AN EVALUATION OF THE USE OF G-CSF AS AN ADJUNCT TO IVF IN WOMEN WHO HAVE PREVIOUSLY FAILED ATTEMPTS AT PREGNANCY WITH IVF

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfillment of the requirements for the degree of Master of Medicine in the branch of Obstetrics and Gynaecology.

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DECLARATION

I, Dr Tasneem Mohamed, declare that this research report is my own work. This report is being submitted to the University of the Witwatersrand (WITS) for the Master of Medicine degree (MMed). It will also be submitted to the College of Medicine of South Africa (CMSA) as part of the qualification requirements for the Fellowship of the College of Obstetrics and Gynaecology, South Africa (FCOG) (SA).

Dr Tasneem Mohamed

14 July 2017

DEDICATION

This research report is dedicated to my amazing children, Yahya and Mahdiya; and to my dearest husband Bilal. Without your help and understanding, writing this report would not have been possible.

PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

I presented this research in the form of a poster at the Faculty of Health Sciences biennial research day which was held on 1 September 2016.

An abstract has also been submitted for consideration for an oral presentation at the annual RCOG World congress held in Cape Town, South Africa on 20-22 March 2017.

ABSTRACT

Background

Recurrent IVF failures may result from implantation defects of which a thin endometrium is often implicated. Studies show that improved endometrial thickness increases the probability of successful IVF.

Objectives

To evaluate the effects of transcervical instillation of G-CSF as an adjunct to IVF. The study looked at the influence of G-CSF on the endometrium and on the achievement of pregnancy.

Methods

A retrospective cross-sectional study of women attending BioART Fertility Centre, who had two or more failed IVFs previously.

Results

There were a total of 49 women studied with a mean age of 38.9. Mean number of previous IVFs were 3.1. Comparison between those that achieved pregnancy and those that did not showed that age was a statistically significant factor (p-value 0.0005). Mean endometrial thickness pre and post-GCSF between the groups was not statistically significant (p-values >0.05).

Conclusion

With the use of G-CSF we achieved a clinical pregnancy rate of 34.69% and a statistically significant overall expansion of endometrial thickness (p-value 0.0029). However we failed to show any association between endometrial expansion and pregnancy outcome.

ACKNOWLEDGMENTS

I would like to thank the following people who assisted me in completing this research report:

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LIST OF ABREVIATIONS AND DEFINITIONS

AMH Anti-Müllerian Hormone
A hormone produced by granulosa cells in ovarian follicles.

ART Assisted Reproductive Technology
All treatments or procedures that include the in vitro handling of both oocytes and sperm, or embryo for the purpose of establishing a pregnancy.[1]

ß-HCG Beta unit of Human Chorionic Gonadotropin (see hCG)

FSH Follicle Stimulating Hormone
A hormone produced in and secreted by the pituitary gland, which promotes the development of sperm and ova.

G-CSF Granulocyte Colony Stimulating Factor
A glycoprotein/haematopoetic growth factor, also known as colony-stimulating factor 3, which stimulates the production and release of granulocytes and stem cells from the bone marrow.

GnRH Gonadotropin Releasing Hormone
A peptide hormone produced in the hypothalamus and transported through the blood stream to the pituitary gland, where it stimulates the synthesis and release of pituitary gonadotrophins.

hCG Human Chorionic Gonadotropin
A hormone that is produced by placental tissue. It maintains the secretion of progesterone by the corpus luteum in early pregnancy.

ICSI Intracytoplasmic Sperm Injection
A procedure whereby a single spermatozoon is injected into the cytoplasm of the oocyte.[1]

IQR	Interquartile range A measure of variability based on dividing a set of data into quartiles. The formula is the third quartile minus the first quartile or looking at the data between the 25th and 75th percentile with the 50th percentile being the median.
IVF	In Vitro Fertilisation The technique whereby an ovum is fertilised outside of the body. The zygote is then incubated in a laboratory until an embryo is formed which is then implanted in the uterus.
PRP	Platelet-rich Plasma
PCOS	Polycystic Ovarian Syndrome
RCT	Randomised Control Trial An intervention study to compare the outcome between two randomly allocated groups that are deliberately subjected to different regimes
SART	Society for Assisted Reproductive Technology
SD	Standard Deviation A measure of the amount of dispersion or variance of a set of data values from the mean or average

1. CHAPTER ONE - INTRODUCTION

1.1 GENERAL INTRODUCTION

Approximately 15% of couples will not fall pregnant within the first year of trying. This has sparked the emergence of novel therapies to try and assist with achieving pregnancy. The causes of infertility are multifactorial and manifold. Broadly they can be broken down into four main groups: (i) female factor, (ii) male factor, (iii) both female and male factor and (iv) unknown.

In Vitro Fertilisation ("**IVF**") is a useful modality to overcome some of these female factors in that super-ovulation can be induced with hormonal treatment and the in-vitro produced embryo can be placed directly into the uterus thus bypassing the fallopian tubes. For males, the semen is analysed and sorted, thus ensuring that only suitable sperm is used. In cases of severe male factor infertility where sperm morphology, number and motility are a problem, the sperm can be injected directly into the ovum ("**ICSI**").

For some infertile woman, pregnancy by standard IVF protocols is still not achieved, even if ovulation induction and embryo development is successful. This may be as a result of defective implantation. One common reason mentioned in the literature is a thin endometrium which can adversely affect receptivity of the endometrium to the transferred embryo and hence results in failure of the embryo to implant.

1.2 LITERATURE REVIEW

1.2.1 Assisted Reproductive Technology

The World Health Organisation describes Assisted Reproductive Technology ("**ART**") as all treatments or procedures that include the in vitro handling of both human oocytes and sperm, or embryos, for the purpose of establishing a pregnancy. IVF is an ART procedure most commonly used in the management of infertility. To date, millions of babies have been born successfully through the use of assisted reproductive technology ("**ART**").[2] Not only can ART be used for the treatment of infertility but also for enhancing the productivity of food animals, the conservation of endangered species,[3] and even more recently; limiting the

transmission of HIV in sero-discordant couples.[4] Certain genetic illnesses can also potentially be eliminated through the use of ART and pre-implantation genetic testing of embryos.

1.2.2 History of IVF

The first "IVF baby", Louise Brown was born in Britain in 1978. However, experiments into IVF had begun as early as a century before, in 1878.[3] The ability to fertilise oocytes in vitro has enabled major advances in reproductive technology and has expanded our knowledge and understanding regarding the process of fertilisation and early embryo development. This technology, though expensive and not readily available to all, has afforded many infertile couples the chance of pregnancy.

1.2.3 Infertility and causes thereof

Infertility is a common presenting problem in the practice of Obstetrics and Gynaecology with approximately one in six couples being affected.[5] Most texts defines infertility as the failure to achieve pregnancy after a period of 12 months with regular sexual intercourse without contraception. There appears to be an increasing prevalence of infertility in recent years. The exact reason for this is difficult to determine. It is speculated that the increase could be due to the fact that women nowadays are focusing more on their careers and hence delaying marriage and childbearing until they are older. It has been demonstrated through the evaluation of Follicle Stimulating Hormone ("**FSH**") and Anti-Müllerian Hormone ("**AMH**") levels that advancement in age is associated with reduced ovarian reserve and decreased reproductive potential.[6]

Habits such as smoking and alcohol consumption are on the rise and this can negatively affect semen and oocyte quality.[5,7] Also not to negate environmental factors such as radiation, with the increased use of cell phones, and its effect on semen and oocyte quality and number.[7]

According to The Society of Assisted Reproductive Technology ("**SART**") database of 2013[8], the causative factors and frequency are as follows:

Table 1.1: Causes of infertility and frequency thereof

Tubal Factor	6%	Male Factor	16%
Ovulatory Dysfunction	7%	Other Factor	7%
Diminished Ovarian Reserve	18%	Unknown Factor	13%
Endometriosis	3%	Multiple Female Factor	12%
Uterine Factor	1%	Female and Male Factor	17%

1.2.4 Granulocyte Colony Stimulating Factor ("G-CSF") in ART

G-CSF was initially identified as a growth factor for neutrophils. It is encoded for by a single gene located on chromosome 17 q11-22 and is produced mainly by cells of monocyte or macrophage origin.[9] In recent years, many additional effects of G-CSF on cell mediated immunity have been uncovered. It has been suggested that the use of G-CSF may in fact be beneficial in the treatment or prevention of immune mediated diseases such as graft-vs-host disease, multiple sclerosis, systemic lupus erythematosus, inflammatory bowel disease and diabetes.[10]

Studies have shown that G-CSF also has an important role to play in human reproduction and this has been the basis for further studies which have combined the use of G-CSF and ART. G-CSF through its "action on neutrophilic granulocyte proliferation and macrophages of decidual cells" has been shown to positively affect implantation.[11,12] In addition, "G-CSF recruits dendritic cells, promotes Th-2 cytokine secretion, activates T regulatory cells and stimulates various proangiogenic effects".[10,11] A study by Scarpellini F *et al.* has shown success with the use of G-CSF in the treatment of patients with recurrent and unexplained pregnancy losses.[13]

G-CSF has been found to be synthesized de novo in the female reproductive tract and hence it is hypothesized to have some influence on the growth and development of the embryo.[11] Spandorfer *et al.* conducted a study to determine whether G-CSF produced by autologous endometrial co-culture was associated with an improved outcome. The study looked at 53 patients with a history of multiple IVF failures. It involved analyzing the culture media which

contained endometrial cells either exposed or unexposed to human embryos. Their results showed that the level of G-CSF was not influenced by embryo exposure. Interestingly however, it showed that the level of G-CSF present did affect pregnancy outcomes, i.e. patients that had G-CSF levels > 130pg/ml were more likely to fall pregnant than those that had levels < 130pg/ml, and this result was statistically significant ($P<0.05$). [11,14]

Ziebe *et al.* in 2013 published a multicenter, randomized, placebo-controlled, double-blind prospective study, to evaluate the effect that G-CSF in embryo culture medium has on ongoing implantation rates. They looked at the ongoing implantation rate at 7 weeks gestation, then at 12 weeks and lastly at actual birth rate. It was concluded by their study that the addition of G-CSF to culture media increased the survival of the transferred embryos to week 12 and actual live birth rate.[11,15]

1.2.5 The effect of endometrial thickness on fertility

There are various factors to consider when determining the possibility of successful pregnancy with IVF therapy. These include the age of the patient, the number of oocytes aspirated, the quality of the embryos cultured and also the receptivity of the endometrium onto which the embryos are to implant. Problems with any of these factors can result in failed IVF therapy, and the presence of more than one of these factors will further decrease the likelihood of achieving pregnancy.

The measurement of endometrial thickness is an indirect measure of endometrial receptivity and is measured in the midsagittal plane by transvaginal ultrasound.[16,17] There is no evidence to state what is considered an exact ideal endometrial thickness, but it is thought to be between 7mm and 14mm. There are studies that show that an endometrial thickness of < 7mm or > 14mm correlates negatively with implantation rates.[16,18]

Al-Ghamdi *et al.* conducted a retrospective cohort study looking at the correlation of endometrial thickness on the day of hCG administration and pregnancy outcomes. It was a large study analysing 2464 cycles of IVF. The result of the study showed a positive linear relationship in pregnancy rate from 29.4% in patients with an endometrial thickness $\leq$ 6mm, to 44.4% in patients with an endometrial thickness $\geq$ 17mm.[16]

Richter *et al.* in a retrospective study looking at 1294 IVF cycles, investigated the relationship between endometrial thickness and implantation. They found a significant

correlation between clinical pregnancy and live birth rates with increasing endometrial thickness.[19] Importantly, they noted that this correlation was independent of the patients' age or the quality of the embryo transferred. Both of these are confounding factors and can also affect implantation success.

1.2.6 Therapies to improve endometrial thickness and/or receptivity

There are various therapies being employed to try and induce endometrial receptivity and thus improve implantation and pregnancy rates with IVF. These therapies are either hormonal/chemical or mechanical and will be discussed further.

Hormonal/ chemical

Sher *et al.* showed that vaginal sildenafil improved endometrial development in 70% of their patients studied, which resulted in higher implantation and ongoing pregnancy rates.[20] Another study by Takasaki *et al.* showed that in addition to sildenafil, vitamin E and l-arginine also has the potential to increase endometrial thickness by increasing uterine radial artery blood flow.[21] Sildenafil more commonly known by its popular trade name "Viagra" is a phosphodiesterase 5 (PDE5) inhibitor and a potent vasodilator.

Exogenously administered oestrogen and progesterone in a variety of regimens can be used to induce endometrial receptivity.[22] Chen *et al.* showed that extended oestrogen administration followed by frozen-thawed IVF programs is beneficial for women with thin endometrium.[23]

Qublan *et al.* in a randomised placebo-controlled trial of 120 women with thin endometrium $\leq$ 7mm, showed that women who received GnRH agonist on the day of oocyte retrieval, on the day of embryo transfer and again 3 days later, had significantly higher oestrogen and progesterone levels, a thicker endometrium, and higher implantation and pregnancy rates compared with the placebo group.[24,25]

A study in Leeds, UK, by Acharya S *et al.* looked at using combined pentoxifylline and tocopherol (Vitamin E) in women with a thin endometrium (< 6mm) who were undergoing assisted reproductive therapies. They found that its use was associated with improved endometrial growth.[26]

Mechanical

'Endometrial scratch' has become a colloquial term used to describe a procedure whereby multiple endometrial biopsies are taken.

It is hypothesised that this procedure may enhance endometrial receptivity and thus implantation in the following ways:

- "local injury to the proliferative phase endometrium might induce the decidualisation of the endometrium and increase implantation rates;"[27]
- "local injury to the endometrium might provoke wound healing, involving a massive secretion of different cytokines and growth factors…which are beneficial for embryo implantation;"[27]
- Controlled ovarian hyperstimulation during IVF therapy might result in the endometrium showing histological advancement which can negatively affect embryo implantation.[27] "Local injury to the endometrium during the controlled ovarian hyperstimulation cycle might result in a 'lag' in the development of the endometrium, so that the development of the endometrium is more equivalent with the development of the embryo and also increase its receptivity."[27]

Studies by Barash *et al.*[14] and also by Zhou *et al.*[27] are in keeping with the above theory. Important to note however, that this procedure must be done in the luteal phase of the cycle preceding the IVF treatment.[24]

A recent study published only last year in the International Journal of Clinical and Experimental medicine, evaluated the effectiveness of autologous platelet-rich plasma ("**PRP**') on endometrial growth and pregnancy outcomes. It was a small study group, comprised of 5 infertile women with thin endometrium (≤ 7mm). The conclusion from the study was that PRP is able to promote endometrial growth and improve pregnancy outcomes.[28] However, owing to the small sample size; further study into this experimental treatment is yet required.

1.2.7 Studies on the use of G-CSF with IVF therapy

The pioneer researcher who initially started experimenting with the use of G-CSF in the field of fertility was a specialist from the United States by the name of Nobert Gleicher.

His first publication was in 2011. It was a prospective cohort study of only 4 patients.[29] All 4 of the patients had a thin endometrium (< 7mm). His intervention was the intrauterine perfusion of G-CSF. The main outcome measure was endometrial thickness on the day of embryo transfer with pregnancy as a second outcome. From the study he reported successful expansion of the endometrium to minimal thickness (≥ 7mm) within approximately 48 hours from G-CSF infusion. All 4 patients achieved pregnancy but one required termination as it was a cornual ectopic.[29]

Gleicher then went on to head yet another study, this time with 21 patients. The study design was the same as his previous study with the same intervention and the same outcome measures. Again his study showed a significant change in endometrial thickness ($P < 0.001$). They had an ongoing clinical pregnancy rate of 19.1% with the exclusion of one pregnancy which was an ectopic.[30]

Soon after, a RCT of 141 patients was published by Barad DH *et al.* Interestingly these patients were *not* selected based on their endometrial thickness or history of previous IVF treatment failures. The control group received G-CSF and the placebo group received saline. The conclusion of this study was that G-CSF does *not* affect endometrial thickness, implantation rates or clinical pregnancy rates compared to saline.[31]

Eftekhar M *et al.* later published an intervention study specifically evaluating the effect of transvaginal perfusion of G-CSF on improving endometrial thickness and pregnancy outcomes. The study group consisted of 68 patients with thin endometrium (< 7mm) undergoing assisted reproduction treatment in a frozen embryo transfer program. Thirty-four of these patients received G-CSF. The study failed to show that G-CSF improved endometrial thickness but showed that its use was associated with improved chemical and clinical pregnancy rates. The result however was not found to be statistically significant.[32] The authors "think that exogenous G-CSF infusion as chemical stimuli and intrauterine infusion as mechanical stimuli may induce secretion of endogenous cytokines and activated the endocrine-paracrine pathways that probably contributed to embryo implantation and pregnancy."[32]

Kunicki *et al.*'s study conducted in Poland also looked at the effect of G-CSF on women undergoing IVF who have treatment-resistant thin endometrium (< 7mm). This study was conducted somewhat differently. It involved 37 patients, all of whom received G-CSF. The researchers then divided the patients into two groups based on whether they conceived or not. The clinical pregnancy rate was 18.9%. They then looked at the change in endometrial thickness experienced by the two groups and found significant endometrial expansion in both groups.[33]

1.3 RESEARCH QUESTION

In a certain number of infertile women, pregnancy is not achieved even with multiple attempts of IVF. As shown from the studies described above there is conflicting research as to the effect of G-CSF on the endometrial lining as well as its influence on pregnancy outcomes. Some studies have shown that G-CSF improves endometrial thickness and pregnancy rates while others have shown it to have no effect on the endometrial thickness but is associated with improved clinical pregnancy rates; and still others have shown it to have no effect on endometrial thickness or pregnancy rates when compared to saline. Where improved pregnancy rates have been shown, it is also not clear as to the exact mechanism whereby this improvement is attributed. Literature suggests that G-CSF may exert its positive effect via a chemical, hormonal or a mechanical effect or via an as yet uncertain alternative mechanism enhancing implantation.

BioART used G-CSF in women with failed IVF from January 2015. This study evaluated the outcomes in these women in terms of pregnancy rates and endometrial expansion.

1.4 AIM OF THIS STUDY

To describe the outcomes in women who had transcervical intrauterine instillation of G-CSF as an adjunctive treatment to IVF in the management of infertility. These women all had at least two previous failed attempts at IVF. All of them underwent IVF treatment between January 2015 to August 2015 at BioART fertility centre.

1.5 OBJECTIVES

The objectives of the study were twofold:

1. To describe the effect of transvaginal intrauterine instillation of G-CSF, in adjunct to IVF treatment on pregnancy rates;
2. To describe the effect of transvaginal instillation of G-CSF on endometrial thickness.

2. CHAPTER TWO – METHODS AND MATERIALS

2.1 METHODS

2.1.1 Study design

This was a retrospective cross-sectional study analysing the data of women who had undergone treatment involving G-CSF instillation and IVF.

2.1.2 Setting

BioART Fertility Centre is situated at the Brenthurst Clinic in Johannesburg. It is run by two obstetrician and gynaecologists who are also specialists in reproductive medicine. The complete team is comprised of a clinical manager, nursing staff, psychologist, laboratory technicians and embryologist. All treatments were done on site. It is a busy practice that attracts patients from all over Johannesburg, South Africa and also throughout the African continent including countries like Malawi, Nigeria, Cameroon, Gabon and the Democratic Republic of Congo. They do an average of 800 IVF cycles a year.

2.1.3 Study population

This was a specific subgroup of women who had previously had at least two failed attempts at pregnancy with IVF either at BioART or other fertility centres. There were no other specific inclusion or exclusion criteria. Women who attend BioART Fertility Centre are either on medical aid or are cash paying clients, but presumably have sufficient resources to afford fertility treatment which can be quite expensive. IVF is not freely available in the state setting and is often not covered for by most medical aids.

At BioART, on the first visit the patient or couple is seen by one of the specialists and then also by the psychologist. A complete history is taken, an examination is performed. Any relevant investigations for example an ultrasound is performed. Further investigations like a hysterosalpinogram if necessary are then scheduled. A semen analysis is booked to exclude male factor infertility. Should any surgery be indicated for example a diagnostic/operative laparoscopy, this will also be discussed and scheduled. Once all the relevant investigations have been done and the cause of infertility is established plans for assisted reproduction are made. Should IVF be warranted this will be discussed and the protocol to be used will be decided upon and date for commencement of treatment will be scheduled.

2.1.4 Sampling

A consecutive sample of eligible, consenting women, taken from the date of the introduction of this new treatment adjunct at BioART i.e. 21/01/2015 until the 31/08/2015.

Technique of transvaginal G-CSF instillation

The patient was placed in lithotomy position. The vulva and vagina were then cleansed with a disinfectant solution (Savlon - Cetrimide 3.0% w/v and Chlorhexidine Gluconate 0.3% w/v). A Cusco speculum was inserted into the vagina to visualize the cervix. The cervix was then also swabbed with disinfectant solution. A Tomcat catheter was inserted through the cervical canal into the mid-cavity of the uterus; the exact position was calculated from the most recent ultrasound scan done for endometrial monitoring. If a Tomcat catheter was not available, a semi-rigid embryo transfer catheter was used. One ampoule of G-CSF [Neupogen® (filgrastim)], at room temperature, was drawn up into a 2 ml syringe and attached to the catheter. The G-CSF was injected, no flush was used. The catheter was then withdrawn and the speculum removed. Patient was left to lie supine for a further 10-15 minutes thereafter.

2.1.5 Sample size

Sample size was determined by the number of women who had undergone IVF treatment with adjunctive transvaginal instillation of G-CSF in the above mentioned time frame. These women also satisfied the specific inclusion criteria.

Our study sample consisted of a total of 49 women. Other studies using G-CSF had between 4 and 141 subjects.[29-33]

2.2 EXPLANATORY VARIABLES

Age, parity, gravidity, previous obstetric history, cause of infertility, previous fertility treatments, IVF protocol used, fluid in the endometrial cavity, donor oocytes, embryo grading and cryopreservation.

Four different IVF protocols were used in this study, namely;

1. Long protocol (midluteal GnRHa downregulation protocol),
2. Step up protocol (agonist antagonist protocol),

3. Frozen embryo transfer protocol (natural cycle/ hormonally manipulated cycle),
4. Oral ovulation induction protocol (clomiphene/ clomiphene and letrozole combination)

The protocols have been attached hereto and marked "Annexure A". The protocol chosen for use was patient specific.

Fluid in the endometrial cavity was observed by transvaginal ultrasound pre G-CSF instillation. The volume was not measured and its presence or absence was merely noted.

Embryos were graded on the third day of culture according to the appearance of the embryo under the microscope using Veeck's day 3 scoring criteria. (Attached hereto and marked "Annexure B".)

There are two main methods of cryopreservation, namely the slow cooling and the rapid cooling method. The laboratory at BioART fertility centre uses the rapid cooling method also known as the "Vitrification" method. The protocol used for embryo vitrification as well as for thawing has been attached hereto and marked "Annexure C".

2.3 OUTCOME VARIABLES

The primary outcome measure was the confirmation of pregnancy. The pregnancy result was initially determined 'biochemically' by serum ß-HCG processed at Lancet laboratory at the Brenthurst Clinic on day 10 post embryo transfer. Should the result have been negative and no bleeding per vagina had occurred, the test was repeated on day 12. If the result was positive the patient was then seen after two weeks for an ultrasound to confirm an ongoing pregnancy (clinical pregnancy). The secondary outcome measure was the measurement of endometrial thickness post G-CSF instillation compared with pre G-CSF instillation.

The Lancet laboratory does both qualitative and quantitative testing of serum ß-HCG. The received blood sample in a SST (serum-separating tube) is spun down using a centrifuge at a speed of 4000 RPM (revolutions per minute). For qualitative testing, the "TOYO®" hCG pregnancy cassette test is used. Serum is drawn into a dropper and 3 drops (120µl) are placed into the sample well of the cassette. Results are read within 10 minutes. One coloured line visible indicates a negative result and two coloured lines visible indicate a positive result.

This test has a sensitivity of 99.4% and a specificity of 99.9%. The positive predictive value is 99.9% and the negative predictive value is 98.4%. Quantitative testing is done using the "VIDAS®" hCG test. This is an automated test and uses enzyme linked fluorescent assay technique in order to quantify measurement of hCG. With this test the smallest concentration of hCG detectable with a 95% probability is $\leq$ 2mIU/ml.

Endometrial thickness is determined by transvaginal ultrasound and is measured as the maximum anterior to posterior distance visualized when looking at the uterus in a mid-sagittal view. Endometrium pre G-CSF was measured on the day of "triggering", and post G-CSF on the day of embryo transfer. Transvaginal G-CSF instillation was done 2 - 4 days pre-embryo transfer. Triggering is the final step in oocyte maturation and is usually achieved by administering either a GnRH agonist like Leuprorelin or hCG.

2.4 DATA COLLECTION

Data was collected retrospectively. All women who had undergone ART treatment with transvaginal G-CSF instillation were recorded. Data was extracted from patient files which also contained a G-CSF specific information sheet. (Attached hereto and marked "Annexure D".) Data was entered into an Excel spreadsheet. (Attached hereto and marked "Annexure E".)

2.5 DATA ANALYSIS

The data was exported to Stata 14.1® (StataCorp,4905 Lakeway Drive,College Station, Texas 77845 USA) for data analysis. Categorical variables were described using frequencies and percentages, and continuous variables were described using means (with SD) and medians (with IQR). Comparisons were made using the Chi-squared or Fishers exact test. Continuous variables were compared using the Student T test or the Wilcoxon Mann Whitney test. A difference was noted to be statistically significant when the p- value was < 0.05.

2.6 ETHICS

Approval to conduct this study was sought and granted unconditionally from the University of the Witwatersrand's Human Research and Ethics Committee (Clearance certificate no: M150961). (Attached hereto and marked "Annexure F".) Consent for use of data by BioART was obtained in writing (Attached hereto and marked "Annexure G".) Consent was also implied as Dr Cassim was a supervisor, and both practitioners will be involved in the manuscript for publication. No identifying data was captured. All file numbers were given a study number and this was kept separately in a locked cupboard within the BioART practice.

3. CHAPTER THREE – RESULTS

In this chapter I will begin with a description of the study population, the demographics and the fertility history. I will then look specifically at the endometrial thickness, pre and post G-CSF instillation as well as data specific to the IVF cycle, that being, the type of oocytes used, the number and quality of the embryos transferred, the IVF protocol used and whether there was fluid noted in the endometrium or not. I will then go on to describe the pregnancy outcomes. Lastly I will compare those that achieved pregnancy and those that did not, and will compare the results of the two groups according to demographics, fertility history, the cause of infertility and cycle specific factors.

3.1 Demographics

There were a total number of 103 women who underwent ART therapy with G-CSF in the period between 21/01/2015 – 31/08/2015. Of these the medical notes of 49 of these women were reviewed. These women had at least 2 or more failed attempts at pregnancy with IVF previously and were treated with G-CSF instillation in addition to their IVF protocol. Women that were excluded had either less than 2 previous failed IVFs or had G-CSF instillation with another form of ART other than IVF, for example, ovulation induction and intrauterine insemination.

The ages of women ranged between 28 and 51. The mean age was 38.90 (SD ± 6.11) and the median age 38 (IQR 34 - 42). The distribution of women's ages is illustrated in Figure 3.1 below.

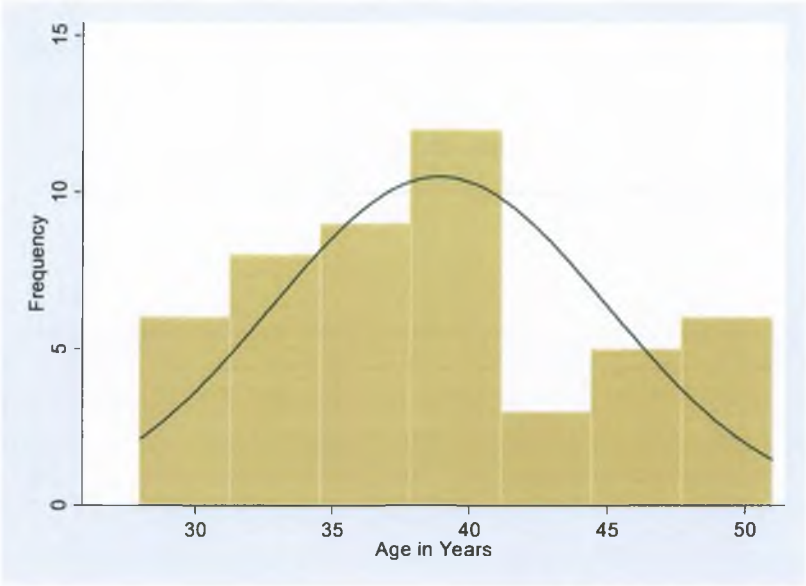


Figure 3.1: Age distribution of study population

The mean parity of the women was 0.35 (SD ± 0.60) and the median was 0 (Range 0 - 2). The mean gravidity of the sample was 1.04 (SD ± 1.09) and the median was 1 (Range 0 - 4). The mean paternal age was 42.70 (SD ± 6.96) and the median was 41.5 (Range 29 - 61). In 3 of the cases the paternal age was not known. The reason being that of theses 3 cases, 2 used donor sperm and the third was a new file and had missing information as the old file had been lost.

The smoking history of the women was not well documented in the files, with only the history of 4 patients being recorded (all of whom were confirmed to be non-smokers) and the history of the other 45 unknown.

3.2 Fertility History

The number of previous IVF treatments ranged between 2 and 10, as shown in Table 3.1 below. The mean number of previous IVF treatments was 3.14 (SD ± 1.77) and the median was 2 (IQR 2 – 4).

Table 3.1: Number of previous IVF cycles

Number of previous IVFs	Frequency	Percentage (%)
2	25	51.02
3	11	22.45
4	6	12.24
5	3	6.12
6	1	2.04
8	2	4.08
10	1	2.04

3.3 Endometrial thickness

The endometrial thickness pre G-CSF treatment ranged between 1.1mm and 12mm. The mean thickness was 7.53mm (SD ± 2.69) and the median was 7.5mm (IQR 6.1 - 9.8). A graphical description of the distribution of endometrial thickness pre G-CSF is depicted in Figure 3.2 below.

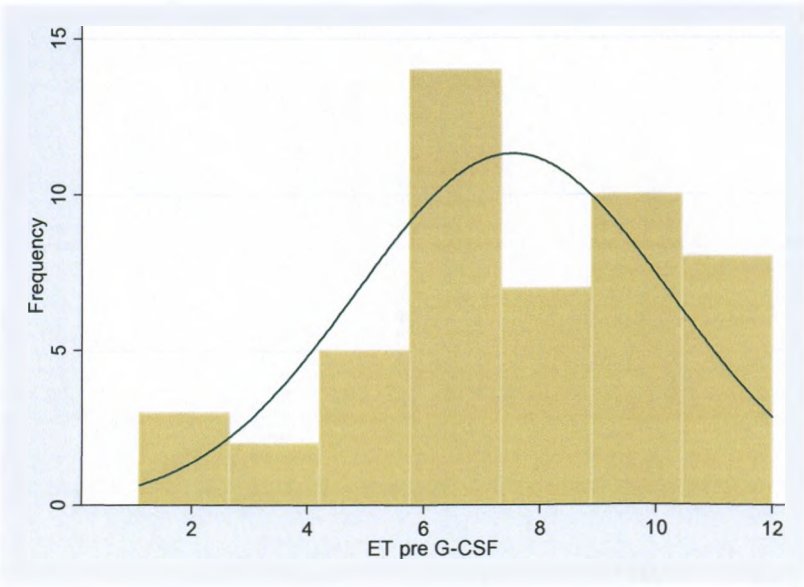
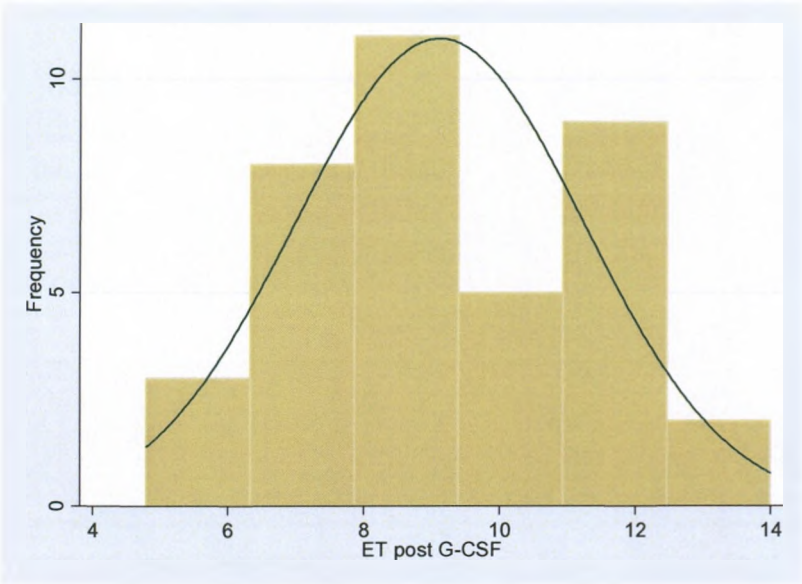


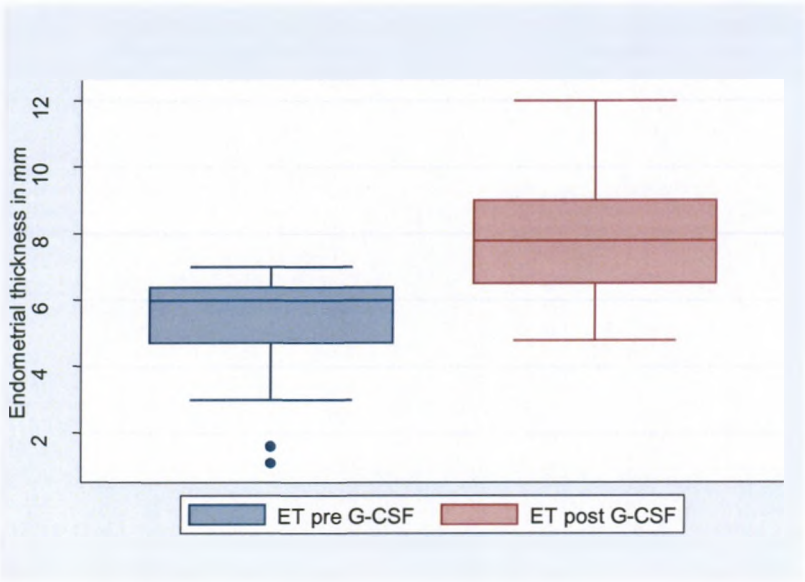
Figure 3.2: Endometrial thickness pre G-CSF in the study population

The endometrial thickness post G-CSF as illustrated in Figure 3.3 below, ranged between 4.8mm and 14mm. The mean thickness was 9.11mm (SD ± 2.12) and the median was 8.8mm (IQR 7.5 – 11).



<u>Figure 3.3:</u> Endometrial thickness post G-CSF in the study population

Comparison of the mean endometrial thickness pre- and post- G-CSF using the paired t-test yielded a p-value <0.001, implying that the difference noted is statistically significant. This difference is illustrated by the box plot shown in Figure 3.4 below.



<u>Figure 3.4:</u> Box plot showing comparison of endometrial thickness pre and post G-CSF in the study population

The box plot in Figure 3.5 below shows graphically the comparison of the mean endometrial thickness pre- and post- G-CSF for specific women with a "thin endometrium" (pre G-CSF ≤ 7 mm).This yielded a p-value = 0.0004, showing statistically significant expansion of the endometrium post G-CSF instillation.

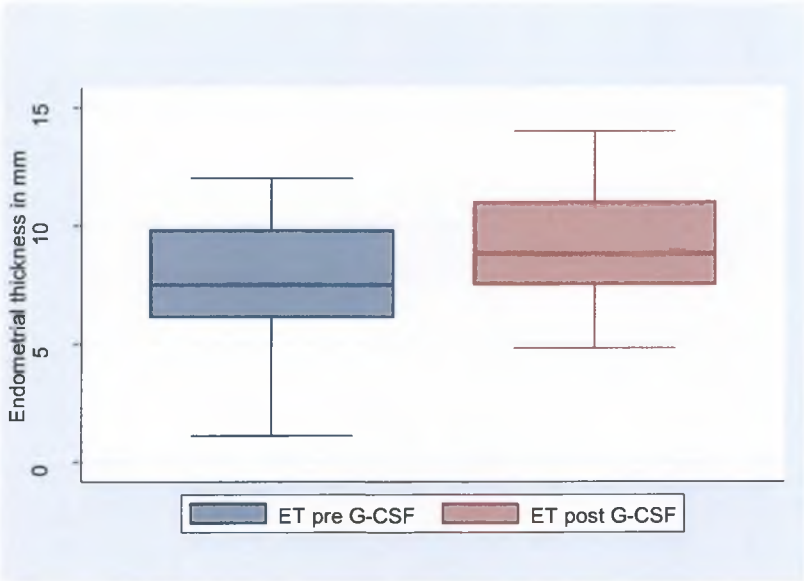


Figure 3.5: Box plot showing comparison of endometrial thickness pre and post G-CSF in the women with "thin endometrium" (pre G-CSF ≤ 7mm)

3.4 Cycle specific data

Eleven of the 49 women used donor oocytes. One women used both own and donor oocytes as shown in Table 3.2 below.

Table 3.2: Types of oocytes used

Oocyte type (donor/own)	Frequency	Percentage (%)
Both	1	2.04
Donor	10	20.41
Own	38	77.55

Embryos transferred were then either fresh embryos or thawed from previously cryopreserved embryos. Two women had both fresh and thawed embryos transferred. Of

these 2 women, one used embryos produced from her own oocytes (fresh) in addition to embryos produced from donor oocytes which had been cryopreserved. The second women used own fresh embryos from the current IVF cycle and in addition used thawed embryos that had been cryopreserved from a previous IVF cycle. Table 3.3 below shows the types of embryos transferred.

Table 3.3: Types of embryos transferred

Embryos transferred (Fresh/thawed)	Frequency	Percentage (%)
Fresh and thawed	2	4.08
Thawed	27	55.10
Fresh	20	40.82

The mean number of fresh embryos transferred was 2.83 (SD ± 1.14) and the median number was 3 (IQR 2 - 4).

The mean number of frozen embryos transferred was 3.77 (SD ± 1.31) and the median number was 3.5 (IQR 3 - 5).

For the purpose of analysis we divided the embryos into good, intermediate and poor quality. Good embryos were either grade 1or 2. Intermediate embryos were grade 3 and poor quality embryos were grade 4 and 5.

The majority (47) had at least 1 good quality embryo as shown in Table 3.4 below.

Table 3.4: Embryo quality

Embryo quality	Frequency	Percentage (%)
Good	47	95.92
Intermediate	1	2.04
Poor	1	2.04

3.5 Fluid in the endometrial cavity

Of the 49 women 3 of them had fluid noted in the endometrial cavity on ultrasound pre G-CSF instillation.

3.6 IVF protocol used

There were a total of 4 different protocols used. The most commonly used protocols were the long protocol and the frozen embryo transfer protocol as shown in Table 3.5 below.

Table 3.5: IVF protocol used

IVF protocol used	Frequency	Percentage (%)
Frozen embryo transfer protocol	25	51.02
Long protocol	18	36.73
Step up protocol	4	8.16
Oral ovulation induction protocol	2	4.08

3.7 Pregnancy outcomes

As shown in Table 3.6 below, 17 women had a positive clinical pregnancy and 32 failed to fall pregnant.

Table 3.6: Pregnancy outcome

Pregnancy outcome	Frequency	Percentage (%)
Positive	17	34.69
Negative	32	65.31

3.7.1 Pregnancy outcomes related to demographics and fertility history

Table 3.7 below shows that younger women were more likely to fall pregnant and this was statistically significant. There was no statistically significant difference in the other demographic factors. Similarly there was no statistically significant difference observed with regards to the causes of infertility or cycle specific factors as shown in Table 3.8 and 3.9 respectively.

Table 3.7: Pregnancy outcomes related to demographics and fertility history

	Pregnancy N=17 (34.69%)	**No pregnancy N=32 (65.31%)**	**p-value**
Demographics			
Age – mean (SD)	34.94 (± 1.15)	41 (± 5.76)	<0.001
Age – median IQR)	34 (31 – 37)	40 (36.5 – 46)	0.001
Age<35	10 (62.5%)	6 (37.5%)	0.004
Age<37	13(45.52%)	10 (43.48%)	0.003
Parity - mean (SD)	0.44 (±0.16)	0.31 (± 0.10)	0.495
Gravidity - mean (SD)	1.06 (±.023)	1.03 (± 0.21)	0.925
Paternal age - mean (SD)	41.29 (± 2.15)	43.31 (± 1.14)	0.362
Fertility history			
Number of previous IVF's –mean (SD)	3.18 (±0.40)	3.13 (± 0.33)	0.922

Comparison of the mean age using the two sample t-test yielded a p-value of <0.001, affirming that the mean age difference between those women that achieved pregnancy and those that did not, was statistically significant. This comparison is illustrated graphically in Figure below.

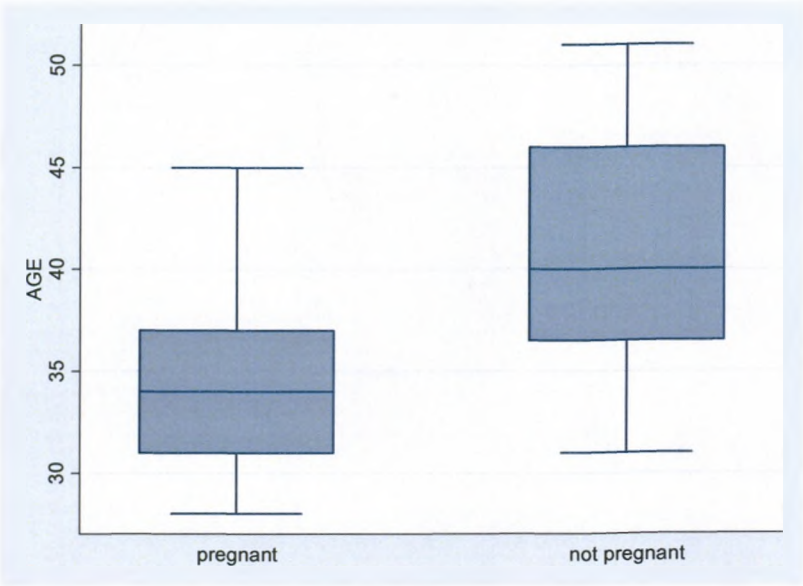


Figure 3.6: Box plot showing comparison of age between women who achieved pregnancy verses those that did not

3.7.2 Pregnancy outcomes related to cause of infertility

Table 3.8 displays the causes of infertility and the pregnancy outcome. There was however no statistically significant association noted for any particular cause of infertility and the attainment of pregnancy. Of the 49 women, 3 of them had pelvic structural abnormalities which were contributory to their infertility. More specifically, one of these women had severe pelvic adhesions, the other had asherman's syndrome and the third woman had a uterine septal defect. A reduced ovarian reserve was determined by the measurement of AMH levels. For the women said to have chronic endometritis, this diagnosis was made histologically following a diagnostic dilatation and curettage. One these two women, one was found to have TB endometritis.

Table 3.8: Pregnancy outcomes related to cause of infertility

	Pregnancy N=17 (34.69%)	No pregnancy N=32 (65.31%)	p-value
Female causes of infertility			
PCOS n=7 (14.29%)	1 (14.29%)	6 (85.71%)	0.397
Adenomyosis n=5 (10.2%)	1 (20%)	4 (80%)	0.646
Endometriosis n=11 (22.45%)	3 (27.27%)	8 (72.73%)	0.725
Fibroids n=6 (12.24%)	1 (16.67%)	5 (83.33%)	0.650
Pelvic structural n=3 (6.12%)	0 (0%)	3 (100%)	0.542
Tubal n=14 (28.7%)	6 (42.86%)	8 (5.14%)	0.516
Ovulatory dysfunction n=4 (8.16%)	0 (0%)	4 (100%)	0.284
Reduced ovarian reserve n=19 (38.78%)	6 (31.58%)	13 (68.42%)	0.767
Chronic endometritis n=2 (4.08%)	0 (0%)	2 (100%)	0.537
Male causes of infertility			
Teratozoospermia n=10 (20.41%)	3 (30%)	7 (70%)	1.000
Azoospermia n=2 (4.08%)	1 (50%)	1 (50%)	1.000
Oligospermia n=4 (8.16%)	0 (0%)	4 (100%)	0.284
Decreased motility n=2 (4.08%)	0 (0%)	2 (100%)	0.537

3.7.3 Pregnancy outcome related to cycle specific data

Table 3.9: Pregnancy outcome related to cycle specific data

	Pregnancy	No pregnancy	p-value
All women	**n=17 (34.69%)**	**n=32 (65.31%)**	
Number of Embryos transferred (Fresh/thawed) – mean (SD)	3.82 (± 1.29)	3.13 (± 3.13)	0.081
ET pre-GCSF – mean (SD)	7.6 (± 0.72)	7.49 (± 0.46)	0.891
ET post-GCSF – mean (SD)	9.14 (± 0.57)	9.10 (± 0.44)	0.954
	n=13 (34.21%)	**n=25 (65.79%)**	
ET expansion (Post G-CSF – Pre G-CSF)	1.76 (± 2.61)	1.70 (± 1.58)	0.933
Women with ET pre-GCSF ≤ 7 mm	**n=6 (31.58%)**	**n=13 (68.42%)**	
ET pre-GCSF – mean (SD)	5.48 (± 2.20)	5.68 (± 1.20)	0.147
ET post-GCSF – mean (SD)	8.03 (± 1.54)	8.05 (± 2.11)	<0.001
ET expansion (Post G-CSF – Pre G-CSF)	2.55 (± 3.64)	2.36 (± 1.87)	0.882

Table 3.10 below compares pregnancy outcomes and the various IVF protocols used.

Table 3.10: Pregnancy outcome related to protocol used

	Pregnancy [N, (%)]	No pregnancy [N, (%)]
Frozen embryo transfer protocol	6 (35.29%)	12 (37.5%)
Long protocol	1 (6.88%)	3 (9.38%)
Step up protocol	9 (52.94%)	16 (50.00%)
Oral ovulation induction protocol	1 (6.88%)	1 (3.13%)

3.7.4 Univariate analysis

Table 3.11: Univariate analysis of factors associated with a clinical pregnancy

	Odds ratio	95% Confidence interval
Age	0.80	0.69 - 0.93
Parity	1.41	0.53 – 3.75
Gravidity	1.03	0.59 – 1.79
Paternal age	0.96	0.89 – 1.05
Post thickness ET	1.01	0.73 - 1.39
IVF cycle	1.02	0.73 – 1.42
Use of donor eggs	2.45	0.59 - 10.2
Number of embryos transferred	1.50	0.92 – 2.45

4. CHAPTER FOUR – DISCUSSION AND CONCLUSION

This is the first study in South Africa describing the pregnancy rates with the use of G-CSF as an adjunct to IVF in the treatment of infertility. Our study was unique in that all woman included had failed two more IVF cycles previously and not all of them had a thin endometrium. This study adds to the growing pool of research looking at the various effects of G-CSF and its use in reproductive health.

4.1 Pregnancy outcomes

IVF treatment was successful in 17 of the 49 women resulting in a pregnancy rate of 34.69%. These women had a positive pregnancy test followed by ultrasound confirmation.

Most studies to date have shown good pregnancy rates with the use of G-CSF.[29,30,32]

In our study, the only statistically significant difference noted between those that achieved pregnancy and those that did not, was age (p-value <0.001). The average age of those who fell pregnant was 35 and 41 for those who did not.

The pregnancy rate achieved at BioART in the year 2014 was 45.68%.Stratifying this data according to age the pregnancy rates in those less than 35 years, between 35-37, 38-40, 41-42, and more than 42 years; were 52.00%, 42.85%, 32.89%, 33.33% and 46.42% respectively. With the use of G-CSF, we now found pregnancy rates in the same age categories of 62.29%, 44.44%, 12.50%, 33.33% and 8.33% respectively. We are unable to make direct comparisons with regards to these results as the 2014 rates are the overall rates at BioART for the year not taking into account the woman's previous fertility treatments. In our study the women in whom G-CSF was used were a particular subset in that they already had failed 2 or more previous IVF treatments.

Table 4.1 below is a comparison according to age of the pregnancy rates achieved at BioART in 2014 compared with the United States national 2014 rates as displayed in the summary report on the SART website.

Table 4.1: Comparison of pregnancy outcomes according to age

Age	BioART 2014	SART National summary report 2014[54]
< 35	52.00	31.90
35-37	42.85	26.50
38-40	32.89	18.30
41-42	33.33	10.50
>42	46.42	3.60

The pregnancy rates with G-CSF adjunctive therapy are higher than the rate previously obtained at BioART and is approximately double the US national rates in the age groups < 35 years and 35-37 years. However the true difference can only be determined using a RCT.

4.2 Endometrial thickness

Some studies have shown G-CSF to have an effect on endometrial expansion whilst others have shown it to have no effect on the endometrium. As part of the second objective in this study, we set out to describe the effect that G-CSF had on our study population. A thin endometrium was however not a criteria for the study or for the use of G-CSF.

In our study sample, the mean endometrial thickness before G-CSF instillation was 7.35 mm and post G-CSF instillation, it was 9.11 mm. The overall expansion in endometrial thickness post G-CSF was found to be statistical significant. (P-value < 0.001)

However a comparison of the mean endometrial thickness pre G-CSF and post G-CSF between those that fell pregnant and those that failed to fall pregnant showed no statistically significant difference between the 2 groups. (P-value 0.891 and 0.954 respectively)

It has been shown previously that endometrial thickness is an important independent factor in predicting the outcome of pregnancy with IVF. A recent systematic review and meta-analysis of 22 studies assessed the clinical significance of endometrial thickness and IVF outcome. 260 of the 10 724 reported cases had a thin endometrium ≤7 mm. The probability of a clinical pregnancy for patients with a thin endometrium was significantly lower when compared to

patients with endometrial thickness ≥ 7 mm [23.3% vs 48.1%, OR=0.42 (95% CI 0.27-0.67)].[45]

In our study, of the 49 women, 22 had a pre G-CSF endometrial thickness of ≤ 7 mm. By looking specifically at these 22 women, comparisons regarding endometrial expansion post G-CSF were made. From these 22 women, 3 had to be excluded from analysis as the post G-CSF measurement was not recorded in the medical notes. Comparison of the means of pre and post G-CSF endometrial thickness showed statistical significance endometrial expansion. (P-value <0.001).

4.3 Study demographics and fertility history

4.3.1 Age, parity and gravidity

The median age of our study population was 38 years. The median gravidity was 1 and the median parity was 0.

We found that younger women were more likely to fall pregnant and that the rate decreased by 20% with every additional year of age.

Our study like others has shown that age is an independent predictor of IVF success.[34-37] A retrospective and comparative study by Kdous M *et al,* of 500 women who underwent an ICSI cycle, found significantly lower pregnancy rates in older women (> 38 years).[34] Tsafrir A *et al,* also in a retrospective study looked specifically at patients aged 40 years and older at the start of IVF treatment. The study had a sample size of 381 women and a total number of 1217 IVF cycles. This study showed that success rates declined with each year after age 40. Pregnancy and delivery rates were 13.9 and 9.1% respectively at age 40 and 2.8 and 0.7% respectively at age 45.[35]

Dhillon RK *et al,* in their study to determine which pretreatment patient variables have an effect on live birth rates following assisted reproduction, found that increasing age (>36 years) was associated with reduced chances of IVF/ICSI success.[36] The reduction in IVF success with advancing age may be attributed to reduced ovarian reserve evidenced by declining AMH levels. However, a retrospective study by Revelli *et al,* looking at the outcome of 448 IVF cycles in 361 women with low AMH levels (<0.5 ng/ml) found that

these women still had reasonable chances of achieving pregnancy, but that their prognosis was significantly affected by chronological age.[37]

Our study showed no statistically significant difference when comparing means for parity and gravidity between the 2 groups. (P-value 0.5 and 0.93 respectively)

This finding is in keeping with the study by Tsafrir *et al* mentioned above, which stated that prior pregnancy did not influence success of IVF treatment.[35] Rabinson J *et al,* in their study evaluating gravidity on the results of IVF also found that it has no influence on the likelihood of achieving pregnancy through IVF.[38]

However, a large analysis of 174 909 ART procedures taken from the German IVF registry looked at the impact of reproductive history on IVF and ICSI outcomes. They found that a former successful ART procedure resulting in a live birth or a miscarriage was an important prognostic indicator of ART success (p-value <0.0001).[39]

4.3.2 Paternal age

The median paternal age in our study was found to be 41.5 years. Studies have shown that paternal age does have an effect on reproductive outcomes. By comparing the mean paternal age between our 2 groups we did not find any association between paternal age and pregnancy. (P-value 0.37)

A study by Gromoll J *et al,* which aimed to evaluate the effect that paternal age has on the integrity of germinal cells, showed that sperm integrity is affected on both a genetic and epigenetic level by age.[40] Sharma *et al,* in their extensive literature review demonstrated negative effects on sperm quality and testicular function with increasing paternal age affecting reproductive outcomes and IVF/ICSI success.[41]

Another study by Ghuman *et al*, looked at the effect that the age of the sperm donor has on the outcomes of IVF. Their results did not show any significant effect with advancing age. However a limitation of their study was that a sperm donor would seemingly have good semen indices and are thus not representative of the general population. Also, none of the donors were > 45 years of age.[42]

4.3.3 Smoking history

In our sample population only 4 patients were confirmed non-smokers. In the other 45 patients, the majority, this history was unknown. We also did not know the smoking history of the partner. Owing to the fact that our study is retrospective, this is an expected limitation. It is thus difficult to draw any conclusions regarding the effect of smoking and the pregnancy outcomes observed in our study.

From the literature, it has been shown that smoking is associated with a reduction in reproductive potential for both male and females. A review article by Firns *et al,* looking at the effect of various lifestyle factors on IVF outcomes found that male smoking was associated with an increased risk of pregnancy loss, and female smoking had an adverse effect on ovarian reserve.[43]

4.3.4 Number of previous IVF cycles

It is difficult to determine the effect that previous failed IVF cycles may have on one's chances of success with further IVF therapy. There are models to predict IVF success pre-first IVF cycle and even after one IVF cycle, but having failed more than one cycle this becomes difficult. One may assume that the pathology may be so severe that future attempts are also likely to fail, but on the other hand the chance of a success may be higher having seen what has not worked previously and where to adjust with subsequent therapies and protocols.

The mean number of previous IVF treatments in our sample was 3.14. Our study did not reveal any association between the number of previous IVF cycles and the pregnancy outcome. (P-value 0.922)

Van Loendersloot *et al,* attempted to develop a model to predict the chance of pregnancy with subsequent IVF cycles, taking into account 13 other variables which could also affect pregnancy outcome. The calculated probability in their model ranged between 0.01-0.56. They noted that a diagnosis of diminished ovarian reserve, endometriosis and a greater number of failed IVF cycles were associated with reduced chance of pregnancy.[44]

4.4 Cycle specific data

4.4.1 Fresh vs frozen embryos

There is still much uncertainty surrounding the debate on whether fresh embryo transfer vs cryopreserved/thawed embryo transfer is superior or vice versa. In our study, we found no association between the type of embryo used and the pregnancy outcome. (P-value = 1)

Other studies have shown that specifically in cases with PCOS, where there is an increased risk of pregnancy complications, owing to superovulation, and where abnormal implantation and placentation has been described; elective cryopreservation and thawed embryo transfer in a subsequent cycle is advocated.[46] A cohort study by Roque M *et al,* compared IVF outcomes between fresh embryo and frozen-thawed ("freeze-all policy") transfer. Fresh embryo transfer was only performed in cases with progesterone levels $\leq$1.5ng/ml at the time of ovulation triggering. The study concluded that outcomes were significantly better in the group using the freeze-all policy compared with the group using the fresh embryo transfer.[47]

4.4.2 Donor oocytes vs own oocytes used

Same as with donor sperm, one would assume that the quality of donor oocytes would be good. Patients who choose to use donor oocytes either have severely diminished ovarian reserve that they are unable to produce enough oocytes or the quality of their own oocytes is not adequate.

Our study showed that there was an increased likelihood of pregnancy when using donor oocytes. (OR 2.45, 95% CI 0.59 - 10.2), but we failed to show any statistically significant difference between using either type of oocyte and pregnancy outcome. (P-value = 0.27)

4.4.3 Number of embryos transferred

In our study the average number of embryos transferred was 3.37 (SD ± 1.33) and ranged between 1 and 7. We did not show any statistically significant association between the number of embryos transferred and pregnancy outcome. (P-value 0.081)

By increasing the number of embryos transferred in an IVF cycle, one hopes to increase the chance of successful pregnancy. Coughlan *et al,* in their review article on recurrent implantation failure states that the probability of a single embryo to implant is approximately 30%, hence the probability of it failing to implant is approximately 70%. Following the

transfer of 2 embryos this probability is 49% ($0.70^2 = 0.49$). Similarly, the transfer of 3,4,5 or 6 embryos will reduce the probability of all embryos failing to implant to 34%, 24%, 17% and 12% respectively.[24] However, the transfer of multiple embryos has its downfall, with the increased risk of multiple pregnancies. Women with multiple pregnancies are at higher risk of pregnancy related complications and preterm delivery with its associated perinatal problems.

Recently there has been a move towards advocating of single embryo transfers especially for women over the age of 35as these women are even more at risk of developing pregnancy related complications. To date, studies looking at well selected single embryo transfers show promising results in terms of pregnancy outcome as well as reduction of multiple pregnancy rates.[48,49]

4.4.4 Embryo Grade

Embryos are graded so that they may be differentiated in terms of quality. It goes without saying that good quality embryos would fare better compared to poorer quality embryos when it comes to reproductive success. The probability of failed implantation with a poor quality embryo is much higher than that with a good quality embryo. It has been proposed that in this case, transferring a greater number of embryos will reduce the probability of all embryos failing to implant.[24]

47 of the 49 patients had at least one good quality embryo (grade 1 or 2). Of the other 2 patients, 1 had only one grade 3 embryo and the other had three grade 5 embryos. Both these patients did not fall pregnant.

As the majority of patients in both groups had good quality embryos, further analysis for comparison between the two groups was not done.

4.4.5 Fluid in the cavity

The presence of fluid in the endometrial cavity is thought to affect the outcome of ART by negatively affecting implantation rates and is often a reason for cycle cancellation.

Endometrial cavity fluid, $\geq$ 3.5 mm in anterior-posterior diameter, when associated with tubal factor infertility rather than other entities is specifically noted to impair ART outcomes.[50,51]

Of the 49 patients in our study, only 3 were noted to have fluid in the endometrial cavity.

Of these 3 patients, only 1 achieved clinical pregnancy. Interestingly, however it was the patient with tubal factor infertility that conceived. Owing to the small number of cases, we are unable to draw any conclusion on the use of G-CSF and fluid in the endometrial cavity and hence no further analysis was done. Additional study in this regard is required.

In the original case report by N.Gleicher *et al*, of the 4 patients described, one of them was noted to have fluid in the endometrial cavity. This patient had the fluid aspirated immediately prior to G-CSF instillation and good endometrial expansion was noted thereafter with no fluid re-accumulation, and successful implantation and ongoing pregnancy.[29]

A literature search on Pubmed has yielded no further reports on the use of G-CSF for the treatment of fluid in the endometrial cavity.

4.4.6 IVF protocol used

The protocol used was patient specific, based on the individual pathology at hand and also on experience from previous IVF failures in that patient. Comparison using Fischer's exact test yielded a P value =1 implying that there was no statistically significant association between the protocol used and the pregnancy outcome.

4.4.7 Relation to cause of infertility

Our study showed similar frequency of the different causes of infertility as other studies. Adenomyosis, tubal factors, unexplained infertility and ovarian problems have been shown to be associated with reduced success for women undergoing infertility treatments.[36,52,53]

We did not show any statistically significant difference in pregnancy outcome for any of the causes of infertility. (P-values > 0.05)

4.5 STRENGTHS AND LIMITATIONS

The strengths of the study are:

1) There were only two practitioners involved in this study both of which are enthusiasts and specialists in the field of infertility. The overall technique of G-CSF instillation is as described above in chapter 2 and was the same for both these practitioners.
2) With regard to the measurement of endometrial thickness between pre and post G-CSF instillation, both researchers had their own patients, thus observer bias should be negligible.

There were important limitations of this study that need to be mentioned:

1) The measure of pregnancy outcome was based on the result of the pregnancy test i.e. either positive or negative as described above. If the pregnancy test was positive, this was followed by ultrasound confirmation of an intrauterine gestational sac and later by the presence of a fetus with fetal cardiac activity noted. The overall success of the treatment however can only be determined by looking at the live birth rate. Unfortunately, at BioART, patients are only followed up until 10 weeks of gestation, thereafter they continue their antenatal follow up at their chosen / regular obstetrician. Information regarding live births was not available at the time of data analysis.
2) This was a retrospective cross sectional study, so some information was missing. Some data was not recorded in the files, like smoking history, paternal age and endometrial thickness. One file had been lost and a new file had been opened but some of the demographic data was then unknown. My impression was that the medical notes in most cases were woman rather than couple based unless there was a specific male factor for the cause of infertility identified.
3) Our study did not take into account additional variables such as BMI, ethnicity and duration of infertility which are known to affect outcomes of IVF treatment.[34,36,55]
4) We did not set out to determine the difference between women who became pregnant and those that did not. Even where differences between the 2 groups had been observed this was not shown to be statistically significant as this study was not sufficiently powered to determine such differences

4.6 CONCLUSION

In this study the pregnancy rate was 34.69%. Only a RCT will be able to answer whether this rate is indeed superior in women who have had previous failed IVF procedures. Whilst we showed an increase in endometrial thickness we were unable to show any statistically significant association between endometrial expansion and pregnancy outcome. Again this study was underpowered to determine this.

We were unable to show any benefit regarding the use of G-CSF in relation to any particular cause of infertility and clinically patients will have to be individualised.

The use of G-CSF and fluid in the endometrial cavity is important to note, however, owing to the small number of cases, we are unable to draw any conclusions from our study. Further study in this area is needed before the use of G-CSF for the treatment of fluid in the endometrial cavity can be advocated.

There is still much research being conducted on the benefits of G-CSF in reproductive health. A randomized control trial published recently looked at the effect of the subcutaneous use of G-CSF in women with repeated IVF failures. They also reported statistically significant improved implantation rates and clinical pregnancy rates even after adjustments for participant's age, endometrial thickness, good quality oocyte counts, number of transferred embryos and AMH levels.[56]

This study is evidence in favour of other workers in this field who have alluded to the fact that G-CSF may have other effects on the achievement of pregnancy other than just by the expansion of the endometrium.

The overall effect of the use of G-CSF with ART seems promising.

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6. ANNEXURES

ANNEXURE A

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Femara PROTOCOL IVF / ICSI

NB Please ensure that you collect your medication well in advance so that there is no delay in starting treatment on the correct days.

DAY OF MENSTRUAL CYCLE	Instructions	Things to remember
1.	This is the first day of your menstruation / period. • The first day of the period is the first day of full menstrual flow. • If bleeding commences after 6pm (18h00), the next day will be day 1 of your period. • If a brown discharge or spotting continues for more than 2 days, take day 3 as your day 1 of your period.	• Call the centre or e-mail the sisters to book your day 10 scan. If this falls over a weekend please call on the Monday.
2.	• Do nothing	
3.	• Do nothing	
4.	• Start taking your femara tablets. Dosage: 5mg daily (2 tablets a day)	• The Femara tablets should be taken at the same time every day
5.	• Continue with femara tablets	
6.	• Continue with femara tablets	
7.	• Continue with femara tablets	
8.	• Continue with femara tablets	• This is the last day of taking Femara tablets
9.	• Do nothing	

10.	• You need to be at the centre at 08h30 for your first scan. • After the scan you will be advised when your second scan will be. You will be monitored until you are ready for the aspiration.	• You will be advised continually during this procedure.
When you are ready for aspiration	• You will be advised when the aspiration will take place. • You will be given a trigger injection. • At this point your husband /partner will be given a script for antibiotics (Zithromax).	• Abstain from intercourse from this point.
Day of aspiration (retrieval of eggs)	• On this day you will be told what time to be at the centre. • Aspirations are usually done in the mornings. • You will need the following: 1.) sperm sample 2.) Pay the remainder of the fee	• Do not have anything to eat or drink 6 hours before the aspiration. • The aspiration is done under conscious sedation and you may therefore feel drowsy after the procedure. Please make arrangements for someone to drive you home
After aspiration	• You may telephone the centre daily after 1pm to check on the embryos • We will contact you and inform you when the embryo transfer will take place.	
Day of embryo transfer	• The patient may eat and drink	• Preferably take the day off work • After the embryo transfer you may resume back to your usual activities but avoid strenuous work • You must however abstain from intercourse after the embryo transfer. • If the pregnancy test is positive you would have to abstain until further advised.
After the embryo transfer	• You must wait for 12 days, and then do a beta quantitative pregnancy test.	• Do not stop any medication without first consulting the centre.

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OCP + HRT CYCLE (for Irregular Cycles)

NB Please ensure that you collect your medication well in advance so that there is no delay in starting treatment on the correct days.

Start with the Oral Contraceptive Pill when you start bleeding.	If you do not have regular menstrual cycles, or haven't seen your menstrual cycle for some time; do a pregnancy test and if its negative use Provera for 5 days and when you start bleeding start the Oral Contraceptive Pill when your menstrual cycle start.	If you use Femodene start in the silver line on the packet.
Count 21 days after you've started with the first Oral Contraceptive Pill and on the 21 Pill you start with the Lucrin Injections. You must finish the packed of Oral Contraceptive Pills. If bleeding commences prior to completing the packet of pills, you may stop the pills. **You will have a period when you stop the pills.**	Start Lucrin injections. Dosage: _____units @ 18h00 daily. Lucrin is administered subcutaneously under the skin	
Day1 of cycle	You may stop the OCP pill	Call to book your **day 14 scan**
Day 4 of menstrual cycle start **- Estrofem ___ mg daily.** **- Meticortin 10mg (2 tablets per day after breakfast)**	Carry on with the Lucrin	

Day 14 After 7 – 10 days of taking Estrofem you need to come in for a scan to assess the endometrial thickness of the uterus	You need to be at the centre at _____H_____ for your first scan.	The tablets may be altered depending upon your response to the medication and further ultrasound scans may be performed depending on the thickness of the uterus lining.
When you are ready for your transfer, you stop the Lucrin and the next day you start with the cyclogest. You do continue with the Estrofem tablets.	You need to take at least 5 days of prontogest. Then continue Cyclogest/Uterogestan thereafter. **The number of embryos / straws which are thawed will be at the discretion of the embryologist. Determining factor being the post thaw embryo survival.**	Start using the Cyclogest 200mg vaginal pessories 8 hourly after the 5 days of prontogest Start using the Cyclogest 200mg vaginal pessories 8 hourly. Use the following times as a guide: morning – afternoon – evening 06h00 - 14h00 - 22h00
Embryo Transfer Day	Embryo transfers usually take place in the morning. The embryo transfer is a relatively easy procedure, whereby the embryos are gently placed into the uterus using a very fine catheter (tube). The procedure feels similar to a cervical smear test. **You will be given 2 tablets (Dormicum 7.5mg and Buscopan 10mg) to take which will make you drowsy.**	You may have something to eat or drink on the morning of embryo transfer. Preferably take the day off work. After the embryo transfer you may resume back to your usual activities but avoid strenuous work **You must however abstain from intercourse after the embryo transfer. If the pregnancy test is positive you would have to abstain until further advised.** The Dormicum 7.5mg and Buscopan 10mg tablets will make you feel drowsy. Please make arrangements for someone to drive you home.
After the embryo transfer	You must wait for 12 days, and then do a beta quantitative pregnancy test.	Remember to use the cyclogest three times a day and you continue with the Estrofem tablets. Do not stop any medication without first consulting the centre.

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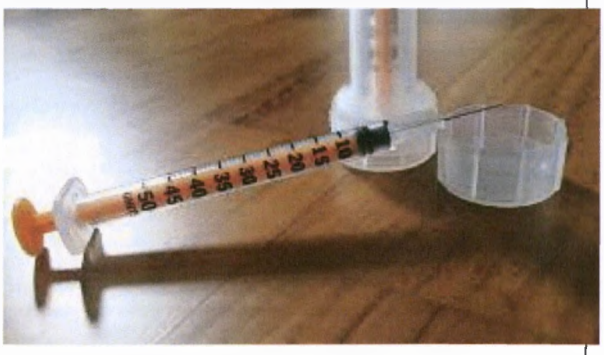
STEP Up Protocol- Trigger with Lucrin

NB Please ensure that you collect your medication well in advance so that there is no delay in starting treatment on the correct days

Day 1 of menstrual cycle • The first day of the period is the first day of full menstrual flow. • If bleeding commences after 6pm (18h00), the next day will be day 1 of your period. • If a brown discharge or spotting continues for more than 2 days, take day 3 as your day 1 of your period.		• **Call the centre** or e-mail the Sisters to **book your day 7 scan.** If this falls over a weekend please call on the Monday. 
Day 2 of menstrual cycle start with gonadotrophins injections e.g. Gonal F, Menonys, fostimon or _____ Dosage: _____	**You need to do the following:** • Start the hormone injections (gonadotrophins injections e.g. Gonal F or Menonys or _____)	• Injections (gonadotrophins injections e.g. / Menonys or fostimon_____) should be administered roughly at the same time every day – give or take within 2 hours • If you need to take Meticortin, please start today.

Day 3 :		•
Day 4:		•
Day 5: increase dose to _____		•
Day 6:		•
Day 7 of menstrual cycle	• You need to be at the centre at _____H_____ for your first scan. • After the scan you will be advised how many more injections you have to take • We will advise you when to start with the antagonist (Cetrotide 0.25mg daily) • At this point you make another appointment for your second scan. You will be monitored until you are ready for the aspiration.	• Do not take your gonadotrophins injections on this day until after the scan. • You will continually be advised during this procedure.
When you are ready for aspiration	• You will be advised when the aspiration will take place. • You will be given a trigger injection. Lucrin 35units  At this point your husband /partner will be given a script for antibiotics (Zithromax) and you will be given a script for cyclogest (progesterone) vaginal pessaries, or alternate progesterone.	• Abstain from intercourse from this point. • At this point you will be informed when to stop your Antagonist (Cetrotide) and gonadotrophins injections e.g. Gonal F, Menonys or _____

Day of aspiration (retrieval of eggs) • ***Take the day off work***	• On this day you will be told what time to be at the centre. • Aspirations are usually done in the mornings. • You will need the following 1) **Sperm sample** 2) **Pay the remainder of the fee**	• Do not have anything to eat or drink 6 hours before the aspiration. • The aspiration is done under conscious sedation and you may therefore feel drowsy after the procedure. Please make arrangements for someone to drive you home.
The day after the aspiration.	• Start using the Cyclogest /utrogestan vaginal pessories • Use one pessory (200mg) three times a day; every 8 hours. • If you are using prontogest, please continue 1 per day.	Use the following times as a guide: morning – afternoon – evening 06h00 - 14h00 - 22h00
After aspiration	• You may telephone the centre daily after 1pm to check on the embryos • We will contact you and inform you when the embryo transfer will take place.	
Day of embryo transfer	• The patient may eat and drink	• Preferably take the day off work • After the embryo transfer you may resume back to your usual activities but avoid strenuous work • You must however abstain from intercourse after the embryo transfer. • If the pregnancy test is positive you would have to abstain until further advised.
After the embryo transfer	• You must wait for 12 days, and then do a beta quantitative pregnancy test.	• Remember to use the cyclogest/utrogestan three times a day. • Do not stop any medication without first consulting the centre.

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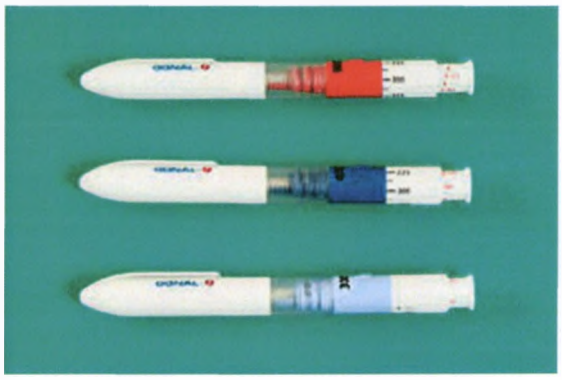
Long Protocol IVF/ICSI - Gonal F

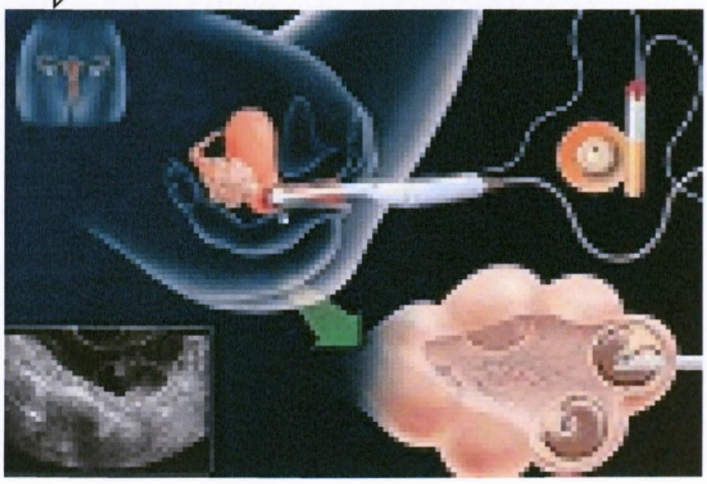
NB: Please ensure that you collect your medication well in advance so that there is no delay in starting treatment on the correct days. Please advise the centre of the date that you plan to start the Lucrin injections.

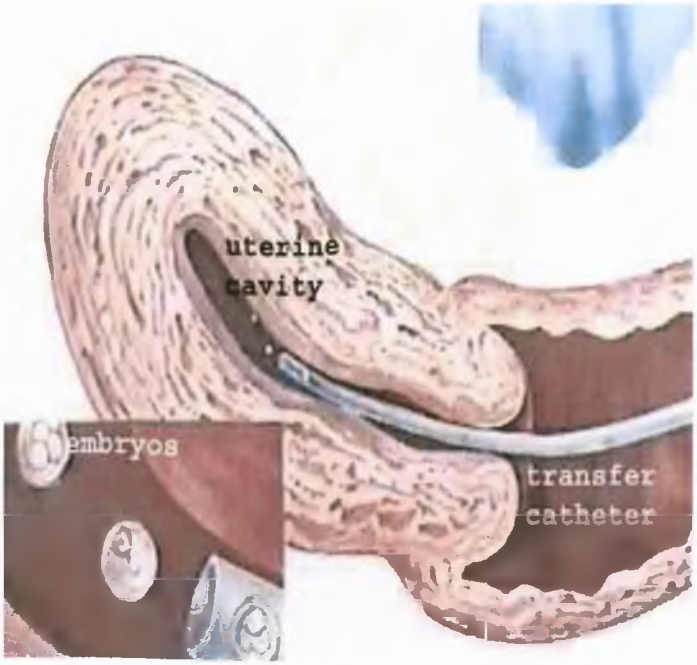
File No: _____

Sisters@bioartfertility.co.za 0114845119

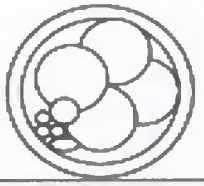
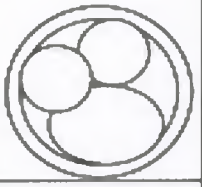
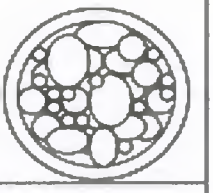
Day 21 of cycle i.e. 21 days from the first day of menstruation - • The first day of the period is the first day of full menstrual flow. • If bleeding commences after 6pm (18h00), the next day will be day 1 of your period. • If a brown discharge or spotting continues for more than 2 days, take day 3 as your day 1 of your period.	• Start Lucrin injections. • Dosage: _____units @ 18h00 daily. Lucrin is administered subcutaneously under the skin  • If you do not see your period within two weeks of starting the Lucrin injections PLEASE CONTACT THE CENTRE.	• Please ensure that a sperm analysis has been done. • DO NOT STOP THE LUCRIN UNLESS INSTRUCTED TO DO SO. **Gonal F Pens should be stored in the fridge (not Freezer) until used, then it can be stored outside the Fridge for 28 days** • You may continue to have intercourse while on the treatment; you will be instructed on when to abstain.

Day 1 of next menstrual cycle	• Carry on with the Lucrin	• Call the centre or e-mail the nurses to book your day 9 scan. If this falls over a weekend please call on the Monday.
Day 2 of menstrual cycle start with Gonal F PEN Dosage: _____ 1. Turn the dial to the prescribed dose. 2. Remove cap of needle, and tighten the needle onto the Gonal F pen. 3. Inject into the tummy pushing the medication into the tummy. 4. Remove needle, and close pen with the cap, and store in fridge. **Gonal F pens should be stored in the fridge until used, then it can be stored outside the fridge for 28 days**	• You need to do the following: • Take blood test. (FSH, LH, E2, PROGESTERONE) (If this day falls on a Sunday you must still have the bloodtest taken, there are laboratories open.) • **Start the hormone injections (gonadotrophins injections e.g. Gonal F) only after you had the blood test done.** • Continue with the Lucrin injections. 	• Injections (gonadotrophins injections e.g. Gonal F) should be administered roughly at the same time every day – give or take within 2 hours • Doing both injections at 18h00 is also acceptable. • **If you have to use Meticortin, please start today.**
Day 9 of menstrual cycle	• You need to be at the centre at 08h00 for your first scan. • After the scan you will be advised how many more injections you have to take. At this point you make another appointment for your second scan. You will be monitored until you are ready for the aspiration.	• Do not take your gonadotrophins injections e.g. Gonal F on this day until after the scan. • You will continually be advised during this procedure.

When you are ready for aspiration	• You will be advised when the aspiration will take place. • You will be given a trigger injection. At this point your husband /partner will be given a script for antibiotics (Zithromax) and you will be given a script for cyclogest (progesterone) vaginal pessaries, or alternate progesterone.	• Abstain from intercourse from this point. • At this point you will be informed when to stop your Agonist injections e.g.Lucrin and Gonadotrophins injections e.g. Gonal F
Day of aspiration (retrieval of eggs) ***Take the day off work***	• On this day you will be told what time to be at the centre. • Aspirations are usually done in the mornings. • You will need the following: 1.Sperm sample 2. Pay the remainder of the fee 	• Do not have anything to eat or drink 6 hours before the aspiration. • The aspiration is done under conscious sedation and you may therefore feel drowsy after the procedure. Please make arrangements for someone to drive you home.
The Day after the aspiration.	• Start using the Cyclogest vaginal pessories. Use one pessory (200mg) three times a day; every 8 hours.	• Use the following times as a guide: morning – afternoon evening(06h00 - 14h00 - 22h00
After aspiration	• You may telephone the centre daily after 1pm to check on the embryos. We will contact you and inform you when the embryo transfer will take place.	

Day of embryo transfer	• The patient may eat and drink  	• **Preferably take the day off work** • After the embryo transfer you may resume back to your usual activities but avoid strenuous work. You must however abstain from intercourse after the embryo transfer. If the pregnancy test is positive you would have to abstain until further advised.
After the embryo transfer	• You must wait for 12 days, and then do a beta quantitative pregnancy test.	• Remember to use the cyclogest three times a day. • Do not stop any medication without first consulting the centre.

ANNEXURE B

Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
				
Even blastomeres, no fragmentation	Even blastomeres, slight fragmentation	Uneven size blastomeres, no fragmentation	Even or uneven size blastomeres, moderate fragmentation	Unrecognizable blastomeres, severe fragmentation

Embryo Grading – Veeck's scoring criteria

ANNEXURE C

Embryo Vitrification – Cook Blastocyst kit

Fill out patient vitrification form

Directions for preparation and use of vitrification kit:

The blastocyst vitrification kit contains solution1, solution2, solution 3 and solution 4.

Solution 1 - is a cryobase buffer

Solution 2 - contains cryobase buffer with 8% ethelene glycol with 8 % DMSO. Solution 2 is prepared by **adding 400 ul of solution 4 (DMSO) to 4.6 ml of vitrification solution 2 and mix well**

Solution 3 - contains cryobase buffer with Trehalose, 16% ethylene Glycol and 16 % DMSO. Solution 3 is prepared by **adding 1ml of solution 4 (DMSO) to 5.25 ml of vitrification solution 2**

Solution 4 - Contains DMSO

Preparation of vitrification dish – 4 well Nunc dish

Take a 4 well Nunc dish. Take 800 ul of Solution 1 and add them into well 1 and 2. Add 2800 ul of prepared solution 2 in well. Add 800 ul of solution 3 into well 4.

Equilibrate the three vitrification solutions to 37 ^{0}C for 10 mins.

While you let the solutions to equilibrate allocate the canister, goblet and straws for the patient according to the availability on the LN storage tanks that are available for cryopreservation by looking into the vitrification patient file.

Label the straws with patient surnames; file ID on both the sleeve and straw.

Procedure:

Place the embryos selected for vitrification into well 1 containing solution 1 – ES and give them a wash and transfer them to well 2 containing solution 1. Transfer the embryos from well 2 to well 3 containing solution 2 for 2 mins. Make a small drop of solution 3 – VS in the middle of Nunc well dish and transfer the embryos into the small drop without making bubbles. Aspirate embryos with the pipette from that small drop of solution 3 and load embryos into the hook with minimum quantity of media within 1 minute. Now carefully touch the tip of loaded hook with embryos on the cryobath block until the drop turns crystal and put them inside the sleeve placed on the LN block.

Now carefully transfer the hooks into the allocated canister and goblet.

Make sure you have made a note of the allocation on the patient forms prior to transfer to LN tanks

Embryo Thawing

The day before prepare a 4 well Nunc culture dish. The first 2 wells contain universal and the last 2 wells contain D1-5 culture media, depending on media embryos were cultured in before.

10min before thawing, place 4thawing mediums in a 4 well Nunc dish respectively and leave on heated stage (30˚C) to warm.

Set timer for 1min and remove straw from liquid nitrogen (double check the name) and place on paper towel on heated stage. Remove the coloured tip. While holding the straw over the lid of the nunc dish, and using clean (cleaned with an alcohol swab) scissors cut the top of straw just under the cotton plug. Push the final amount of media out using a syringe. Make sure all the embryos are out of the straw.

In the lid of the nunc dish make dilutions of the media from straw media to thawing media1. Embryos are gradually moved from drops until in media 1, this is over 5mins.

Dilutions of thawing media 1 to thawing media 2. Embryos are gradually moved from drops until in media 2, this is over 5mins.

After embryos are completely in thawing media 2 set heated stage up to 37˚C.

Dilutions of thawing media 2 to thawing media 3. Embryos are gradually moved from drops until in media 3, this is over 10mins.

Dilutions of thawing media 3 to thawing media 4. Embryos are gradually moved from drops until in media 4, as quickly as possible.

After thawing media 4 make 1 dilution drop of media 4 and universal. Then place embryos into universal media in culture dish and finally well 4 of culture dish containing D1-5 culture media.

Place dish containing embryos in incubator for transfer the following day.

ANNEXURE D

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GRANULOCYTE - COLONY STIMULATING FACTOR *(G-CSF)* NEUPOGEN

Name : _____

Age : _____

File NO. : _____

Batch NO. : _____

Treatment Date : _____

PREVIOUS CYCLE HISTORY						
Previous Number of Biochem Pregnancies						
Previous Number of failed IVFs						
Cycle Number	1	2	3	4	5	
Size of Endometrium						
Fluid in the Cavity						
Number of Embryos Transferred						
Grade of Embryos						

Dr. M.I. Cassim

Reg No: 2003/015157/23
Pr No: 016 000 0204 153

Dr. Y.M. Dasoo

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Fertility Centre
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CURRENT CYCLE HISTORY		
Number of Embryos Transferred		
Grade of Embryos		
Transfer Catheter		
Ease Level		
FHS (Yes / No)		
Endometrial Thickness Before		
Fluid In Cavity	(Yes/No)	
Endometrial Thickness Post		
Fluid Still Present Post	(Yes/No)	
Pregnancy	(Yes/No)	
Repeated IVF/Failure	(Yes/No)	
How Many ?		

Dr. M.I. Cassim

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Pr. No: 016 000 0204 153

Dr. Y.M. Dasoo

ANNEXURE E

STUDY NUMBER	AGE	PARITY	PATERNAL AGE	GRAVIDITY	SMOKING	CAUSE OF INFERTILITY	PROTOCOL	NO. OF PREV TREATMENTS	E-PRE-G-CSF	DONOR EGGS	ASP	ET	THWD	FET	G-I	G-II	G-III	G-IV	G-V	E-POST G-CSF	PREG (POS/NEG)
1																					
2																					
3																					
4																					
5																					
6																					
7																					
8																					
9																					
10																					
11																					
12																					
13																					
14																					
15																					
16																					

DATA COLLECTION SHEET

1. Study Number : _____
2. Age : _____
3. Parity : _____
4. Gravidity : _____
5. Smoker : YES _____ NO _____
6. Paternal Age : _____
7. Cause of infertility : _____
8. IVF protocol used : _____
9. Donor eggs : YES _____ NO _____
10. Number of previous treatments : _____
11. Date of G-CSF instillation : _____
12. Date of Embryo transfer : _____
13. Endometrial thickness pre-G-CSF instillation : _____
14. No of oocytes aspirated : _____
15. No of embryos transferred : _____

    FRESH _____ THAWED _____
16. Fluid in endometrial cavity : YES _____ NO _____
17. Embryo grade : _____
18. Endometrial thickness post G-CSF : _____
19. Pregnancy outcome : POSITIVE _____ NEGATIVE _____

ANNEXURE F

R14/49 Dr Tasneem Mohamed et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150961

NAME: (Principal Investigator)	Dr Tasneem Mohamed et al
DEPARTMENT:	Obstetrics and Gynaecology BioART Fertility Centre,Johannesburg
PROJECT TITLE:	An Evaluation of the Use of Granulocyte-Colony Stimulating Factor as an Adjunct to In Vitro Fertilisation in Patients who have Previously Failed Attempts at Pregnancy with In Vitro Fertilisation
DATE CONSIDERED:	02/10/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Yasmin Adam
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	05/10/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

ANNEXURE G

BioART.
Fertility Centre
Reg No: 2005/015157/23

• Dr. M.I. Cassim
MBBch (Wits) F.C.O.G (SA),
Mphil (Repro) Stellenbosch,
Dip B.A. (Edinburgh)

• Dr. Y.M. Dasoo
MBBch (Wits) F.C.O.G (SA),
MSC (Repro Physiology)
Stellenbosch

Pr. No 016 000 0204 153

03 September 2015

To whom it may concern

This letter serves to confirm that Dr M.I. Cassim will be the co-supervisor (along with Dr Y. Adam) for the dissertation of Dr Tasneem Mohamed.

For her MMed, Dr Mohamed will be evaluating the effects of the use of granulocyte-colony stimulating factor ("**G-CSF**") as an adjunct to in vitro fertilization and the achievement of pregnancy.

This study will be done retrospectively and will require her to analyse the data of patients that have received treatment with G-CSF.

Dr M.I. Cassim and Dr Y. Dasoo have been using this treatment on consenting patients since January this year. The literature available thus far has shown varying results regarding this new treatment modality.

We, Dr Cassim and Dr Dasoo allow Dr Mohamed access to our patients files so that she may collect the necessary data for analysis and write-up. We do however stress on the anonymity of our patients such that all files will be allocated a study number.

Should you have any queries, please do not hesitate to contact us.

Regards

Dr M.I. Cassim

Dr Y. Dasoo

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