------------------------------------------------------------------
    diff = mean(Forma) - mean(Mincs)                              t =  -0.1840
Ho: diff = 0                                     degrees of freedom =      281

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.4271         Pr(|T| > |t|) = 0.8542          Pr(T > t) = 0.5729
```

16. Frequency of good quality embryos on cycle days 2, 3 & 5 – Distribution between two incubators

Modeling the probability of having a good embryo quality on days 2,2 and 5 for the two incubators where the denominator is the total number of ova. The model is adjusted for the age of the ova, where seom donor ova was involved and the number of mii ova available.
Binomial regression estimating the risk ratio mincs compared to Forma

Descriptives first

```
. generate pergd2= GQDAY2/ NrOva
(10 missing values generated)

. generate pergd3= GQDAY3/ NrOva
(12 missing values generated)

. generate pergd5= GQDAY5/ NrOva
(104 missing values generated)

. tabstat pergd2 pergd3 pergd5, statistics( mean count sd ) by(inc2) varwidth(14) columns
> (statistics)

inc2          variable |      mean         N        sd
----------------------+------------------------------
Forma           pergd2 |  .5589354       318  .1869036
                pergd3 |  .3931441       317  .2063107
                pergd5 |  .2360079       243  .1735365
----------------------+------------------------------
Mincs           pergd2 |   .574494        57  .2032567
                pergd3 |  .4458132        56  .2201563
                pergd5 |  .2176929        40  .1705393
----------------------+------------------------------
Total           pergd2 |  .5613003       375  .1892781
                pergd3 |  .4010516       373  .2089919
                pergd5 |  .2334192       283  .1729345
--------------------------------------------------------

. xi: binreg GQDAY2 i.inc2 ageova i.donova mii, rr n(NrOva) vce(cluster ptnr)
i.inc2            _Iinc2_1-2          (naturally coded; _Iinc2_1 omitted)
i.donova          _Idonova_1-2        (_Idonova_1 for donova==0 omitted)

Generalized linear models                         No. of obs      =        374
Optimization     : MQL Fisher scoring             Residual df     =        369
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  652.4588348                   (1/df) Deviance =   1.768181
Pearson          =  592.2582469                   (1/df) Pearson  =   1.605036

Variance function: V(u) = u*(1-u/NrOva)           [Binomial]
Link function    : g(u) = ln(u/NrOva)             [Log]

                                                  BIC             =  -1533.592

                              (Std. Err. adjusted for 374 clusters in ptnr)
------------------------------------------------------------------------------
             |             Semirobust
      GQDAY2 | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Iinc2_2 |   1.015209   .0504566     0.30   0.761     .9209797    1.119079
      ageova |   1.002432   .0053321     0.46   0.648     .9920358    1.012938
  _Idonova_2 |   .9830849   .0562256    -0.30   0.765     .8788368    1.099699
         mii |    .990021   .0026132    -3.80   0.000     .9849125     .995156
       _cons |   .5595437   .0989846    -3.28   0.001     .3955983     .791432
------------------------------------------------------------------------------
No difference in good probability at day 2 p=.761

. xi: binreg GQDAY3 i.inc2 ageova i.donova mii, rr n(NrOva) vce(cluster ptnr)
i.inc2            _Iinc2_1-2          (naturally coded; _Iinc2_1 omitted)
i.donova          _Idonova_1-2        (_Idonova_1 for donova==0 omitted)
```

```
Generalized linear models                         No. of obs      =        372
Optimization     : MQL Fisher scoring             Residual df     =        367
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  804.3363556                   (1/df) Deviance =   2.191652
Pearson          =  740.8271866                   (1/df) Pearson  =   2.018603

Variance function: V(u) = u*(1-u/NrOva)           [Binomial]
Link function    : g(u) = ln(u/NrOva)             [Log]

                                                  BIC             =  -1367.898

                                (Std. Err. adjusted for 372 clusters in ptnr)
------------------------------------------------------------------------------
             |               Semirobust
      GQDAY3 | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Iinc2_2 |   1.154268   .0790091     2.10   0.036     1.009351    1.319991
      ageova |   .9961983   .0079541    -0.48   0.633     .9807299    1.011911
  _Idonova_2 |   .9581891   .0841636    -0.49   0.627     .8066498    1.138197
         mii |   .9855162   .0041316    -3.48   0.001     .9774515    .9936474
       _cons |    .497486   .1320792    -2.63   0.009       .29566    .8370842
------------------------------------------------------------------------------
```

Significant difference in good probability on day 3. rr=1.15 (95%CI 1.01 to 1.32), p=.036. Thus the Minc incubator has a 15% (relative) probability of having good embryos on day 3 compared to the Forma type. From the descriptive statistics we see that the mean proportion good embryos on day 3 is .393 in the Forma incubators versus .445 in the Minc incubator. The crude ratio is 1.13 which is close to the adjusted ratio from the model.

```
. xi: binreg GQDAY5 i.inc2 ageova i.donova mii, rr n(NrOva) vce(cluster ptnr)
i.inc2            _Iinc2_1-2          (naturally coded; _Iinc2_1 omitted)
i.donova          _Idonova_1-2        (_Idonova_1 for donova==0 omitted)


Generalized linear models                         No. of obs      =        282
Optimization     : MQL Fisher scoring             Residual df     =        277
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  684.6339911                   (1/df) Deviance =   2.471603
Pearson          =   612.658972                   (1/df) Pearson  =   2.211765

Variance function: V(u) = u*(1-u/NrOva)           [Binomial]
Link function    : g(u) = ln(u/NrOva)             [Log]

                                                  BIC             =  -878.1743

                                (Std. Err. adjusted for 282 clusters in ptnr)
------------------------------------------------------------------------------
             |               Semirobust
      GQDAY5 | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    _Iinc2_2 |   1.016109   .1397514     0.12   0.908     .7760137    1.330488
      ageova |   .9966452   .0126569    -0.26   0.791     .9721443    1.021764
  _Idonova_2 |   .9181337   .1211473    -0.65   0.517     .7089087    1.189109
         mii |   .9785701   .0079925    -2.65   0.008     .9630298    .9943611
       _cons |   .3322401   .1460856    -2.51   0.012     .1403394    .7865466
------------------------------------------------------------------------------
```

No difference

One days 2 and 5 there is no effect whereas on day 5 there is some effect. Not sure why one do not see a consistent effect. Drop of in number at day 5 n=282.

17. Pregnancy Rate

```
. . tabulate PR inc2, exact col


           |         inc2
        PR |     Forma      Mincs |     Total
-----------+----------------------+----------
         0 |       129         20 |       149
           |     40.69      37.74 |     40.27
-----------+----------------------+----------
         1 |        24          5 |        29
```

```
           |      7.57        9.43 |       7.84
-----------+---------------------+-----------
         2 |         1           0 |         1
           |      0.32        0.00 |       0.27
-----------+---------------------+-----------
         3 |         1           0 |         1
           |      0.32        0.00 |       0.27
-----------+---------------------+-----------
         4 |         3           0 |         3
           |      0.95        0.00 |       0.81
-----------+---------------------+-----------
         5 |        15           4 |        19
           |      4.73        7.55 |       5.14
-----------+---------------------+-----------
         6 |       111          16 |       127
           |     35.02       30.19 |      34.32
-----------+---------------------+-----------
         7 |         6           2 |         8
           |      1.89        3.77 |       2.16
-----------+---------------------+-----------
         8 |        12           1 |        13
           |      3.79        1.89 |       3.51
-----------+---------------------+-----------
         9 |         3           0 |         3
           |      0.95        0.00 |       0.81
-----------+---------------------+-----------
        31 |         4           2 |         6
           |      1.26        3.77 |       1.62
-----------+---------------------+-----------
        32 |         5           3 |         8
           |      1.58        5.66 |       2.16
-----------+---------------------+-----------
        33 |         3           0 |         3
           |      0.95        0.00 |       0.81
-----------+---------------------+-----------
     Total |       317          53 |       370
           |    100.00      100.00 |    100.00

           Fisher's exact =                 0.575
```

No difference – all the categories are close

17.1 Foetal Heart

```
. tabulate FH inc2, exact col
```

```
+--------------------+
| Key                |
|--------------------|
|     frequency      |
| column percentage  |
+--------------------+
```

```
           |         inc2
        FH |     Forma      Mincs |     Total
-----------+---------------------+-----------
         0 |       136         22 |       158
           |     49.45      46.81 |     49.07
-----------+---------------------+-----------
         1 |        89         20 |       109
           |     32.36      42.55 |     33.85
-----------+---------------------+-----------
         2 |        47          5 |        52
           |     17.09      10.64 |     16.15
-----------+---------------------+-----------
         3 |         3          0 |         3
           |      1.09       0.00 |      0.93
-----------+---------------------+-----------
     Total |       275         47 |       322
           |    100.00     100.00 |    100.00

           Fisher's exact =                 0.484
```

```
. tabulate FH inc2, exact col

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

           |         inc2
        FH |     Forma      Mincs |     Total
-----------+----------------------+----------
         0 |       136         22 |       158
           |     49.45      46.81 |     49.07
-----------+----------------------+----------
         1 |        89         20 |       109
           |     32.36      42.55 |     33.85
-----------+----------------------+----------
         2 |        47          5 |        52
           |     17.09      10.64 |     16.15
-----------+----------------------+----------
         3 |         3          0 |         3
           |      1.09       0.00 |      0.93
-----------+----------------------+----------
     Total |       275         47 |       322
           |    100.00     100.00 |    100.00

           Fisher's exact =                 0.484

No difference
```

18. Clinical Pregnancy Rate

```
. tabulate inc2 clin_pr, chi2 row

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

           |        clin_pr
      inc2 |         0          1 |     Total
-----------+----------------------+----------
     Forma |       173        144 |       317
           |     54.57      45.43 |    100.00
-----------+----------------------+----------
     Mincs |        28         25 |        53
           |     52.83      47.17 |    100.00
-----------+----------------------+----------
     Total |       201        169 |       370
           |     54.32      45.68 |    100.00

          Pearson chi2(1) =   0.0557   Pr = 0.813

No difference
Some more modelling of risk difference (rd). Adjusted for age of ova, procedure used
(IVF,PICSI ,ICSI),donor ova used,surrogate used and number of mii.

. xi: binreg clin_pr i.inc2 ageova i.proc3 i.donova i.surr mii, rd
i.inc2            _Iinc2_1-2          (naturally coded; _Iinc2_1 omitted)
i.proc3           _Iproc3_0-2         (naturally coded; _Iproc3_0 omitted)
i.donova          _Idonova_1-2        (_Idonova_1 for donova==0 omitted)
i.surr            _Isurr_0-1          (naturally coded; _Isurr_0 omitted)

Generalized linear models                         No. of obs      =        369
Optimization     : MQL Fisher scoring             Residual df     =        361
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  494.2326504                   (1/df) Deviance =   1.369066
Pearson          =  369.0002449                   (1/df) Pearson  =   1.022161

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

BIC = -1639.565

clin_pr	EIM Risk Diff.	Std. Err.	z	P>|z|	[95% Conf.	Interval]
_Iinc2_2	.0482779	.0733129	0.66	0.510	-.0954128	.1919686
ageova	.0085928	.0077341	1.11	0.267	-.0065657	.0237514
_Iproc3_1	-.152674	.0871449	-1.75	0.080	-.3234748	.0181268
_Iproc3_2	-.0766151	.0860183	-0.89	0.373	-.2452078	.0919777
_Idonova_2	.1953642	.0808181	2.42	0.016	.0369636	.3537648
_Isurr_1	-.0306542	.1606965	-0.19	0.849	-.3456136	.2843051
mii	.0077962	.0042126	1.85	0.064	-.0004604	.0160528
_cons	.1486018	.2748562	0.54	0.589	-.3901065	.6873101

Estimated difference in pregnancy rate between minca and Forma incubators is 4.8% (95%CI: -9.5 to 19.2%)
,p=.51. This adjusted difference is larger than the crude difference (47.2 versus 45.4%) and is due to the differences in adjusted factors. Confidence interval is wide reflecting the uncertainty of the sample size and estimate. No evidence based on the current study to say that the incubators have different performance.
Intesting to note that there is a huge difference in clinical pregnancy rate between donated and orginal ova. 19.5% difference , p=.016. Suppose this is well known.

Appendix XVII - Prospective Data Results

Clinical Pregnancy [Positive Foetal Sac]

1. Endometriosis

```
. tabulate fd_2 clin_pr, chi2 row

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

   FDiag== |        clin_pr
    2.0000 |         0          1 |     Total
-----------+----------------------+----------
         0 |       387        164 |       551
           |     70.24      29.76 |    100.00
-----------+----------------------+----------
         1 |        48         20 |        68
           |     70.59      29.41 |    100.00
-----------+----------------------+----------
     Total |       435        184 |       619
           |     70.27      29.73 |    100.00

          Pearson chi2(1) =   0.0036   Pr = 0.952
```

Endometriosis and cpr by site (already done previously with comments – see above)

```
. by site, sort : tabulate fd_2 clin_pr, chi2 row

-------------------------------------------------------------------------------------------
-> site = aev

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

   FDiag== |        clin_pr
    2.0000 |         0          1 |     Total
-----------+----------------------+----------
         0 |       242        140 |       382
           |     63.35      36.65 |    100.00
-----------+----------------------+----------
         1 |        36         15 |        51
           |     70.59      29.41 |    100.00
-----------+----------------------+----------
     Total |       278        155 |       433
           |     64.20      35.80 |    100.00

          Pearson chi2(1) =   1.0255   Pr = 0.311

-------------------------------------------------------------------------------------------
-> site = tbh

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

   FDiag== |        clin_pr
    2.0000 |         0          1 |     Total
-----------+----------------------+----------
         0 |       145         24 |       169
           |     85.80      14.20 |    100.00
-----------+----------------------+----------
         1 |        12          5 |        17
```

```
           |     70.59      29.41 |    100.00
-----------+----------------------+----------
     Total |       157         29 |       186
           |     84.41      15.59 |    100.00

          Pearson chi2(1) =   2.7155   Pr = 0.099
```

2. Tubal Factor

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

   FDiag== |        clin_pr
   14.0000 |         0           1 |     Total
-----------+----------------------+----------
         0 |       368         164 |       532
           |     69.17      30.83 |    100.00
-----------+----------------------+----------
         1 |        67          20 |        87
           |     77.01      22.99 |    100.00
-----------+----------------------+----------
     Total |       435         184 |       619
           |     70.27      29.73 |    100.00

          Pearson chi2(1) =   2.1993   Pr = 0.138
```

Tubal factor . by site, sort : tabulate fd_6 clin_pr, chi2 row

```
-------------------------------------------------------------------------------------------
-> site = aev

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

   FDiag== |        clin_pr
   14.0000 |         0           1 |     Total
-----------+----------------------+----------
         0 |       252         144 |       396
           |     63.64      36.36 |    100.00
-----------+----------------------+----------
         1 |        26          11 |        37
           |     70.27      29.73 |    100.00
-----------+----------------------+----------
     Total |       278         155 |       433
           |     64.20      35.80 |    100.00

          Pearson chi2(1) =   0.6480   Pr = 0.421

-------------------------------------------------------------------------------------------
-> site = tbh

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

   FDiag== |        clin_pr
   14.0000 |         0           1 |     Total
-----------+----------------------+----------
         0 |       116          20 |       136
           |     85.29      14.71 |    100.00
-----------+----------------------+----------
         1 |        41           9 |        50
           |     82.00      18.00 |    100.00
-----------+----------------------+----------
```

```
     Total |       157         29 |       186
           |     84.41      15.59 |    100.00

          Pearson chi2(1) =   0.3014   Pr = 0.583
```

---

3. Descriptive Statistics

3.1 Overall

```
. tabstat PatientAge DonorAge OocyteAge BMI count1 count2 mot1 mot2 NROocytes NrMII NrFer
> t GQED2 GQED3 GQED4 GQED5 GQED6 GQED7 DayET NrET GQEET NrET, statistics( count min p25
> p50 p75 max ) by(site) varwidth(8) columns(statistics)
```

site	variable	N	min	p25	p50	p75	max
1	Patien~e	572	23	34	37	40	54
	DonorAge	108	19	23	24	28	44
	Oocyte~e	572	19	30	35	39	46
	BMI	468	15.12	20.855	23.31	26.5	45.36
	count1	430	0	15	40	70	150
	count2	429	0	3	12	30	101
	mot1	430	0	30	50	55	80
	mot2	429	0	50	90	95	99
	NROocy~s	463	0	4	8	13	66
	NrMII	460	0	3	6	11	46
	NrFert	427	0	3	5	9	39
	GQED2	415	0	2	4	7	28
	GQED3	366	0	2	4	7	22
	GQED4	44	0	0	1.5	3	10
	GQED5	247	0	1	3	5	17
	GQED6	28	0	0	0	1.5	6
	GQED7	8	0	0	0	0	1
	DayET	479	2	3	5	5	7
	NrET	533	0	1	2	2	8
	GQEET	478	0	1	2	2	5
	NrET	533	0	1	2	2	8
2	Patien~e	248	25	32	35	38	45
	DonorAge	6	20	22	24.5	35	35
	Oocyte~e	248	20	31	35	38	45
	BMI	232	17.8	23.59	26.97	31.075	47.78
	count1	224	0	4.5	40	65	300
	count2	225	.01	.5	10	20	101
	mot1	218	0	30	50	50	90
	mot2	219	0	40	90	95	99
	NROocy~s	241	0	2	3	5	30
	NrMII	224	0	2	3	4	30
	NrFert	225	0	1	2	3	28
	GQED2	198	0	1	2	3	14
	GQED3	173	0	1	1	2	10
	GQED4	40	0	0	1	2	4
	GQED5	19	0	0	1	3	9
	GQED6	1	0	0	0	0	0
	GQED7	0	.	.	.	.	.
	DayET	185	1	3	3	3	6
	NrET	248	0	0	2	2	4
	GQEET	185	0	1	2	2	4
	NrET	248	0	0	2	2	4
Total	Patien~e	820	23	33	36	40	54
	DonorAge	114	19	23	24	28	44
	Oocyte~e	820	19	31	35	38	46
	BMI	700	15.12	21.625	24.71	28.13	47.78
	count1	654	0	10	40	70	300
	count2	654	0	2	10	30	101
	mot1	648	0	30	50	55	90
	mot2	648	0	50	90	95	99
	NROocy~s	704	0	3	5	11	66
	NrMII	684	0	3	5	9	46
	NrFert	652	0	2	4	7	39
	GQED2	613	0	2	3	6	28
	GQED3	539	0	1	3	5	22

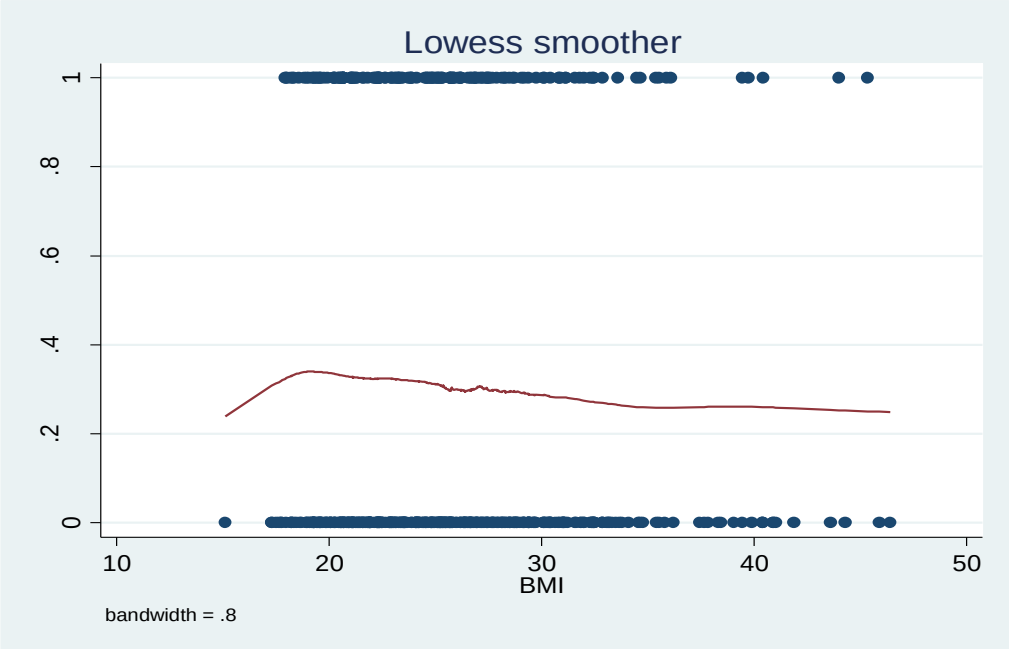
```
            GQED4 |         84          0          0          1          3         10
            GQED5 |        266          0          1          3          5         17
            GQED6 |         29          0          0          0          1          6
            GQED7 |          8          0          0          0          0          1
            DayET |        664          1          3          4          5          7
             NrET |        781          0          1          2          2          8
            GQEET |        663          0          1          2          2          5
             NrET |        781          0          1          2          2          8
-------------------------------------------------------------------------------------

. tabstat AMH BMI OocyteAge  NROocytes NrMII DayET NrET GQEET, statistics( count min p25 p
> 50 p75 max ) by(site) varwidth(8) columns(statistics)

site     variable |          N        min        p25        p50        p75        max
------------------+--------------------------------------------------------------------
1  aevitas    AMH |        195        .08        .38        1.2       2.53      12.25
              BMI |        468      15.12     20.855      23.31       26.5      45.36
         Oocyte~e |        572         19         30         35         39         46
         NROocy~s |        463          0          4          8         13         66
            NrMII |        460          0          3          6         11         46
            DayET |        479          2          3          5          5          7
             NrET |        533          0          1          2          2          8
            GQEET |        478          0          1          2          2          5
------------------+--------------------------------------------------------------------
2  TBH        AMH |         85        .16        .72       1.38       3.48      18.88
              BMI |        232       17.8      23.59      26.97     31.075      47.78
         Oocyte~e |        248         20         31         35         38         45
         NROocy~s |        241          0          2          3          5         30
            NrMII |        224          0          2          3          4         30
            DayET |        185          1          3          3          3          6
             NrET |        248          0          0          2          2          4
            GQEET |        185          0          1          2          2          4
------------------+--------------------------------------------------------------------
Total        AMH |        280        .08       .505        1.3       2.78      18.88
              BMI |        700      15.12     21.625      24.71      28.13      47.78
         Oocyte~e |        820         19         31         35         38         46
         NROocy~s |        704          0          3          5         11         66
            NrMII |        684          0          3          5          9         46
            DayET |        664          1          3          4          5          7
             NrET |        781          0          1          2          2          8
            GQEET |        663          0          1          2          2          5
```

3.2 BMI

Non-parametric smooth of cpr on bmi.



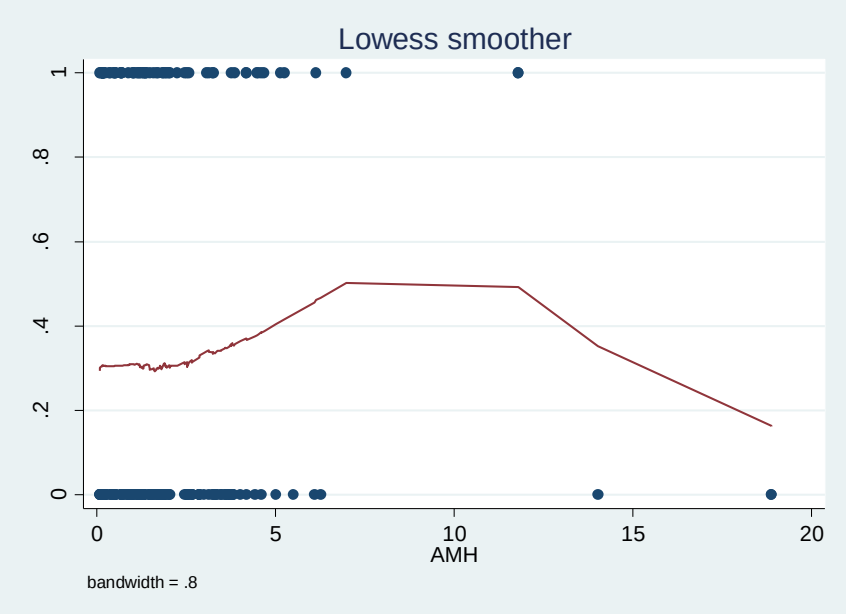
Excluding one case with BMI<16

Small decrease in cpr over increase in bmi. Data sparse above BMI>40.

Number of participants over BMI=40 is small

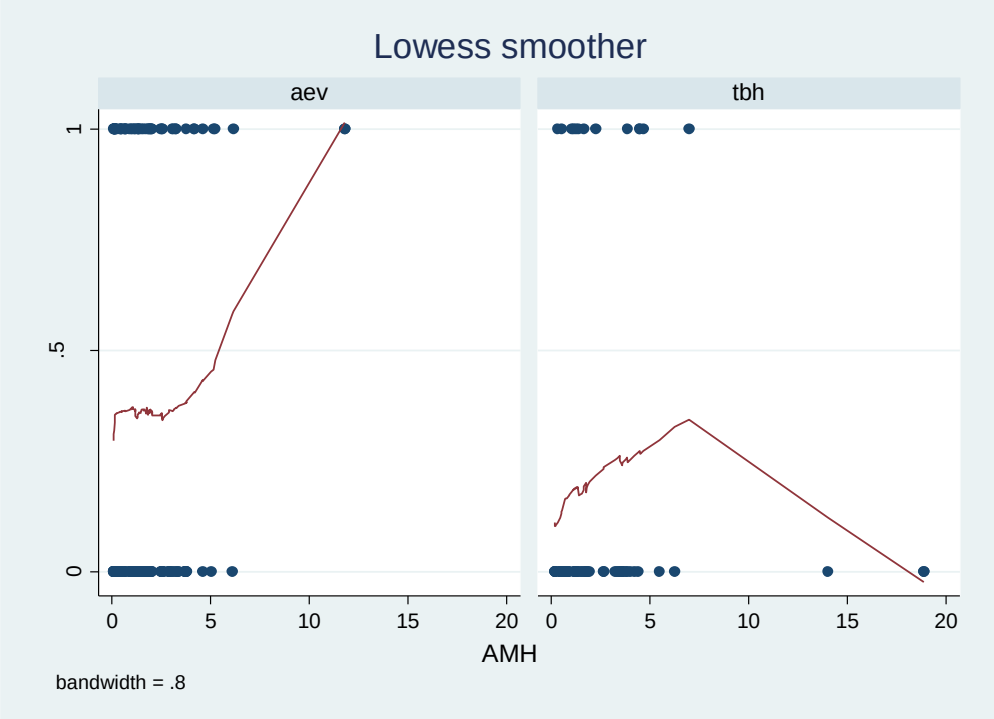
BMI and cpr by site and endometriosis. Endo has lower cpr but association the same across BMI.

3.3 AMH



Restricted to AMH <8

No association up to AMH=4 then an increase – number above 4 small



Outlying values

Restricted to <10

Trend is the same by at different levels. **Lots of missing data** which limits it use in the models.

3.4 Age of Oocyte

Very few young patients in tbh. Overall trends are the same confirming results from pooled analysis. No interaction between oocyteage and site with respect to the outcome clinical pregnancy.

4. Regression models for CPR using the above factors as determinants in the model.

Doing a binomial regression model with each of the determinants with CPR adjusted for **site** and **age** of oocytes

4.1 BMI

```
. xi: binreg clin_pr BMI OocyteAge i.site , rr
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)


Generalized linear models                         No. of obs      =        538
Optimization     : MQL Fisher scoring             Residual df     =        534
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  626.5595503                   (1/df) Deviance =   1.173332
Pearson          =  537.6985313                   (1/df) Pearson  =   1.006926

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u)                   [Log]

                                                  BIC             =  -2731.157

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         BMI |   1.010109   .0124765     0.81   0.415     .9859497    1.034861
   OocyteAge |   .9771112   .0100395    -2.25   0.024     .9576311    .9969877
    _Isite_2 |   .3976129   .0773101    -4.74   0.000     .2716168    .5820555
       _cons |   .6415825      .2856    -1.00   0.319     .2681271    1.535198
------------------------------------------------------------------------------
```

BMI not associated in the basic adjusted model

4.2 AMH

```
. xi: binreg clin_pr AMH OocyteAge i.site , rr
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)
Generalized linear models                         No. of obs      =        215
Optimization     : MQL Fisher scoring             Residual df     =        211
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  256.7494394                   (1/df) Deviance =   1.216822
Pearson          =  215.4731523                   (1/df) Pearson  =     1.0212

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u)                   [Log]

                                                  BIC             =  -876.4552

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         AMH |   1.044782   .0333424     1.37   0.170     .9814338    1.112219
   OocyteAge |   .9785973       .014    -1.51   0.130      .951539    1.006425
    _Isite_2 |   .5059629   .1417104    -2.43   0.015     .2922225    .8760395
       _cons |   .7039241    .339798    -0.73   0.467     .2732965    1.813083
------------------------------------------------------------------------------
```

AMH not associated in this basic adjusted model. AMH not further used due to the extensive missing values in the data >65%!. This level of missingness can introduce severe bias in any result.

4.3 Endometriosis

```
. xi: binreg clin_pr fd_2 OocyteAge i.site , rr
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)


Generalized linear models                         No. of obs      =        619
Optimization     : MQL Fisher scoring             Residual df     =        615
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  717.3802174                   (1/df) Deviance =   1.166472
Pearson          =  617.9320284                   (1/df) Pearson  =   1.004768

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u)                   [Log]

                                                  BIC             =  -3235.905

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        fd_2 |   .9321431   .1841266    -0.36   0.722     .6329136    1.372843
   OocyteAge |   .9726291   .0094621    -2.85   0.004     .9542594    .9913524
    _Isite_2 |   .4482482   .0819427    -4.39   0.000     .3132661    .6413922
       _cons |   .9022013   .2851496    -0.33   0.745     .4855935    1.676232
------------------------------------------------------------------------------
Not associated
```

4.4 Tubal Factor

```
. xi: binreg clin_pr fd_6 OocyteAge i.site , rr
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)


Generalized linear models                         No. of obs      =        619
Optimization     : MQL Fisher scoring             Residual df     =        615
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  717.3898132                   (1/df) Deviance =   1.166488
Pearson          =  617.3270852                   (1/df) Pearson  =   1.003784

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u)                   [Log]

                                                  BIC             =  -3235.895

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
        fd_6 |   .9311696   .1893196    -0.35   0.726     .6251263    1.387043
   OocyteAge |   .9726314   .0094657    -2.85   0.004     .9542548    .9913618
    _Isite_2 |   .4555812   .0849225    -4.22   0.000     .3161534    .6564985
       _cons |   .8999005   .2852886    -0.33   0.739     .4834422    1.675114
------------------------------------------------------------------------------
Not associated
```

5. Combined binomial regression model

```
. xi: binreg clin_pr BMI fd_2 fd_6 OocyteAge i.site  , rr
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)

Generalized linear models                         No. of obs      =        538
Optimization     : MQL Fisher scoring             Residual df     =        532
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  626.4162669                   (1/df) Deviance =   1.177474
Pearson          =  537.9328769                   (1/df) Pearson  =   1.011152

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u)                   [Log]

                                                  BIC             =  -2718.724
```

```
------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         BMI |   1.010287   .0125675     0.82   0.411     .9859533    1.035222
        fd_2 |   1.002177   .1997848     0.01   0.991     .6780419    1.481263
        fd_6 |   .9246829   .1969432    -0.37   0.713      .609116    1.403737
   OocyteAge |   .9772056   .0100608    -2.24   0.025     .9576844    .9971247
    _Isite_2 |   .4025823   .0794859    -4.61   0.000     .2733967    .5928107
       _cons |   .6408761   .2864065    -1.00   0.319     .2669153    1.538773
------------------------------------------------------------------------------
```

- BMI, endo, tubal not associated with cpr
- Age of oocytes is significantly associated rr=.98 (95%CI: .96 to .99), p=.025. Thus for every extra year the probability is 2% less than of the base year (relative).
- Site TBH: negatively associated with cpr. Site is a proxy for the patient populations seen.

6. Extended model

Due to the strong impact of site the interaction of site with each of the determinants were investigated. There was a significant interaction between site and endometriosis. This term was therefore included in the final model. There were no other interactions: no interactions between oocyteage and site for example. Therefore the parameters estimated for oocyte age for example is the pooled estimate from both sites.

```
. xi: binreg clin_pr BMI i.site*fd_2 fd_6 OocyteAge i.site  , rr
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)
i.site*fd_2       _IsitXfd_2_#        (coded as above)
note: _Isite_2 omitted because of collinearity


Generalized linear models                         No. of obs      =        538
Optimization     : MQL Fisher scoring             Residual df     =        531
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  623.2882881                   (1/df) Deviance =   1.173801
Pearson          =  537.9222429                   (1/df) Pearson  =   1.013036

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u)                   [Log]

                                                  BIC             =  -2715.565

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         BMI |   1.010459   .0125411     0.84   0.402     .9861754     1.03534
    _Isite_2 |   .3552575   .0774896    -4.74   0.000     .2316739    .5447653
        fd_2 |   .8778275   .2006671    -0.57   0.569     .5608267    1.374009
_IsitXfd_2_2 |   2.573437   1.230724     1.98   0.048     1.007938    6.570425
        fd_6 |   .9467217   .2006154    -0.26   0.796     .6249542    1.434156
   OocyteAge |   .9766213   .0099948    -2.31   0.021     .9572271    .9964085
    _Isite_2 |          1  (omitted)
       _cons |   .6584946    .292872    -0.94   0.348      .275405    1.574463
------------------------------------------------------------------------------.
 lincom fd_2+ _IsitXfd_2_2 , eform

 ( 1)  fd_2 + _IsitXfd_2_2 = 0
------------------------------------------------------------------------------
     clin_pr |      exp(b)   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         (1) |   2.259034   .9565067     1.92   0.054     .9851722    5.180044
```

- Impact endo on cpr significantly different between the two sites.
  For the Aviates site endo is not associated with cpr rr=.88 (95%CI: 1.01 to 6.57), p=.569. For TBH endometriosis diagnosis is positively associated with cpr, rr=2.26 (95%CI: .99 to 5.18) p=.054. Both these estimates are adjusted for the other factors in the model.
- Oocyte age is negatively associated with cpr. The rr=.98 (95%ci: .96 to .99) , p=.021 indicates that the probability of having a clinical pregnancy decreases by a relative 2% for every year. The association is linear over age – non-linear effects not significant (tests not shown)
- BMI not associated with cpr. Again the same effect over both sites.

- Tubal factor not a determinant for cpr, p=.796. Again there was no association with site and hence this estimate of the rr is a stable one across both sites.

7. CPR tabulated by ENDOMETRIOSiS by site to understand results from the model.

. by site, sort : tabulate fd_2 clin_pr, chi2 row (endo)

---

site = 1 Private Clinic

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

   FDiag== |        clin_pr
    2.0000 |         0          1 |     Total
-----------+----------------------+----------
         0 |       242        140 |       382
           |     63.35      36.65 |    100.00
-----------+----------------------+----------
         1 |        36         15 |        51
           |     70.59      29.41 |    100.00
-----------+----------------------+----------
     Total |       278        155 |       433
           |     64.20      35.80 |    100.00

          Pearson chi2(1) =   1.0255   Pr = 0.311
```

---

site = 2 – Public Clinc

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

   FDiag== |        clin_pr
    2.0000 |         0          1 |     Total
-----------+----------------------+----------
         0 |       145         24 |       169
           |     85.80      14.20 |    100.00
-----------+----------------------+----------
         1 |        12          5 |        17
           |     70.59      29.41 |    100.00
-----------+----------------------+----------
     Total |       157         29 |       186
           |     84.41      15.59 |    100.00

          Pearson chi2(1) =   2.7155   Pr = 0.099
```

7.1 Aevitas model

```
. xi: binreg clin_pr BMI fd_2 fd_6 OocyteAge if site==1 , rr

Generalized linear models                         No. of obs      =        362
Optimization     : MQL Fisher scoring             Residual df     =        357
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  475.0962933                   (1/df) Deviance =   1.330802
Pearson          =  361.2208737                   (1/df) Pearson  =   1.011823

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u)                   [Log]

                                                  BIC             =  -1628.221
```

```
------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         BMI |   1.010718   .0137547     0.78   0.433     .9841155    1.038039
 endo   fd_2 |   .8672684   .1983603    -0.62   0.534     .5539467     1.35781
 tubal  fd_6 |   .8274202   .2187221    -0.72   0.474     .4928532    1.389104
   OocyteAge |   .9776321    .010385    -2.13   0.033     .9574883    .9981997
       _cons |   .6400714   .3050133    -0.94   0.349      .251539    1.628739
------------------------------------------------------------------------------
```

7.2 TBH model

```
. xi: binreg clin_pr BMI fd_2 fd_6 OocyteAge if site==2 , rr

Generalized linear models                         No. of obs      =        176
Optimization     : MQL Fisher scoring             Residual df     =        171
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  147.0799297                   (1/df) Deviance =   .8601165
Pearson          =  176.9143285                   (1/df) Pearson  =   1.034587

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u)                   [Log]

                                                  BIC             =  -737.0728

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Risk Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         BMI |    1.01028   .0315699     0.33   0.743     .9502609     1.07409
        fd_2 |   2.599755   1.164539     2.13   0.033     1.080547    6.254912
        fd_6 |   1.363064   .5574569     0.76   0.449     .6115013    3.038331
   OocyteAge |   .9688466    .037524    -0.82   0.414     .8980231    1.045256
       _cons |   .2733733   .4179925    -0.85   0.396     .0136538    5.473411
------------------------------------------------------------------------------
```

8. Models with MII Oocytes

8.1 Model with all interactions

For formal analysis of the risk factors see models above.

Also table with descriptive stats above by site done previously.

Models with NR MII returned

The relative risk models did not converge and I therefore used a binomial regression model with odds ratios to quantify the risk.

Started with a model that had all the intereactions. This is to test if any association with cpr is different between sites.

The first model shows that there is no factor with an interaction with site. Took out some of the interactions but in the end none were significant. The final model is therefore a main effects model where there is an adjustment for site only. The site effect adjusts for the level of response in cpr but not in the type of association. In the final model only site and number of MII oocytes available were significant factors. This is true across both sites One get a very stable estimate by doing a pooled analysis. With adjusting for Nr MII also in the model the interaction of endometriosis and site disappear. None of the other factors are significant. Nr MII is therefore such a dominant factor that it swamps all other risk factors

Model with all interactions(yellow)

```
.  xi: binreg clin_pr i.site*BMI i.site*fd_2 i.site*fd_6 i.site*OocyteAge i.site  i.site*N
> rMII , or
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)
i.site*BMI        _IsitXBMI_#         (coded as above)
i.site*fd_2       _IsitXfd_2_#        (coded as above)
```

```
i.site*fd_6       _IsitXfd_6_#        (coded as above)
i.site*Oocyte~e   _IsitXOocyt_#       (coded as above)
i.site*NrMII      _IsitXNrMII_#       (coded as above)

Generalized linear models                         No. of obs      =        457
Optimization     : MQL Fisher scoring             Residual df     =        445
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  507.3379559                   (1/df) Deviance =   1.140085
Pearson          =  455.0601087                   (1/df) Pearson  =   1.022607

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -2218.146

------------------------------------------------------------------------------
             |                 EIM
      clin_pr | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
     _Isite_2 |   2.242697   4.992907     0.36   0.717     .0285591    176.1153
          BMI |   1.042624   .0274358     1.59   0.113     .9902142    1.097808
  _IsitXBMI_2 |   .9763252   .0443222    -0.53   0.598     .8932079    1.067177
     _Isite_2 |          1  (omitted)
         fd_2 |   .9152843   .3522441    -0.23   0.818     .4305009    1.945979
 _IsitXfd_2_2 |   3.293774   2.394945     1.64   0.101     .7920898    13.69662
     _Isite_2 |          1  (omitted)
         fd_6 |   .6717523    .316736    -0.84   0.399     .2666008     1.69261
 _IsitXfd_6_2 |   2.201427   1.492286     1.16   0.244     .5830404    8.312084
     _Isite_2 |          1  (omitted)
    OocyteAge |   .9974493   .0241534    -0.11   0.916     .9512154     1.04593
_IsitXOocyt_2 |   .9670134   .0527008    -0.62   0.538     .8690471    1.076023
     _Isite_2 |          1  (omitted)
     _Isite_2 |          1  (omitted)
        NrMII |   1.089135   .0269443     3.45   0.001     1.037585    1.143246
_IsitXNrMII_2 |   .9437219   .0593856    -0.92   0.357     .8342198    1.067598
        _cons |   .1233397    .140649    -1.84   0.066     .0131961    1.152818
------------------------------------------------------------------------------
```

8.2 Model with only certain interactions

```
.  xi: binreg clin_pr BMI i.site*fd_2 i.site*fd_6 i.site*OocyteAge i.site  NrMII , or
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)
i.site*fd_2       _IsitXfd_2_#        (coded as above)
i.site*fd_6       _IsitXfd_6_#        (coded as above)
i.site*Oocyte~e   _IsitXOocyt_#       (coded as above)
Generalized linear models                         No. of obs      =        457
Optimization     : MQL Fisher scoring             Residual df     =        447
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  508.4742871                   (1/df) Deviance =   1.137526
Pearson          =  458.2456187                   (1/df) Pearson  =   1.025158

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -2229.259

------------------------------------------------------------------------------
             |                 EIM
      clin_pr | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
          BMI |   1.034378   .0220338     1.59   0.113     .9920812    1.078477
     _Isite_2 |   .8078238   1.534963    -0.11   0.911     .0194954    33.47357
         fd_2 |   .9125414   .3491958    -0.24   0.811     .4310516    1.931861
 _IsitXfd_2_2 |   3.314479   2.411893     1.65   0.100      .796177    13.79815
     _Isite_2 |          1  (omitted)
         fd_6 |   .6847531   .3188442    -0.81   0.416      .274907     1.70562
 _IsitXfd_6_2 |   2.198688   1.488462     1.16   0.245      .583337      8.2872
     _Isite_2 |          1  (omitted)
    OocyteAge |   .9935871   .0234946    -0.27   0.786     .9485892    1.040719
_IsitXOocyt_2 |   .9693866     .05291    -0.57   0.569     .8710391    1.078838
     _Isite_2 |          1  (omitted)
        NrMII |   1.078187   .0236195     3.44   0.001     1.032873    1.125488
        _cons |   .1862943   .1943539    -1.61   0.107     .0241086     1.43955
------------------------------------------------------------------------------
```

8.3 Model with interactions with diagnostic factors

```
.  xi: binreg clin_pr BMI i.site*fd_2 i.site*fd_6 OocyteAge i.site  NrMII , or
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)
i.site*fd_2       _IsitXfd_2_#        (coded as above)
i.site*fd_6       _IsitXfd_6_#        (coded as above)
note: _Isite_2 omitted because of collinearity
note: _Isite_2 omitted because of collinearity

Generalized linear models                         No. of obs      =        457
Optimization     : MQL Fisher scoring             Residual df     =        448
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  508.7987489                   (1/df) Deviance =   1.135711
Pearson          =  456.5784715                   (1/df) Pearson  =   1.019148

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -2235.059

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         BMI |   1.033769   .0219799     1.56   0.118     .9915743    1.077759
    _Isite_2 |    .277525   .0958186    -3.71   0.000     .1410637    .5459952
        fd_2 |   .9130412   .3495845    -0.24   0.812     .4311049    1.933739
_IsitXfd_2_2 |   3.166845   2.286743     1.60   0.110     .7691018    13.03977
    _Isite_2 |          1  (omitted)
        fd_6 |   .6835159   .3181832    -0.82   0.414     .2744771    1.702123
_IsitXfd_6_2 |    2.24028   1.513321     1.19   0.232     .5960891    8.419641
   OocyteAge |   .9879087   .0211498    -0.57   0.570     .9473136    1.030243
    _Isite_2 |          1  (omitted)
       NrMII |   1.075949   .0232241     3.39   0.001      1.03138    1.122444
       _cons |   .2338454   .2251485    -1.51   0.131     .0354311    1.543382
------------------------------------------------------------------------------
```

8.4 Final model with all interaction omitted since they were not significant

```
.  xi: binreg clin_pr BMI fd_2 fd_6 OocyteAge i.site  NrMII , or
i.site            _Isite_1-2          (naturally coded; _Isite_1 omitted)

Generalized linear models                         No. of obs      =        457
Optimization     : MQL Fisher scoring             Residual df     =        450
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  512.0612712                   (1/df) Deviance =   1.137914
Pearson          =    456.50605                   (1/df) Pearson  =   1.014458

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -2244.046

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         BMI |   1.030499   .0216113     1.43   0.152     .9890006    1.073739
        fd_2 |    1.22024   .4015392     0.60   0.545     .6402421    2.325662
        fd_6 |   .9506806   .3169077    -0.15   0.879     .4946386    1.827179
   OocyteAge |   .9894298   .0211367    -0.50   0.619      .948858    1.031736
    _Isite_2 |   .3855531   .1062295    -3.46   0.001     .2246761    .6616244
       NrMII |   1.077137   .0231901     3.45   0.001     1.032631    1.123561
       _cons |   .2229341   .2132613    -1.57   0.117     .0341907    1.453602
------------------------------------------------------------------------------
```

- Tbh site has significantly lower cpr or=.39 (95%CI:.22 to .66), p=.001
- The number of MII oocytes available are significantly associated with cpr or=1.08 (95%CI: 1.03 to 1.12) p=.001. Nr MII used a a continuos variables and or increases by 8% for every additional mature occyte available. From the scatter plot and smooth the strong association is clear, linear and the same across both sites
- By testing for interactions and founding none we can pool the data and do a better analysis of the risk factors. The patient population at tbh has a lower cpr profile

but the risk profile with respect to other factors is the same as for AEV. By having more MI oocytes in tbh will offset the risk profile of tbh. Site is a proxy for many elements.

To reflect the association with Nr MII a lowess smooth and categorised table are presented. In the tbh table the cpr is lower but you still have the positive association. Thus taking the difference due to site into account the association of nR Mii is the same across both sites.

```
generate nummii= NrMII
(239 missing values generated)

. recode nummii (1/10=1) (11/20=2) (21/30=3) (30/max=4)
(nummii: 608 changes made)
```

9. Separate models for sites

9. 1 Site 1

```
xi: binreg clin_pr BMI fd_2 fd_6 OocyteAge  NrMII if site==1, or


Generalized linear models                         No. of obs      =        287
Optimization     : MQL Fisher scoring             Residual df     =        281
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  362.3304316                   (1/df) Deviance =   1.289432
Pearson          =  284.7727901                   (1/df) Pearson  =   1.013426

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -1227.984

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         BMI |   1.042624   .0274358     1.59   0.113     .9902142    1.097808
        fd_2 |   .9152843   .3522441    -0.23   0.818     .4305009    1.945979
        fd_6 |   .6717523    .316736    -0.84   0.399     .2666008     1.69261
   OocyteAge |   .9974493   .0241534    -0.11   0.916     .9512154     1.04593
      NrMII |   1.089135   .0269443     3.45   0.001     1.037585    1.143246
       _cons |   .1233397    .140649    -1.84   0.066     .0131961    1.152818
------------------------------------------------------------------------------
```

9.2 Site 2

```
.  xi: binreg clin_pr BMI fd_2 fd_6 OocyteAge  NrMII if site==2, or


Generalized linear models                         No. of obs      =        170
Optimization     : MQL Fisher scoring             Residual df     =        164
                   (IRLS EIM)                     Scale parameter =          1
Deviance         =  145.0075243                   (1/df) Deviance =   .8841922
Pearson          =  170.2873186                   (1/df) Pearson  =   1.038337

Variance function: V(u) = u*(1-u/1)               [Binomial]
Link function    : g(u) = ln(u/(1-u))             [Logit]

                                                  BIC             =  -697.2634

------------------------------------------------------------------------------
             |                 EIM
     clin_pr | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
         BMI |    1.01794   .0376562     0.48   0.631     .9467476    1.094486
        fd_2 |    3.01474   1.859843     1.79   0.074      .899766    10.10113
        fd_6 |   1.478814   .7202162     0.80   0.422      .569323    3.841211
   OocyteAge |   .9645469   .0470923    -0.74   0.460     .8765262    1.061407
      NrMII |   1.027841   .0594708     0.47   0.635     .9176461    1.151268
       _cons |   .2766136   .5289054    -0.67   0.502      .006521    11.73364
------------------------------------------------------------------------------
```

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