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RESULTS AND CLINICAL OUTCOMES OF PGT-A FROM A SINGLE FERTILITY CENTRE IN JOHANNESBURG, SOUTH AFRICA

Kajel Somaroo

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A DISSERTATION SUBMITTED TO THE FACULTY OF HEALTH SCIENCES, UNIVERSITY OF THE WITWATERSRAND, IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICINE.

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Declaration

I hereby declare that this dissertation is my unaided original intellectual work which has not been submitted before to any institution for assessment purposes. Furthermore, I have acknowledged all sources used and have given due reference to these in the text of this dissertation.

Signature:…KSomaroo…….

Date: …24/08/2022……

Abstract

Background

The use of preimplantation genetic testing for aneuploidy (PGT-A) as part of assisted reproductive technology (ART) has become routine practice in some settings as it is hypothesized that the selection of a euploid embryo should increase the probability of achieving a pregnancy. However, PGT-A as a universal screening tool for all ART cycles is yet to be determined as there are insufficient studies that support or refute its use.

Aims

The fundamental aim of this study was to investigate the effectiveness of PGT-A on ART cycles by evaluating the clinical outcomes at one ART centre in Johannesburg, South Africa. The study also aimed to quantify the chromosomal status of the blastocyst biopsies. In addition, the maternal age effect on ART and clinical outcomes was also evaluated.

Methods

A total of 1448 ART cycles fulfilled the inclusion criteria of the retrospective case-control record review. The PGT-A cases compr-*ised 854 ART cycles and the control group included 594 ART cycles. The next-generation sequencing (NGS) results for 2518 embryo biopsies subject to PGT-A analysis were available for investigation.

Positive beta-human chorionic gonadotropin (β-hCG) levels, implantation rates and clinical pregnancy were evaluated in the case and control groups to determine whether PGT-A improved clinical outcomes.

Results

The overall maternal ages ranged from 21 to 48 years, the mean ± standard deviation maternal age in the case group was 36 ± 4.50 years and was 35 ±4.32 years in the control group. The PGT-A results of the blastocysts identified chromosomes 22, 16, 15, 21, 4, 2, 19, X, 9 and 13 (incidence is reflected by the order) as having the highest number of abnormalities overall.

A logistic regression analysis revealed that the ART cycles in the PGT-A case group were 2.07 more likely to establish a clinical pregnancy ($p < 0.0001$) compared to the control group in the overall dataset. Furthermore, in the >40-year maternal age category the odds of establishing a clinical pregnancy increased by three times in the case group compared to the control group ($p = 0.0015$).

Discussion

The study is novel in its contribution to the current body of PGT-A knowledge as it is the first evaluation of PGT-A conducted in South Africa. This study also draws its main findings per ART cycle which reduce patient bias.

The findings of this study support the use of PGT-A across all maternal age groups as the odds of achieving a clinical pregnancy with the transfer of a euploid embryo is significantly higher than in the group that used morphological assessment alone.

Conclusion

This study shows that PGT-A is an effective tool for embryo selection which promotes single embryo transfers and improves clinical outcomes.

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List of Abbreviations

aCGH	Array comparative genomic hybridization
AMA	Advanced maternal age
ART	Assisted reproductive technology
ASRM	American society for reproductive medicine
CCS	Comprehensive chromosome screening
CP	Clinical pregnancy
DET	Double embryo transfer
ESHRE	European society of human reproduction and embryology
ESTEEM	ESHRE study into the evaluation of oocyte euploidy by microarray analysis
FISH	Fluorescence *in situ* hybridization
ICM	Inner cell mass
ICMART	International committee for monitoring assisted reproductive technology
ICSI	Intracytoplasmic sperm injection
IVF	*In vitro* fertilization
PGT-A	Preimplantation genetic testing for aneuploidy
PGT-M	Preimplantation genetic testing for monogenic disorders
qPCR	Quantitative polymerase chain reaction
RCT	Randomized control trial
SET	Single embryo transfer
TE	Trophectoderm
WGA	Whole-genome amplification
WHO	World health organization
β-hCG	Beta human chorionic gonadotropin

Chapter 1

1 Literature review

1.1 Historical background

"God blessed them, and said unto them, Be fruitful and multiply, fill the earth, and subdue it" was the first instruction to be given to Adam and Eve in the Bible, which solidified mankind's inherent and deep-seated desire to procreate. Despite the blessings, the inclusion of barrenness within the Biblical narrative makes it clear that infertility is anticipated for a part of society (Levine, 2016).

The practice of aiding fertility is found in ancient Vedic literature dating back 4000 years, where enhancement concoctions made by priests were used for queens of childless kings to assist in conception (Kalra, Baruah & Kalra, 2016). Western medical practices for infertility find their primary sources in Greece, Hippocrates, known as the "father of medicine" recognized infertility as a medical problem in antiquity and formulated early treatments based on medical reasoning and rational thinking (Johnston, 1963; King, 2002).

Spermatozoa were first identified under the microscope in 1677 by Dutch scientist Antonie van Leeuwenhoek, which aided advancements in understanding reproductive physiology and gamete formation (Kovacs, Brinsden & DeCherney, 2018). Tremendous advancement in the treatment of infertility came in the nineteenth century with the discovery that pregnancy was a result of the union of an ovum and sperm (Bavister, 2002).

Meanwhile, in the field of genetics, the nineteenth century saw the early studies of reproductive genetics when Gregor Mendel famously experimented with peas to prove the Laws of Heredity (Hoßfeld et al., 2017). He proposed the principles of segregation and independent assortment where gametes were first classified as haploid and genes were shown not to influence each other during gametogenesis (Henig, 2000). Mendel's work was rediscovered by scientists in 1902, who went on to prove the theories of cell division in somatic cells, gamete formation through meiosis, and the process of fertilization (Opitz, 2016).

Later in the twentieth century, the first baby created through *in vitro* fertilization (IVF) was born in London, England (Steptoe & Edwards, 1978). A mere 12 years later, Handyside et al.

(1990) performed testing on preimplantation embryos to identify female embryos free of sex-linked disease and X-linked mental retardation. The authors biopsied one cell from an embryo on day three of development and performed DNA amplification by quantitative polymerase chain reaction (qPCR) of repeat sequences found on the Y chromosome. This launched the field of preimplantation genetic testing which has now become an important part of assisted reproductive technology (Penzias., et al., 2018).

Fluorescence *in situ* hybridization (FISH) was later applied to screen embryos for an abnormal number of chromosomes, termed aneuploidy, and structural rearrangements from reciprocal translocation carriers, while qPCR was applied for the detection of monogenic disorders (Parikh et al., 2018). Preimplantation genetic testing for monogenic disorders (PGT-M), structural rearrangements (PGT-SR) and aneuploidy (PGT-A) have each developed into their independent fields of study.

Preimplantation genetic testing for aneuploidy is used to screen the chromosome complement of embryos prior to transfer. Traditionally morphology assessment alone was used for embryo selection however the introduction of PGT-A has provided an alternative tool that can be added as part of ART treatment for the selection of chromosomally normal embryos.

This section will focus on infertility, the treatment of infertility in the form of ART, and the use of PGT-A as part of treatment, followed by the rationale for this study.

1.2 Infertility

1.2.1 **Definitions of infertility**

The burden of infertility is significant and has not displayed any signs of decline over the past 20 years (World Health Organization, 2020a). The healthcare community uses three definitions to classify infertility: clinical, epidemiological, and demographic (Jacobson et al., 2018).

The World Health Organization (WHO) and International Committee for Monitoring Assisted Reproductive Technology (ICMART) **clinically** define infertility as a "disease of the reproductive system defined by the failure to achieve a clinical pregnancy (CP) after 12 months or more of regular unprotected sexual intercourse" (Zegers-Hochschild et al., 2017). The clinical definition is intended for early detection and treatment management.

The American Society for Reproductive Medicine (ASRM) proposed a revised clinical definition of infertility to include age-defined thresholds, especially for women over the age of

35 years, which are considered to be of advanced maternal age (AMA), as there is a proven decline in fertility, discussed in detail in Section 1.4.2. The ASRM, therefore, defines infertility as, “the failure to achieve a successful pregnancy after 12 months or more of regular, unprotected sexual intercourse” and suggests that “treatment should be initiated at 12 months for women under the age of 35 years, at 6 months for women over the age of 35 years and immediate assessment and treatment for those over 40 years” (Practice Committee of the American Society for Reproductive Medicine, 2013).

The **epidemiological** definition of infertility is the "inability of women of reproductive age (15–49 years) who are at risk of becoming pregnant (i.e. sexually active, not using contraception and not lactating) and report trying unsuccessfully to conceive for 24 months or more" (World Health Organization, 2020a). Extending the timeframe minimizes the risk of overestimation of infertility rates by decreasing false positives (Jacobson et al., 2018) The clinical and epidemiological definitions are more closely in line with clinical practice than the demographic definition.

The **demographic** definition applies a five-year timeframe for the inability to conceive for women of reproductive age (15–49 years) who are at risk of becoming pregnant and maintaining a longing for a child (Larsen, 2005; Gurunath et al., 2011). The five-year period allows for a longer period between previous live births and further reduces the risk of false positives (Mascarenhas et al., 2012), however, the definition does not account for individuals with symptoms of infertility. Demographers use population-based surveys to evaluate reproductive outcomes for comparison between countries.

Infertility can be further classified as **primary** or **secondary** infertility. A woman who is unable to ever conceive a child, with the inability to become pregnant or to achieve a live birth, is classified as having primary infertility. Whereas a woman unable to bear a child, due to the inability to fall pregnant or achieve a live birth following a previous successful pregnancy would present with secondary infertility (The ESHRE Guideline Group on RPL et al., 2018; World Health Organization, 2020a).

Although there are data available on infertility from high-income countries, more research needs to be done in developing countries, especially in lower resource settings worldwide. There is a need to generate consistent data to formulate treatments and interventions based on the types and causes of infertility (Mascarenhas et al., 2012; Inhorn and Patrizio, 2015; Dyer et al., 2019).

1.2.2 **Prevalence of Infertility**

Worldwide estimates suggest that globally, 48 million couples are affected by infertility, however, the main challenge in producing estimates on a global scale is conceptual and methodologically heterogeneous, making standardization of data difficult (Gurunath et al., 2011; Mascarenhas et al., 2012; World Health Organization, 2020b).

The global statistics on infertility are mostly based on two studies. An early study conducted by Boivin et al., (2007) evaluated 25 population-based surveys of 172 413 married women and found a clinical infertility rate in developed countries of 3.5%-26.4% and developing countries of between 1.3% and 25.7%. They published a global rate of infertility of 9%, affecting approximately 72.4 million couples with only just over half of those couples, seeking medical treatment.

The second more recent study, funded by the Bill and Melinda Gates Foundation and supported by the WHO, analysed 277 health surveys on demographics and reproductive health from 1990 through 2010 from 190 countries and territories and estimated that 48.5 million couples worldwide were infertile in 2010 (Mascarenhas et al., 2012). However, when applying the demographic definition of infertility, the duration would be adjusted for the five-year timeframe, resulting in an increase in the prevalence of infertility by 2.5-fold; estimating couples carrying the disease burden at 121 million worldwide (World Health Organization, 2014; Lee, 2018).

1.2.2.1 Infertility in Africa

The study conducted by Mascarenhas et al., (2012), revealed that in developing countries one in four couples are affected by infertility. Of the estimated 48.5 million couples affected by infertility, 10 million of those couples are estimated to reside in Sub-Saharan Africa, indicating one of the highest infertility prevalence rates worldwide (Mascarenhas et al., 2012).

Infertility is recognized as a major health issue in Africa, with regional prevalence rates ranging from 20-to 40% (Leke et al., 1993; Ericksen & Brunette, 1996). Some African studies have found that infertility affects greater than 20% of people in Nigeria, Ethiopia, and Gambia with secondary infertility being more prevalent in these settings mainly due to complications with first pregnancies in poor resource settings (Ebomoyi & Adetoro, 1990; Adetoro & Ebomoyi, 1991; Larsen, 2000). However, the limited number of quality assisted reproductive technology centres, high treatment costs, and perceived risk (fetal abnormality, lack of social acceptance), are barriers to seeking infertility treatment in the majority of low-resource African countries (Ericksen & Brunette, 1996; Jegede & Fayemiwo, 2010; Dyer et al., 2019).

Childlessness comprises of two categories, voluntary and involuntary childlessness. Childlessness is defined as voluntary when an individual deliberately chooses not to have children. Involuntary childlessness includes the inability to have children for medical reasons or circumstantial delayed childbearing. Reliable estimates on childlessness are needed to understand true infertility prevalence amongst the involuntary childlessness population (Sappleton, 2018; Razeghi-Nasrabad et al., 2020).

In South Africa, limited data are available on the population prevalence of infertility as countrywide statistics fail to distinguish between voluntary childlessness and involuntary childlessness including the temporary childlessness related to delayed or circumstantial childbearing (Statistics South Africa, 2019). A qualitative study conducted by Dyer et al., (2002) described that women in South Africa with involuntary childlessness had limited knowledge of human reproduction and were not aware of current treatment options for infertility. Awareness and information are essential for improving accessibility and understanding of ART treatment modalities.

The severe psychological, socio-cultural, and economic consequences of being childless in Africa make accessibility to ART treatment an important health issue. Obtaining an accurate diagnosis for infertility provides couples and clinicians with the ability to guide treatment modalities. Agreement on the criteria used for diagnosis is critical for selecting the appropriate interventions to achieve favourable clinical outcomes. Furthermore, accurate data on the prevalence, secular trends, and geographical influences, as well as clear definitions of infertility are essential for fertility specialists and patients to make informed decisions regarding assisted reproductive technologies (Gurunath et al., 2011).

1.3 Overview of Assisted Reproductive Technologies (ART)

Assisted reproductive technologies include all fertility treatments in which embryos are created outside of the body. The technologies are applied to treat several causes of infertility by retrieving gametes, generating embryos in a controlled laboratory environment, and transferring the embryo with the greatest potential to achieve a live birth (Zegers-Hochschild et al., 2017).

The treatment of infertility is complex with each reproduction cycle consisting of several steps. The optimization of any one of the steps can improve outcomes therefore treatment protocols must be established from good quality evidence supported by well-designed studies.

The steps of ART generally include (Farquhar and Marjoribanks, 2018):

a) Controlled ovarian stimulation to induce oocyte maturation
b) Follicle growth monitoring
c) Transvaginal oocyte retrieval
d) Optional: In cases of male infertility, retrieval of sperm is required.
e) *In vitro* fertilization or intracytoplasmic sperm injection (ICSI).
f) Fertilization: the formation of zygotes with two pronuclei (2PN)
g) Embryo culture: strict laboratory conditions are applied to determine the oxygen concentration, culture media, and assisted hatching techniques used
h) Optional: Embryos can be biopsied and genetically screened
i) Optional: Vitrification
j) Embryo transfer: fresh or frozen (thawed after vitrification) embryos selected based on morphology and/or genetic status
k) Implantation
l) Luteal phase support: administration of progesterone, oestrogen, and human chorionic gonadotrophin

These steps have one objective, which is to establish a pregnancy, therefore evidence-based practices must be used to optimize protocols, which can ultimately give favourable outcomes such as increased live birth rates, with reduced multiple gestations and associated adverse events (Derks et al., 2009; Forman et al., 2014).

1.3.1 Measures of ART Success

Success is defined as the achievement of a favourable or desired outcome (Merriam-Webster Dictionary, n.d.). ART treatment involves complex and multistep approaches used to achieve a pregnancy. Consequently, suitable measures at every stage must be used to determine the success of treatment.

A numerator, in the context of medical treatment, represents a clinical measure of interest (e.g., fertilization, pregnancy, live birth). The figure representing the total population, the denominator, provides the context in which the outcome is evaluated (e.g., per treatment cycle or embryo transfer) (Schmutzler, 2019).

1.3.1.1 Embryo Parameters

Treatment steps occurring at different stages of ART provide a plethora of evaluation points. Controlled ovarian stimulation involves the administration of exogenous gonadotropin to retrieve a large number of oocytes for ART (Farquhar & Marjoribanks, 2018).

The successful retrieval of mature oocytes is a prerequisite for ART. Zhao et al., (2020a) published a retrospective study that estimated the optimized number of oocytes retrieved in patients ranged between 21-30 oocytes which increased cumulative live birth rates to 93.8% per ART cycle. This indicated that the number of oocytes retrieved had a positive correlation with the cumulative live birth rate among women under 35 years of age. In women of AMA, studies have shown that the number and quality of mature oocytes decline, with typically eight to ten oocytes retrieved per cycle. Sperm are introduced to the oocytes after retrieval and fertilization is assessed 24 hours later (Sacchi et al., 2019; Seshadri et al., 2020).

Conventional IVF involves the culturing of sperm with oocytes and is considered a traditional fertilization method. Intracytoplasmic sperm injection is a technique used to inject a single sperm into the oocyte, which was first introduced for patients diagnosed with severe male-factor infertility, but has now expanded to groups with unexplained infertility and AMA. However, several studies have shown no advantage in reproductive outcomes of groups using ICSI when compared to conventional IVF techniques (Boulet et al., 2015; Tannus et al., 2017; Li et al., 2018). In cases of PGT-M, the use of ICSI is preferred as it reduces the risk of contamination from remaining cumulus cells or exogenous sperm that is usually introduced when using IVF techniques (De Rycke & Berckmoes., 2020).

The variables that contribute to fertilization rates are numerous. The formation of zygotes with two pronuclei (2PN), one parental and one maternal in origin indicates successful fertilization. The fusion of the two pronuclei forms the primary nucleus of the embryo. The nucleus of cells

contains deoxyribonucleic acid (DNA) molecules packaged in thread-like structures called chromosomes (Ferguson-Smith, 2015; Zegers-Hochschild et al., 2017).

The zygote then undergoes several rounds of cleavage divisions over the first three days leading to the formation of embryos usually containing six to ten cells. The embryo reaches the blastocyst stage by the fifth or sixth day of development, characterized by the fluid-filled blastocyst cavity, termed the blastocoele, the inner cell mass (ICM) and the trophectoderm (TE) (Figure1-1) (Mamas, 2012).

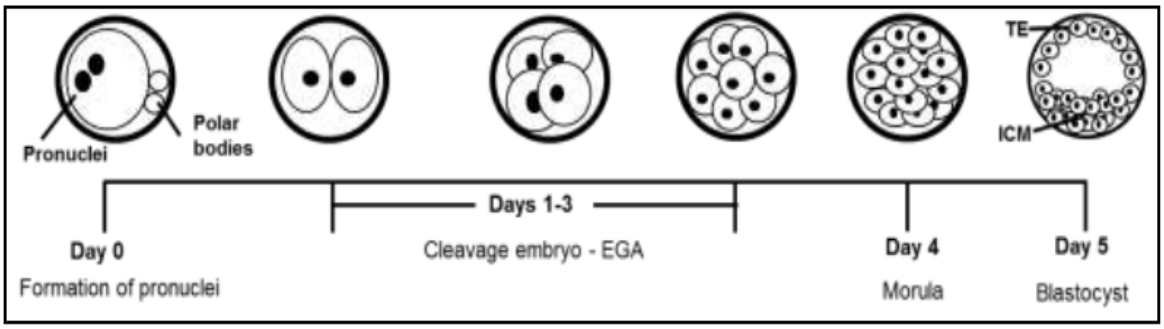


Figure 1-1: The developmental stages of a zygote to the blastocyst stage (Mamas, 2012).

1.3.1.2 Morphological assessment

Despite research efforts beginning in the 19th century, the morphology of mammalian fertilization and embryonic development remained sparse and inadequate as studies had to be conducted *in vivo* impeding research. The creation of embryos *in vitro* gave the ability to observe fertilization and embryo development under controlled laboratory conditions where critical measurements could be assessed (Bavister, 2002).

Since early attempts to evaluate and base clinical decisions on embryo morphology, many grading systems have been described to quantify development potential by describing the embryo beyond the fertilization stage (Hardarson, Van Landuyt & Jones, 2012). The foundation of grading systems was based on the assessment of cleavage stage embryos, as day 3 embryos were transferred for decades at ART centres as a result of the inability to culture embryos to further stages (Bolton, Wren & Parsons, 1991; Jones et al., 1998).

The introduction of routine blastocyst culture in the late 1990s resulted from the change of culture media formulated on the nutrient requirement at different stages of embryo development (Gardner, 1998). The successful blastocyst culturing revolutionized transfer practices and developments of grading systems from day four to day six began (Gardner et al., 2000).

Early approaches to blastocyst grading proposed that low cell count was linked to abnormal development and morphology (Dokras, Sargent & Barlow, 1993). Shapiro, Harris & Richter (2000) demonstrated that the grade of expansion of the blastocyst and timed development of the fluid-filled blastocoele cavity are indicators of implantation potential. The retrospective study showed a 43% implantation rate in the expanded blastocyst group whereas the less developed blastocysts resulted in a 17% implantation rate in women undergoing double embryo transfer (Shapiro, Harris & Richter, 2000).

The most widely used grading system in current practice was developed by Gardner and Schoolcraft., (1999) and was designed to assess three parameters (Table 1-1); the degree of blastocoele expansion and the quality and number of cells in the ICM and trophectoderm TE.

Table 1-1. Embryonic grading system developed by Gardner and Schoolcraft (1999)
This grading system is used for the evaluation of the early blastocyst specifically the expansion, inner cell mass (ICM), and trophectoderm (TE).

Grade	**Blastocyst expansion**	**Description**
Degree of expansion		
1	Early blastocyst	Blastocoele is less than half the volume of the embryo
2	Blastocyst	Blastocoele is greater than or equal to half the volume of the embryo
3	Full blastocyst	Blastocoele is completely filling the embryo
4	Expanded blastocyst	Blastocoele volume is larger than full blastocyst characterised by a thinning of the zona pellucida
Cell morphology		
Grade	**ICM grading**	**TE grading**
A	Tightly packed and many cells	Many cells form a cohesive epithelium
B	Loosely grouped and several cells	Few cells form the loose epithelium
C	Very few cells and disorganised	Very few large cells

This comprehensive alphanumeric system has been validated by different groups who have demonstrated that the embryos with higher grades in two or more parameters achieve higher implantation rates (Gardner et al., 2000, 2004; Balaban, Yakin & Urman, 2006). Consensus between higher scoring blastocysts (>3AA), a fully expanded blastocyst cavity with a compact ICM and a cohesive TE both with adequate cell counts as a preferred choice for transfer has been reached within some groups when selecting the most viable blastocyst (Racowsky et al., 2010; ESHRE Special Interest Group of Embryology, 2011).

Traditionally, assessment of embryos before transfer only comprised of morphological scoring methods however, this technique alone has been shown to be an inefficient measure of reproductive potential of the blastocyst with several studies showing good morphology embryos having weak implantation potential (Alfarawati et al., 2011; Fragouli et al., 2014).

1.3.1.3 Clinical Outcomes

Clinical outcomes are commonly used numerators in measuring ART success. Implantation rates, clinical pregnancy rates, and live birth rates are commonly used as measurable clinical outcomes (Schmutzler, 2019).

1.3.1.3.1 Successful embryo transfer

Increased levels of beta-human chorionic gonadotropin (β-hCG) hormone are secreted primarily by syncytiotrophoblastic cells in the placenta during pregnancy (Zegers-Hochschild et al., 2017; Betz & Fane, 2018). Several studies have investigated the role of β-hCG as an early predictor of pregnancy (Montagnana et al., 2011; Betz & Fane, 2018). However, a positive serum or urinary hormone level without the development of a gestational sac is known as a biochemical loss (Zeadna et al., 2015). In the general population, biochemical pregnancy losses usually go undetected as they occur before the woman reaches the expected date of her menstruation cycle (Kolstad et al., 1999).

Implantation rates are assessed after embryo transfer by testing for quantifiable signs of implantation such as increased levels of β-hCG hormone secreted during pregnancy and the presence of a gestational sac (Zegers-Hochschild et al., 2017).

1.3.1.3.2 Implantation rate

Implantation rates provide an immediate measure of treatment effectiveness, however, transfer success is dependent on the morphology of embryos, ploidy status, the quality of clinical treatment, age, and timing of embryo transfer. Three studies compared the implantation rates of embryos selected after PGT-A and morphological assessment alone. All three studies observed higher implantation rates after PGT-A though the studies varied in design, biopsy stage, PGT-A technique, and patient prognosis. These studies were also largely based on good-prognosis women (Staessen et al., 2004; Greco et al., 2014; Rubio et al., 2017).

Implantation failure is used to describe patients who both never show signs of an increased level of β-hCG hormone produced during pregnancy and those who have increased β-hCG levels without evidence of a gestational sac (Coughlan et al., 2014). Biochemical pregnancies fall into the category of implantation failure which is not uncommon in the general population with incidences varying between 8% and 33% (Maesawa et al., 2015).

Clinical pregnancy follows a positive implantation outcome and is generally assessed between four and seven weeks after transfer. These measures provide information to clinicians and researchers on treatment modalities that favour the establishment of pregnancy (Barnhart, 2014).

1.3.1.3.3 Clinical pregnancy

A successful clinical pregnancy is identified by ultrasonographic visualization of a gestational sac(s) or definitive clinical signs of pregnancy, which include ectopic pregnancy. Clinical pregnancy with a fetal heartbeat is also diagnosed by ultrasonographic and usually recorded as a positive outcome when at least one discernible heartbeat is detected per ART cycle initiated or per embryo transfer (Zegers-Hochschild et al., 2017).

Clinical pregnancy rates can to some extent be predicted based on variables measured up to this point in ART treatment. Implantation rates as a prognosticator of pregnancy outcomes found that women with 100% implantation rates were less likely to suffer a pregnancy loss when compared to women with lower implantation potential (Huisman et al., 2000; Zegers-Hochschild et al., 2005).

Although clinical pregnancy rates have a time advantage for measuring treatment outcomes compared to live birth rates, clinical pregnancies do not guarantee a live birth. A **pregnancy loss** is the spontaneous demise of a pregnancy before the fetus reaches viability and includes all losses until 24 weeks of gestation (The ESHRE Guideline Group on RPL et al., 2018).

This definition encompasses losses with spontaneous conception and through ART. **Recurrent pregnancy loss** is defined as the loss of two or more pregnancies and is reported to affect 1-2% of women but the exact prevalence is difficult to estimate due to the lack of standardized investigations (Egerup et al., 2016; The ESHRE Guideline Group on RPL et al., 2018).

1.3.1.3.4 Live birth rate

The ultimate goal of ART is to achieve a live birth. Live birth is defined by the complete expulsion of a fetus with clear evidence of life after completing 22 weeks of gestation, which refers to the individual newborn with twin and higher-order multiples counted as separate live births (Zegers-Hochschild et al., 2017).

Live birth rates are the longest wait for the clinical measure. It provides a more quantitative and accurate measure of treatment than β-hCG and clinical pregnancy measures are subjective and more open to interpretation (Barnhart., 2014; Yan et al., 2021). There is much debate on the calculation of live birth rates, traditionally live births have been reported per embryo transfer but cumulative live birth rates are becoming the preferred measure, that is, the first live birth

following the transfer of embryos from a single ovarian stimulation nevertheless no consensus has been reached on this measure (Maheshwari, McLernon & Bhattacharya, 2015).

The likelihood of achieving a live birth per ART cycle in patients under 41 years of age is estimated to be 35%, whereas, the chance of achieving a live birth over 41 years of age declines to 8% (Stern et al., 2013). The decline is largely attributed to aneuploidy, it is well-established that the incidence of aneuploidy increases with maternal age (Hassold et al., 1996; Franasiak et al., 2014a).

1.4 Aneuploidy

In eukaryotic cells, mitosis and meiosis are mechanisms of cell division. Mitosis results in two nuclei that are identical to the original nucleus usually partitioned into two new cells. Meiosis occurs in the process of gametogenesis, in which a diploid gamete produces haploid sperm and eggs (Figure 1-2) (Malmquist & Prescott, 2013).

Aneuploidy is the occurrence of one or more extra or missing chromosomes leading to a deviation from the normal number of chromosomes in a cell or organism. In humans, a euploid cell contains 23 chromosome pairs (2n) with one copy of a homologous chromosome typically inherited from each gamete (Hassold et al., 1996). Aneuploidy arises through various mechanisms in preimplantation embryos with most changes in chromosome number occurring during meiosis I (Gabriel et al., 2011). Early pregnancy loss is caused by chromosome abnormalities in 50% of cases, with whole chromosome gains, termed trisomy (2n+1), being more prevalent than whole chromosome losses, termed monosomy (2n-1) in studies evaluating products of conception (Warburton et al., 1978; Rubio et al., 2003).

Meiotic nondisjunction is the term used for the mal-segregation of chromosomes during gametogenesis (Sora, Lucchini & Magni, 1982). The chromosome pairs do not separate, two chromatids are pulled to one cell, giving rise to gametes with two copies of chromosomes and the other with no copies. Consequently, when these gametes fuse, an imbalance in the number of chromosomes occurs in the embryo due to the error in meiosis (LeMaire-Adkins, Radke & Hunt, 1997). Spontaneously occurring aneuploidy is well-recognized in human embryos, it often leads to arrested embryo development, implantation failure, miscarriage, or an aneuploid pregnancy (Hassold et al., 1996).

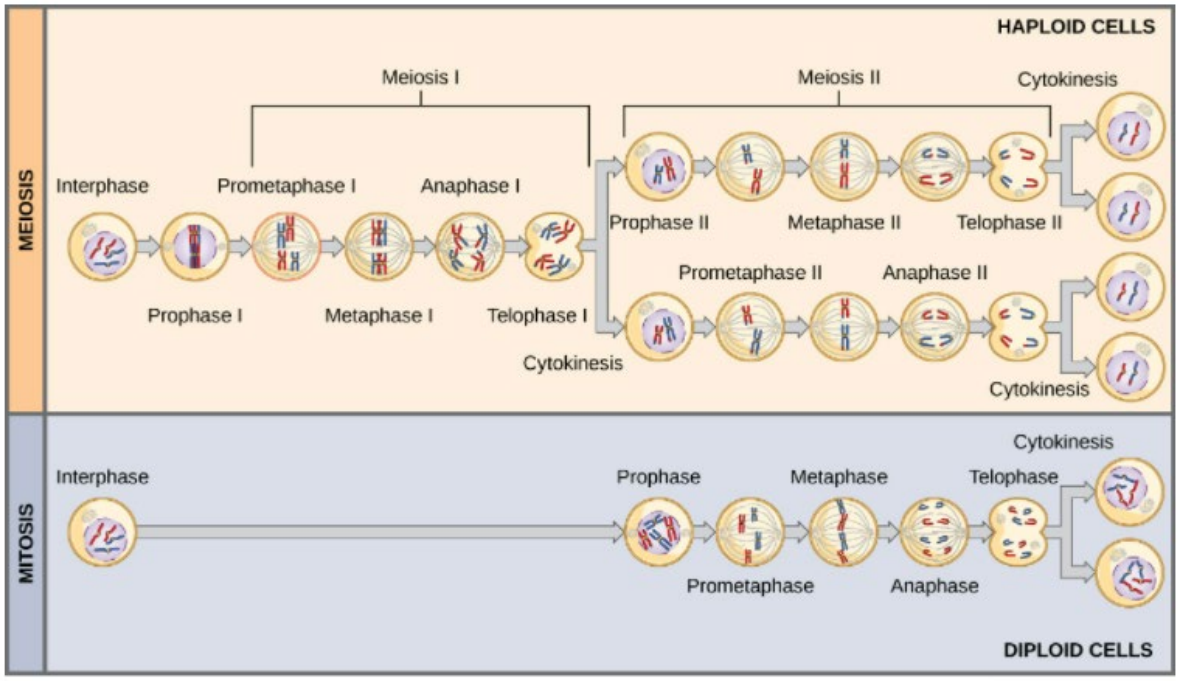


Figure 1-2 Mitosis and meiosis in the nucleus in eukaryotic cells resulting in diploid and haploid cells, respectively (Malmquist & Prescott, 2013)

The majority of data on aneuploidy in human embryos are generated through preimplantation genetic studies and it has been proven to be a common occurrence (Munné et al., 2004). The most common occurrence of aneuploidy at birth is an extra copy of chromosome 21 (trisomy 21), which gives rise to Down syndrome. Besides trisomy 13, 18, 21 and sex chromosome aneuploidies which can cause severe birth defects, most aneuploidies are not compatible with life (Delhanty et al., 1997). Among clinically recognized stillbirths, 4% are attributed to monosomy X and trisomy 16, 21 and 22, and 0.3% of new-borns are born with trisomies 21, 18 and 13 and sex chromosome trisomies; 47 XXX, 47, XXY and 47, XYY (Hassold & Hunt, 2001; Schaeffer et al., 2018).

Comprehensive chromosome screening (CCS) has revealed that aneuploidies of sex chromosomes, and chromosomes 15, 16, 19, 20, 21 and 22, occur most frequently, however, aneuploidies of all chromosomes have been observed (Traversa et al., 2011; Liang et al., 2013). The relationship between chromosomal status and morphological grade remains elusive. Some studies show that genetically normal embryos can be of weak embryo grading and good quality embryos can have aneuploidies (Gardner et al., 2000; Capalbo et al., 2014; Fragouli et al., 2014; Munné et al., 2019).

An extensive study conducted by Capablo et al., (2014) on 956 blastocysts showed a moderate relationship between morphology and ploidy. A euploidy rate of 56.4% was seen in embryos

with excellent morphology and dropped to 25.5% in the blastocysts of poor quality. Complex aneuploid embryos also showed an association with morphology with 6.8%, 15.2%, 17.4%, and 27.5% of excellent, good, average, and poor-quality embryos, respectively, having three or more aneuploidies. This study further indicated that morphology cannot be used to decrease the probability of transferring a chromosomally abnormal embryo as there is still a high proportion of morphologically excellent embryos that exhibit aneuploidies (Capalbo et al., 2014).

Apart from aneuploidies affecting whole chromosomes, structural chromosomal abnormalities, partial gains and losses on the p- or q- chromosomal arms have been found to occur in approximately 7% to 8% of preimplantation embryos (Fragouli et al., 2013; Escribà, Vendrell & Peinado, 2019). Segmental aneuploidies are inherited from balanced rearrangement carriers, or they can occur *de novo* by errors in meiosis during gametogenesis or mitotically during cell division (Babariya et al., 2017; Girardi et al., 2020).

The ability of CCS technologies to detect segmental errors derived from the use of PGT-A for patients who carry balanced translocations. The size of the imbalances can be predicted, based on where the breakpoints are located within the chromosomes involved in the balanced translocation. These studies provided data on the capability of PGT-A technology to detect size-specific segmental aneuploidies (Munné, 2005; Zhang et al., 2016). Studies investigating the origin of segmental aneuploidies revealed higher incidences of segmental aneuploidies were of mitotic origin in blastocysts stage embryos (Babariya et al., 2017; Vera-Rodriguez & Rubio, 2017).

Similar to segmental aneuploidies, mosaicism in human embryos only became reliably detectable with recent advances in technologies which can give further insight into the true incidences and clinical implications (Treff & Franasiak, 2017; Escribà, Vendrell & Peinado, 2019).

1.4.1 **Mosaicism**

Mitotic errors also contribute to the complexities associated with the transfer potential of an embryo. Mitotic errors post-fertilization can give rise to two distinct cell populations within a single embryo, this phenomenon is called embryo mosaicism (Warburton et al., 1978). Unlike meiotic errors which give rise to all aneuploid cells within an embryo, mitotic errors in embryonic development result in a mixture of euploid and aneuploid cells. Euploid gametes and error-free progress of mitosis results in euploid embryos. Meiotic errors result in homogenously aneuploid cells in embryos. Errors in mitosis during embryonic cell division cause diploid-aneuploid mosaicism (Figure 1-3) (Vázquez-Diez & FitzHarris, 2018).

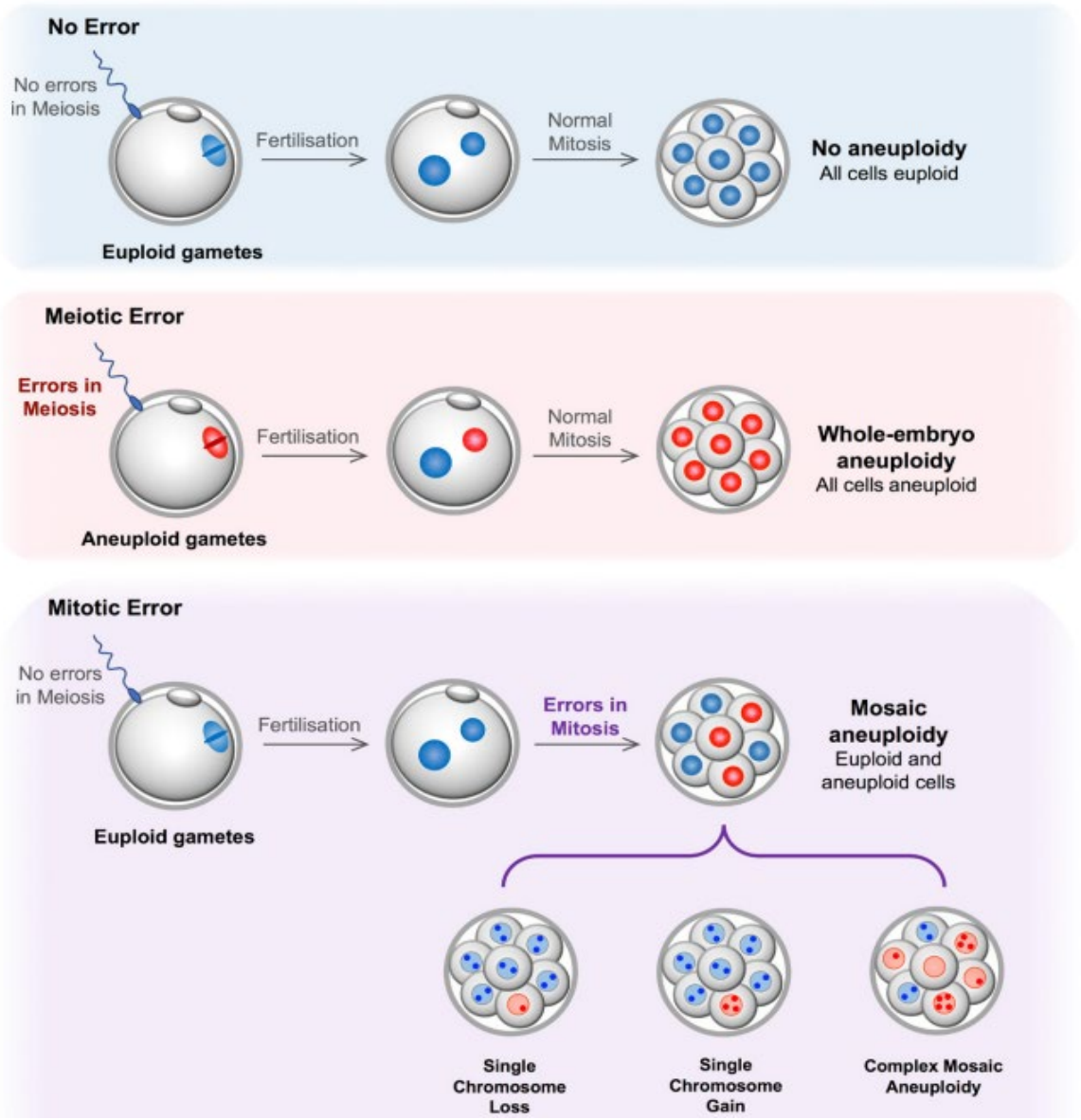


Figure 1-3. Ploidy of preimplantation embryos
This illustration represents errors in meiosis I although errors in meiosis II can also occur (Vázquez-Diez & FitzHarris, 2018).

The mechanisms that cause mosaicism include; anaphase lag which involves delayed movement of chromosomes or nondisjunction during cell division (Coonen et al., 2004). The level of mosaicism detected is dependent on the number of biopsied cells sent for testing. Depending on the location of the biopsy, the distribution of euploid and aneuploid cells may vary. Therefore, the result cannot always be extrapolated to the entire embryo (Vera-Rodriguez & Rubio, 2017).

Preimplantation genetic testing for aneuploidy results is reported as mosaic when 20% - 80% of trophectoderm cells are abnormal, which has become a widely utilized method for result

interpretation amongst laboratories (Cram et al., 2019; PGDIS., 2020). Embryos classified as a mosaic by PGT-A have reduced implantation rates and increased incidence of miscarriage (Fragouli et al., 2017). However, mosaic embryos made available for transfer to 18 women with no euploid embryos showed potential for mosaic embryos to result in healthy babies. Eight transfers resulted in clinical pregnancies of which six progressed to full term with the birth of an infant. These pregnancies were followed up with conventional karyotyping which all revealed a normal chromosome complement indicating that mosaic embryos have the potential to result in live births (Greco, Minasi & Fiorentino, 2015). Subsequently, it is proposed that mosaic embryos can be prioritized for transfer, according to the chromosome involved and level of mosaicism, in the absence of euploid embryos (Cram et al., 2019).

As a result, mosaic embryos are considered as a third group that has intermediate potential to result in pregnancy. Patients without euploid embryos available for transfer may consider implanting a mosaic embryo, a practice supported in statements released by the Preimplantation Genetic Diagnosis International Society (PGDIS) in 2016 and updated in 2020 and Congress on Controversies in Preconception, preimplantation and prenatal genetic diagnosis (CoGEN). These guidelines provide much-needed guidance on prioritizing the selection of mosaic embryos centred around the chromosome involved and the level of mosaicism (COGEN, 2016; PGDIS, 2016, 2020).

The guidelines released by the PGDIS in 2020 were updated to include comments for the laboratory mentioning that biopsy technique, internal validations and trophectoderm representation of surrounding cells and inner cell mass should be taken into consideration (PGDIS, 2020). Results obtained from PGT-A performed on eight to ten trophectoderm cells will vary in the representation of the entire embryo which is made evident by healthy births resulting from mosaic embryo transfers (Treff & Franasiak, 2017; Victor et al., 2019; Munné et al., 2020).

1.4.2 **Maternal Age**

Interestingly, the incidence of mosaicism does not increase with advancing maternal age like aneuploidies of meiotic origin. However, mosaicism coupled with increased risk of aneuploidy with increasing age affects the likelihood of having a euploid embryo in AMA groups (Taylor et al., 2014).

Maternal nondisjunction accounts for 95% of Down syndrome cases with higher incidences recorded with increasing maternal age (Oliver et al., 2008). Aneuploidy levels observed in oocytes are higher than levels observed in sperm, possibly due to the presence of a checkpoint

mechanism that does not allow the development of aneuploid sperm during spermatogenesis, which oogenesis lacks (Uroz & Templado, 2012).

Ageing oocytes are ineffective in correcting mal-segregated chromosomes as the cohesion weakens with age, which leads to deteriorating checkpoint efficiency making errors in maternal meiosis I being the most common source of aneuploidy (LeMaire-Adkins, Radke & Hunt, 1997). These mechanisms are affected in women of advancing maternal age, which contributes to the lower success rates of ART recorded in these groups (Wartosch et al., 2021).

Franasiak and colleagues performed 15169 trophectoderm biopsies on embryos from female patients between the ages of 22 to 49 years, interestingly they found an increase in aneuploidy prevalence in women 23 years and younger, with the lowest risk of aneuploidy between the ages of 26 to 30 and a steady increase of aneuploidy risk from age 31 through to 43, with rates plateauing at roughly 85% (Figure 1-4) (Franasiak et al., 2014b).

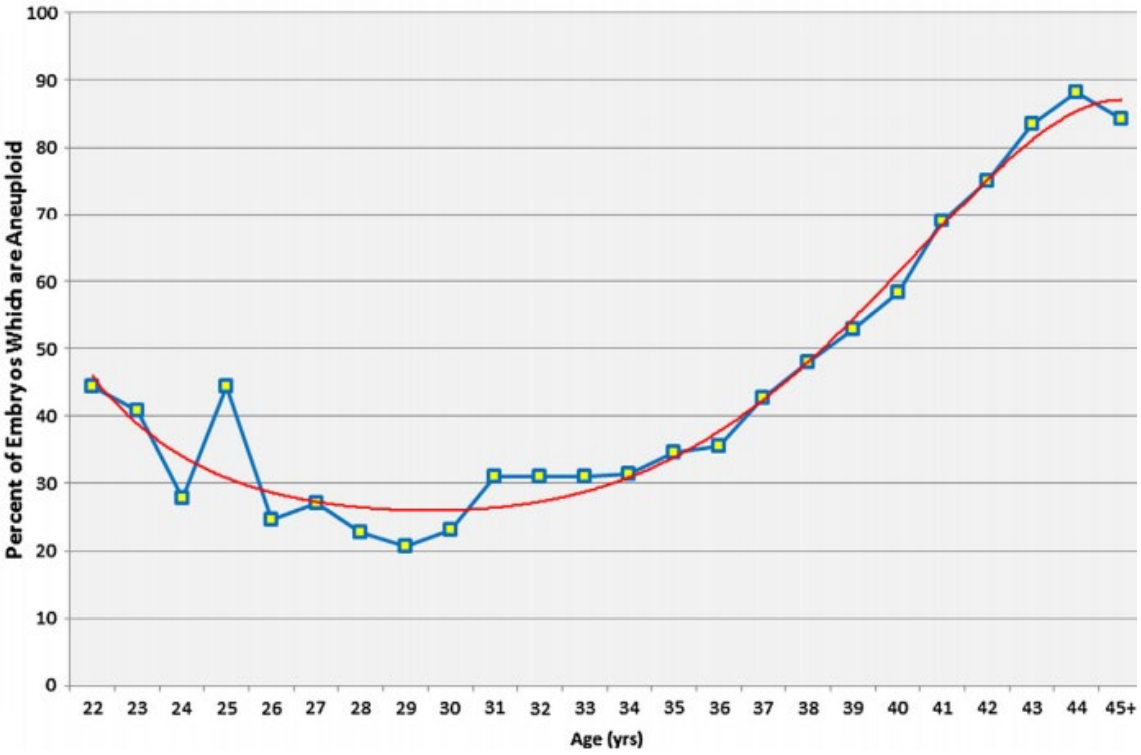


Figure 1-4. Prevalence of aneuploidy with advancing maternal age (Franasiak et al., 2014b)

Establishing aneuploidy-associated risk in relation to maternal age is useful for informing reproductive decisions. Accordingly, PGT-A is recommended as an additional tool for patients

with AMA to reduce repeated implantation failure or recurrent pregnancy loss associated with aneuploidy (ESHRE PGD Consortium Steering Committee, 2002).

1.5 Pre-implantation genetic testing (PGT)

Assisted reproductive treatment is a prerequisite for PGT which is performed at the embryo stage. For those embryos undergoing PGT, five to ten trophectoderm cells are biopsied from a blastocyst-stage embryo, usually on day five of development (ESHRE PGD Consortium Steering Committee, 2002).

The trophectoderm biopsy involves drilling the zona pellucida furthest from the inner cell mass to allow the embryo to hatch, the inner cell mass develops into the fetus, and the trophectoderm forms the placenta. Cells from the trophectoderm are removed using laser pulses, placed in a tube, and frozen for PGT (Veiga et al., 1997).

1.5.1 **Preimplantation genetic testing for aneuploidy (PGT-A)**

In theory, the selection of a euploid embryo should increase the probability of achieving a pregnancy. However, controversy surrounds the clinical effectiveness of PGT-A, with studies highlighting that aneuploid embryos will not typically implant or will miscarry, and those with the correct chromosome complement will eventually be chosen for transfer by a process of elimination (Gleicher et al., 2014).

Adding to the controversy, early techniques used for PGT-A included FISH, which only allowed for the detection of aneuploidy of five to eight chromosomes, those commonly associated with aneuploid pregnancies or frequently seen in pregnancy loss, such as chromosomes 13, 18, 21, 16, 22, 15 and the sex chromosomes (Hassold et al., 1996; Mastenbroek et al., 2007). With no surprise, randomized clinical trials (RCT) involving FISH testing all failed to show improvements in clinical outcomes due to limitations of the technology, as embryos containing aneuploidies of untested chromosomes were inaccurately classified as euploid (Mastenbroek et al., 2007; Mersereau et al., 2008).

Array comparative genomic hybridization (aCGH) for PGT-A testing introduced screening for all chromosomes in a single assay which quickly replaced FISH testing. Randomized control trials conducted by Scott et al., (2012) and Yang et al., (2012) showed that all chromosome screening improved embryo implantation rates and decreased the likelihood of chromosomal-related miscarriages. As a result, clinical use of PGT-A increased with accumulating evidence of its effectiveness (Handyside., 2013a).

High-resolution next-generation sequencing (NGS) increases the detection rate of PGT-A by 20% when compared to aCGH (Munné & Wells., 2017), allowing for the detection of smaller segmental aneuploidies and mosaicism, suggesting that NGS is a more accurate tool for screening when compared to previous technologies used for PGT-A. However, like most technologies applied for PGT-A, NGS cannot determine all forms of polypoid embryos, which are those embryos that contain an extra set(s) of chromosomes (Yan et al., 2021).

For comprehensive chromosome screening by NGS, the principle involves whole-genome amplification (WGA) and then the fragmentation of the WGA products into approximately 100–200 base-pairs fragments. The fragments are sequenced in parallel until adequate sequencing depth (number of sequencing reads covering each base in the genome) is obtained. The chromosomal sequencing data are then counted and compared to a reference genome using specialized software. The number of reads is proportional to the chromosomal copy number, trisomy or monosomy will result in a greater or fewer number of reads, respectively (Handyside, 2013; Fiorentino, et al., 2014).

1.5.2 **Efficacy of PGT-A**

Preimplantation genetic testing for aneuploidy involves a process where embryos created *in vitro* are biopsied in order to determine their chromosomal status, this information is used to prioritize embryos for transfer rather than basing decisions on morphology alone (Dahdouh, Balayla & García-Velasco, 2015; Gleicher, Patrizio & Brivanlou, 2021). This section will highlight some of the impactful investigations performed on PGT-A.

With the expansion of comprehensive chromosome screening spanning all 23 pairs of chromosomes, the theory remains that PGT-A should improve clinical outcomes by increasing implantation rates and reducing the risk of miscarriage and multiple gestations by aiding single-embryo transfers (SET). Earlier RCTs performed on PGT-A usually focus on mainly good prognosis patients seeking ART treatment with PGT-A or morphological assessment alone (Yang et al., 2012; Scott et al., 2013).

Scott et al., (2013) compared a small group of patients with a mean age of 32.2 years with CCS as part of ART treatment to determine whether they showed improved implantation and delivery rates compared to groups with routine care. Significantly higher implantation (66.4% vs 47.9%, p= 0.002) and delivery rates (84.7% vs 67.5%, p = 0.001) were observed in the group that included CCS with ART treatment. However, the improvement in clinical outcomes could only be applied to good prognosis patients with an abundance of embryos to evaluate.

The benefit of PGT-A in AMA patients was highlighted when Rubio et al., (2017) showed higher delivery rates (52.9% vs 24.2%, $p = 0.0002$) after the first attempt of embryo transfer in the PGT-A groups when compared to the control group with a significantly reduced estimated time to pregnancy (7.7 vs 14.9 weeks). The ESTEEM trial was performed in 2018 to determine the effectiveness of PGT-A in clinical practice; this study showed a reduced number of miscarriages, transfers, and double-embryo transfers (DET) in AMA patients using PGT-A in conjunction with ART (Verpoest et al., 2018).

The multinational, multicentre STAR trial had similar findings to the ESTEEM trial in AMA patient groups undergoing ART. The trial included an evaluation of patients between 25-40 years of age in one of the biggest studies performed to assess the clinical outcomes after a single-vitrified-warmed blastocyst transfer of a PGT-A tested embryo using NGS compared to those chosen by morphology alone. No overall improvement in ongoing pregnancy rates and live birth rates were noted in all age groups however the use of PGT-A supported improved outcomes per embryo transfer was noted in patients 35-40 years of age (Munné et al., 2019). The nonreproducible results by larger clinical trials imply that PGT-A may not benefit patients <35 years of age, however, there is increasing evidence that PGT-A does help those of AMA.

Observational studies are not without challenges in methodologies. High-quality studies play an important role in comparative research for effectiveness as these studies can distinguish associations between factors but cannot conclude that one causes the other as in the cases of clinical trials (Dreyer et al., 2010).

The observational study conducted by Greco et al., (2014) investigated the clinical pregnancy rates and implantation rates of patients with a history of recurrent pregnancy loss under the age of 36 with PGT-A compared to good prognosis patients with PGT-A as a positive control and repeated implantation failure without PGT-A patients as a negative control. An improvement in clinical outcomes was shown in groups with multiple failed ART attempts (Greco et al., 2014).

A retrospective study looked at the live birth rate of aCGH tested embryos when compared to non-tested transfers, significantly higher rates were observed in the PGT-A groups although testing was performed on polar body biopsies as opposed to the well-accepted trophectoderm biopsy (Veiga et al., 1997; Feichtinger et al., 2015). As a result, the difference in patient and treatment practices between studies as well as the lack of prospective randomization limit the findings of observational studies.

Table 1-2 summarizes the key findings of randomized control trials and observational studies performed since the introduction of CCS for PGT-A testing. The ASRM advised that the use of PGT-A as a universal screening tool as part of ART treatment is yet to be determined as there are insufficient studies that support or refute its use (Penzias et al., 2018). Despite numerous studies in support of improved clinical outcomes in certain groups using PGT-A, the method remains one of the most controversial practices for the selection of embryos (Gleicher et al., 2021).

The cost of CCS adds to the controversy surrounding PGT-A, which includes additional laboratory expenses such as the costs of trophectoderm biopsy, vitrification and the fee for PGT-A. Patients may believe that PGT-A has implied benefits as chromosomally abnormal embryos are excluded (Facadio Antero et al., 2021). The current cost-effectiveness analysis performed on PGT-A has significant heterogeneity in the methods and results obtained, however, the consensus remains that PGT-A is beneficial in specific patient populations and appropriate counselling should be provided (Somigliana et al., 2019; Neumann et al., 2020; Facadio Antero et al., 2021).

As of 2018, 16.4% of South Africans were registered members of medical aid schemes (Statistics South Africa, 2019), leaving the majority of the population seeking health services from government facilities. A retrospective review of 22 ART cycles which included PGT-M (formally, PGD) revealed that a lack of funding is a barrier to treatment access in South Africa (Carzis et al., 2019). Access to ART and PGT are largely dependent on socioeconomic factors in low- and middle-income countries as the cost of treatment are borne by the patient. Higher-income countries, such as Israel, Australia and the United States have high utilization of ART treatment as the cost of treatment has full or partial reimbursement structures (Adamson et al., 2018).

In low- and middle-income countries the maternal mortality and infant mortality rates can be 50 to 100-fold and tenfold higher than in high-income countries, respectively (Goldenberg, McClure & Saleem, 2018). Understandably, developing countries with limited resources place emphasis on burdens such as mortality, infectious disease and malnutrition with restricted priority on infertility (Akinloye & Truter, 2011; Morshed-Behbahani et al., 2020).

PGT-A is only offered by private fertility centres in South Africa which can be financially overwhelming for patients. A cost-effectiveness analysis must be performed to guide decision-making, especially for patients in countries that face socio-economic inequalities. There is a global scarcity of publications with a comprehensive economic evaluation of PGT-A to

determine the cost-effectiveness in relation to the clinical benefit (Lee, Costello, et al., 2019; Lee et al., 2021).

Table 1-2. Highlighting current randomized control trials and observational studies performed to evaluate PGT-A

Study	Methodology	Collected	Single/multi-centre	Sample	Sample size	Key findings
Randomized control trials						
(Munné et al., 2019) (STAR Trial)	NGS	2014-2016	Multi-	Trophectoderm	662	↑ OPR for 35-40years
(Ozgur et al., 2019)	NGS	2012-2014	Single	Trophectoderm	302	No improvement in LB
(Verpoest et al., 2018) (ESTEEM Trial)	aCGH	2012-2016	Multi-	1st + 2nd PB	396	↑LB ↓ MR
(Rubio et al., 2017)	aCGH	2012-2014	Multi-	Blastomere	205	↑ IR ↑ DR Reduced time to pregnancy
(Coates et al., 2017)	aCGH	2013-2015	Single	Trophectoderm	398	↑ IR, ↑ LB (DET PGT-A group)
(Forman et al., 2014)	qPCR	2011-2012	Single	Trophectoderm	205	↓ MP
(Scott et al., 2013)	qPCR	2009-2013	Multi-	Trophectoderm	72	↑ IR ↑ LB
(Yang et al., 2012)	aCGH	2011-2012	Multi-	Trophectoderm	55	↑CP ↓ MR
Observational studies						
(Sanders et al., 2021)	NGS	2016-2018	Multi-	Trophectoderm	2664	↑ LB Reduced time to pregnancy
(Feichtinger et al., 2015)	aCGH	2013-2015	Single	Polar Body	111	↑ LB
(Greco et al., 2014)	aCGH	2012-2013	Multi-	Trophectoderm	88	↑ IR

OPR = Ongoing pregnancy rate; LB = Live birth; MR = Miscarriage rate, IR = Implantation rate; DR = Delivery rates; DET = Double embryo transfer; MP = Multiple pregnancy; CP = Clinical Pregnancy; NGS = Next-generation sequencing; aCGH = Array comparative genomic hybridization; qPCR = quantitative polymerase chain reaction

1.6 Study rationale

The increasing prevalence of infertility makes reproductive health an important global health concern. The main challenge in producing global estimations of infertility is the lack of a clear definition, limited population-based studies, and high-quality studies (Dyer, 2009; Mascarenhas et al., 2012). In South Africa, the true prevalence of infertility is unknown.

In 2014, the African Network and Registry for Assisted Reproductive Technology (ANARA) reported that twelve Private and three State ART centres in South Africa performed 4995 ART cycles (ANARA, 2014). Based on the population size and global infertility rates, South Africa only meets 6.4% of its predicted ART needs with an overall pregnancy rate of 33.4% per cycle (ANARA, 2014). Accessibility to ART treatment and knowledge of interventions is needed for infertility management (Dyer et al., 2002).

PGT-A services using high-resolution NGS technology were introduced in South Africa in May 2017 by a private genetic testing laboratory. Vitalab Fertility Clinic, Sandton, Johannesburg (hereafter referred to as Vitalab) makes use of PGT-A services for patients who were likely to benefit from testing. Vitalab participated in the 2014 South African Registry for Assisted Reproductive Techniques report on ART facilities and treatments in South Africa (ANARA, 2014). However, the use of PGT-A to improve clinical outcomes of ART treatment has never been evaluated in South Africa.

An evaluation of clinical outcomes of ART cycles using NGS-based PGT-A as a tool for embryo selection is needed to assess the effectiveness and utility of testing, especially in resource-constraint settings such as South Africa. If this tool is not suitable for universal usage, as previously discussed, then it is imperative to identify groups that will benefit from the use of PGT-A.

Furthermore, the true impact of mosaic embryos has not been fully investigated globally. As NGS allows scientists to report on mosaicism more accurately than previous technologies used for PGT-A, more studies are needed to contribute to the true incidences of mosaicism and their clinical impact (Girardi et al., 2020; Viotti et al., 2021).

The introduction of clinical guidelines on the use of PGT-A in developing nations is recommended to overcome treatment barriers and possibly enhance the delivery of services. This study aimed to determine whether euploid embryo transfers improve clinical outcomes compared to the selection of embryos for transfer based on morphology alone. This study also

contributes to the current body of knowledge on the incidence of aneuploidy and mosaicism following comprehensive chromosome screening performed by next-generation sequencing in a South African private healthcare setting.

1.7 Aim and Objectives

The proposed study aimed to assess the effectiveness of PGT-A testing on ART cycles by evaluating clinical outcomes at a single ART centre in Johannesburg, South Africa.

The specific study objectives were:

I. To quantify trisomy, monosomy, and segmental, mosaic aneuploidies observed in preimplantation embryos.
II. To determine whether the selection of euploid embryo improves clinical outcomes namely, β-hCG levels, implantation rate, and clinical pregnancy when compared to a control population.
III. To analyse the effect of maternal age on aneuploidy occurrence and clinical outcomes.

Chapter 2

2 Methodology

This chapter describes the methods used to achieve the three objectives of this study; specific procedures used to collect data as well as statistical analyses performed.

This study was a retrospective case-control record review and analysis of PGT-A laboratory results and clinical outcomes from one ART centre in South Africa. The participant's medical reports used in this study were accessed and extracted from electronic databases held at Vitalab (permission for access was granted by the relevant head of authority as detailed in Appendix A).

All data generated were collected and stored on the data management system Research Electronic Data Capture (REDCap) software available from the University of Witwatersrand (Harris et al., 2009).

2.1 Ethical Approval

Ethics clearance for this Masters study was granted by the University of Witwatersrand's Human Research Ethics Committee (HREC). Ethics clearance certificate M191015 can be found in Appendix B. Signed informed consent forms for PGT-A were collected by Vitalab and stored on the electronic medical record of the patient.

2.2 Medical records

A total of 1504 medical records of patients who received ART treatment at Vitalab Fertility Clinic were evaluated for this study. The ART cycles were further categorized into case and control groups detailed below. Blastocysts were vitrified after trophectoderm biopsy to allow for genetic analysis. Only ART cycles with frozen embryo transfer were included in this study, which is the transfer of a vitrified embryo. Figure 2-1 depicts a flow diagram detailing the information collected from the medical records.

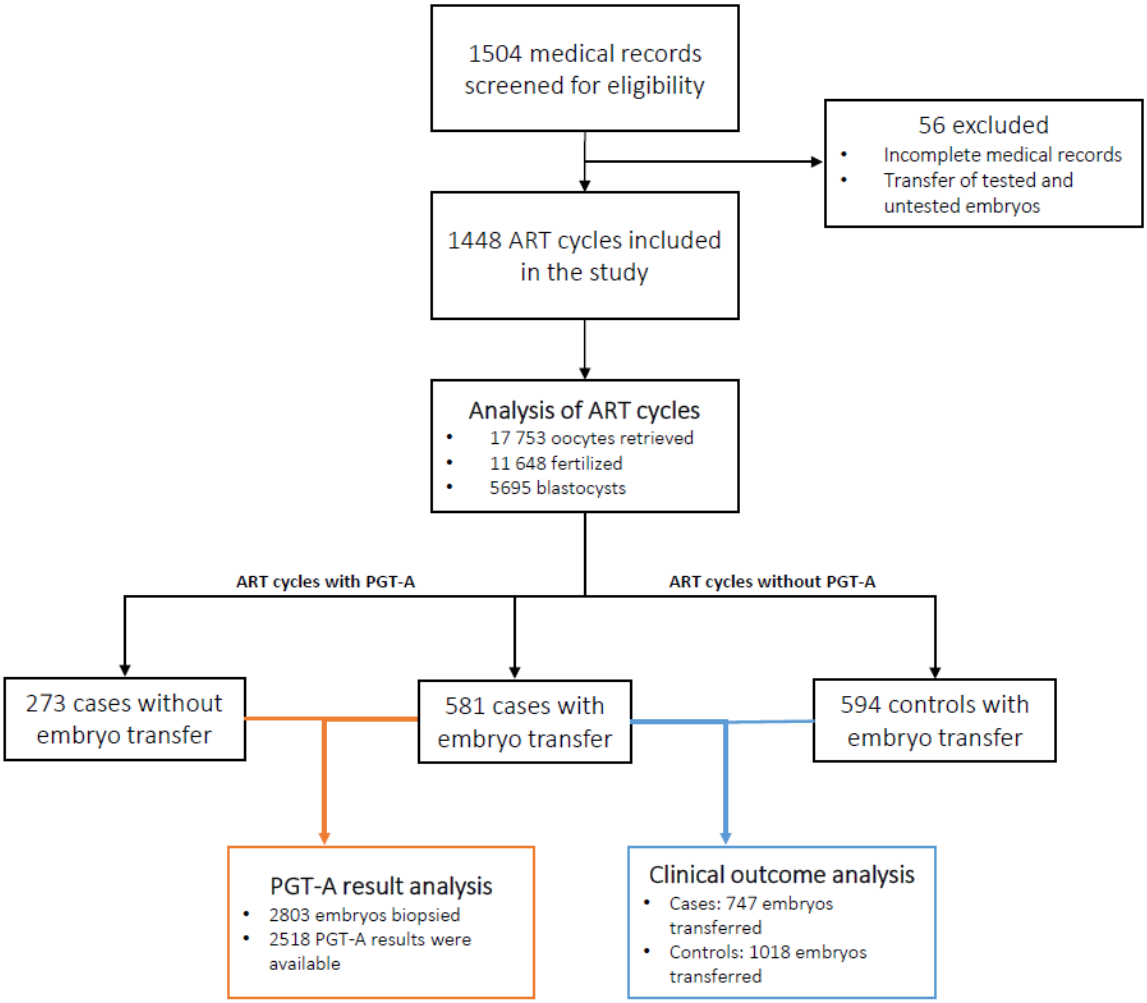


Figure 2-1. Patient characteristics used for study inclusion and the data collected for analysis

After the exclusion criteria were applied, 1448 ART cycles were assessed using the 20 evaluative points detailed in the Participant Data Collection Sheet (Appendix C). The ART outcomes included oocytes were retrieved and the number of embryos developing to the blastocyst stage was also recorded for all cycles.

The maternal age at the time of ART treatment was recorded from all medical records. ART cycles with the use of egg donors represented 204 cycles. The donor age was used for PGT-A analyses for ploidy assessment. Donor cycles were included in the clinical analysis to determine whether the transfer of a euploid embryo, regardless of the age of the oocyte and the age of the maternal environment, improved clinical outcomes.

The ART cycles were further categorized into two groups; those individuals who included PGT-A as part of their ART treatment (cases) and those who performed embryo transfers based on morphology alone (controls).

2.2.1 **Cases**

ART cycles with PGT-A comprised the 854 cases of this study. The cases represented a convenience sample taken from the period of May 2017 and May 2019. The study began in 2019, giving two years of NGS data by end of May 2019.

Generally, five to ten cells are biopsied from the trophectoderm of 2803 embryos on day five or six of development. The embryos are then vitrified after biopsy while the cells are sent for PGT-A evaluation, embryo transfer is planned once the results are received. The PGT-A results are usually available between seven- and ten days post-biopsy. Samples recorded as "amplified and stored" represented 242 PGT-A results, which were part of a batch of tested embryo biopsies, subjected to DNA amplification to increase sample stability, and saved for future testing.

The PGT-A reports detailing the result and specific chromosomal abnormalities were evaluated and recorded as detailed in Section 2.3. This group prioritized euploid embryos for transfer. It was noted that no mosaic or aneuploid embryos were transferred during this period.

2.2.2 **Controls**

The control group consisted of 594 ART cycles without PGT-A testing. In this group, the embryos were transferred based on morphology only, with higher grades prioritized for transfer.

2.2.3 **Exclusions**

Incomplete medical records or transfers of tested and untested embryos, which represented 56 ART cycles, were excluded from this study.

2.3 Evaluation of PGT-A results

2.3.1 **Chromosomal classification of embryos**

PGT-A results for 2518 embryo biopsies were available for analysis from the 854 cases. The incidence of whole chromosome gains and losses, segmental aneuploidy (deletions and duplications of whole chromosome segments) and mosaicism in preimplantation embryos were assessed and recorded.

The process involved accessing the 854 medical files stored on the fertility centre database that recorded individual PGT-A results for the 2518 embryo biopsies. The case and embryo number and the corresponding chromosomal results were evaluated and entered into a study database. The database included analysis for all 22 autosomes and the sex chromosomes. The PGT-A results were collected and categorized as detailed in the PGT-A Data Collection Sheet (Appendix D). The number of biopsies with no results were also recorded, these were either due to insufficient intact DNA for analysis or uninterpretable NGS results. Aneuploid results represent full gains or losses involving a whole chromosome. A segmental aneuploidy represents a subchromosomal aneuploidy. Aneuploidy also includes polyploids, however, NGS is limited to the detection of 69, XXY and 92, XXXY. An aneuploid mosaic result represents those that contain one or two aneuploid events and mosaic events. A complex abnormal result is given to those biopsies that have three or more aneuploidies.

Mosaic embryos contain normal diploid cell lines and a proportion of cells with one or more aneuploidies. NGS technology allows for the detection of aneuploid events that fall between the thresholds used for assigning one, two or three chromosomal copies indicative of a mosaic abnormality. Depending on the exact value, samples are either classified as low- or high-level mosaic monosomy, mosaic trisomy, segmental mosaic or complex mosaics (three or more distinct mosaic chromosomes). Low-level mosaicism lies between the 20% and 40% of abnormal cells and high-level mosaicism represents biopsies showing greater than 40% but less than 80% chromosomal abnormality.

Each PGT-A result was numerically coded for statistical analysis, the aneuploidy events were presented as percentages and frequency of aneuploidy per chromosome.

2.4 Aneuploidy incidence by maternal age

The maternal ages in the cases were grouped in five-year intervals to determine the rate of aneuploidy in relation to age. The groups consisted of ≤25, 26-30, 31-35, 35-40 and ≥41 years of age. Age stratification to assess aneuploidy rates was similarly demonstrated by Demko et al., (2016). Each group was assessed to determine whether aneuploidy rates increase with advancing age and if mosaic events are independent of maternal age.

The 2518 PGT-A results were assessed to determine the highest frequency of PGT-A usage in the different age categories which in turn described the age-specific usage. The rates of euploidy, aneuploidy and mosaicism were assessed per maternal age category. A risk analysis

was performed to determine whether the relative risk of aneuploidy, mosaicism and inconclusive results and stored embryos increases with maternal age.

Aneuploidy patterns observed provide knowledge for better clinical management, and improved decisions on whether PGT-A should be offered to all patients, or those considered to be of high risk.

2.5 Clinical analysis

Clinical outcomes for all the 581 PGT-A cases and 594 controls were assessed after embryo transfer to evaluate the success of ART treatment. The clinical outcomes evaluated in this study can be found in Appendix B.

It is hypothesized that a higher number of embryos are transferred in groups that rely on morphological assessment alone prior to embryo transfer. For double embryo transfers, the transfer of each embryo was counted as one positive β-hCG or clinical pregnancy, therefore the clinical outcomes were measured per ART cycle. The transfer characteristics between the two groups were assessed to determine whether the PGT-A group preferentially opt for single embryo transfers when compared to the control group.

2.5.1 **Clinical outcomes**

The outcomes detailed below were evaluated to determine the clinical effectiveness of PGT-A. The ART cycles without transfer were excluded from the analysis, therefore, 273 ART cycles in the PGT-A case groups were excluded, these were 91 cases with transfers not occurring within the period of this study and 182 ART cycles with no euploid embryos.

A positive value for serum β-hCG levels ≥50 IU/L usually performed twice (at 2–4-week intervals) after implantation indicates a successful embryo transfer. Positive β-hCG with ultrasound confirmation of a gestational sac was used to determine the **implantation rates** per embryo transfer.

The number of gestation sacs with or without fetal heartbeat(s) was measured per embryo transfer. The implantation rate and the number of fetal heartbeat(s) observed are the only two measures evaluated per embryo transfer as the number of embryos transferred correlates to the number of the gestational sac(s) or fetal heartbeat(s) confirmed.

Clinical pregnancy is confirmed by ultrasonographic visualization usually performed between 4 - 7 weeks confirmed the number of gestational sacs present with the additional signs of a fetal

heartbeat. The first-trimester miscarriage rate per embryo transfer was determined from the number of gestational sacs present without a fetal heartbeat at 12 weeks.

The number of clinical pregnancies was the main outcome of this study as live birth data was not assessed due to inconsistent feedback given to the ART centre by patients once pregnancy had been established. Clinical pregnancy is the single measure used in this study to truly determine the clinical effectiveness of PGT-A, which was used to determine whether the selection of a euploid embryo is an effective tool for embryo transfer when compared to groups who used morphology assessment alone.

2.6 Morphological assessment

The morphological information was obtained from all ART records. Vitalab utilizes the Gardner and Schoolcraft (1999) grading system to appraise embryos at the blastocyst stage. The score for the expansion of the blastocyst, inner cell mass, and trophectoderm, was recorded for each embryo generated per ART cycle.

The gradings were then categorized into four groups as previously described by Capalbo et al., (2014). The **excellent** group consisted of embryos with a 3AA and 4AA grading; the **good** group consisted of embryos with 3,4,5,6, AB and BA; the **average** group were embryos graded as 3,4,5,6 BB, AC and CA; and the **poor** group were graded as 3BB or lower.

Overall, 5695 blastocyst grades were recorded in this study and categorized into excellent, good, average and poor groups. The morphology was then assessed per maternal age category to determine whether excellent embryos decrease with increasing maternal age.

Maternal age and euploidy risk were also assessed to determine whether increasing maternal age has an influence on ploidy. The analysis included 2518 biopsies, which also incorporated embryo grades in relation to euploid, aneuploid and mosaic results.

2.7 Statistical Analysis

2.7.1 **Descriptive statistics**

Descriptive statistics were used to summarize and describe variables within the study populations. Categorical variables were represented as frequencies and percentages. The Shapiro-Wilk and Skewness and Kurtosis tests were used to assess the distribution of continuous

data. Normally distributed continuous variables are presented as percentage frequency, mean and standard deviation.

2.7.2 **Analytical statistics**

A distribution frequency analysis was conducted to assess the euploidy rate as well as the most commonly occurring aneuploidies and mosaic events seen in preimplantation embryos. A regression analysis was then performed to investigate whether there is a correlation between maternal age and embryo chromosomal abnormalities.

A t-test was used to determine whether there was a significant difference between continuous normally distributed variables between the case and control groups. These included age and clinical pregnancy rate per ART cycle. Two-proportion z-tests were also used to assess the proportions of normally distributed data. Categorical variables such as maternal age groups, PGT-A results and morphology were compared using the Chi-square test.

Assisted reproductive technology outcomes were assessed using linear regression to determine whether age has a significant impact on the number of harvested oocytes, fertilization rate, and the number of blastocysts formed within the case and control groups.

To identify whether PGT-A improves clinical outcomes, a bivariant analysis was used to understand the relationship between; age and ART outcomes, euploidy rate, and clinical outcomes in the case and control groups. This test identified the variables for a regression analysis to establish the factors that impact the clinical outcomes of patients who opt for PGT-A testing.

A regression analysis was performed to evaluate the relationship between age, aneuploidy, ART cycles, and ART outcomes. The factors with statistically significant differences in the case and control groups can be used as possible new predictors to improve ART outcomes. Statistica version 13.5.0.17 (TIBCO, Palo Alto, California) was used for statistical analysis. For all tests, statistical significance was determined if $p < 0.05$.

Chapter 3

3 Results

3.1 Participant characteristics

A total of 1504 medical records were evaluated; 56 ART cycles were excluded and 1448 met the inclusion criteria. All ART cycles had viable embryos available for evaluation; the patient characteristics are shown in Table 3-1. The PGT-A cases comprised of 854 ART cycles which were performed with PGT-A between May 2017 and May 2019. The control group included 594 ART cycles. Maternal ages ranged from 21 to 48 years with a mean ± standard deviation (SD) age of 35 ± 4.44 years in the overall population group at the time of ART treatment being undertaken.

A two-proportion z test revealed that the control group used a statistically significantly higher number of egg donors compared to the cases (p <0.0001). Significant differences were also observed in; the number of oocytes retrieved, formation of zygotes with two pronuclei (2PN) indicating fertilization, and blastocysts form.

Table 3-1. Summary of participant characteristics and ART outcomes in the PGT-A case and control groups

	PGT-A cases	Controls	P-value
Number of cycles	**854**	**594**	<0.0001ǂ Z
Maternal age (y) (mean ±SD)	36 ±4.50	35 ±4.32	0.2840¥
No cycles using donor oocytes (n)	62	142	0.0186ǂ Z
Donor age (y) (mean ±SD)	26 ± 4.68	26 ± 5.48	1.0000¥
No. of oocytes retrieved (n)	10273	7480	<0.0001ǂ Z
Mean oocytes retrieved per cycle (±SD)	11.97 ± 6.65	12.57 ± 6.87	0.0891¥
No. of embryos fertilized (n)	6509	5139	0.0015ǂ Z
No. of blastocysts formed (n)	2803	2892	0.4542 Z
Mean blastocyst formed per cycle (±SD)	3.81 ± 2.52	4.86 ± 3.19	

ǂ statistically significant p<0.05
Z Chi-squared test
¥ two-sample T-Test

3.2 PGT-A result analysis

The first objective of this study was to assess the chromosomal status of preimplantation embryos. A total of 854 women received ART treatment with PGT-A at Vitalab between May 2017 and May 2019.

The 854 cases had 2803 embryos biopsied, 90% (2518/2803) had conclusive PGT-A results, 9% (242/2803) were stored for future testing, and 1% (43/2803) failed to give an NGS result, these were either due to insufficient intact DNA for analysis or uninterpretable NGS results. Of the 2518 PGT-A results, 57% (1428/2518) were reported as euploid, 33% as aneuploid (828/2518) and 10% (262/2518) as a mosaic (Figure 3-1).

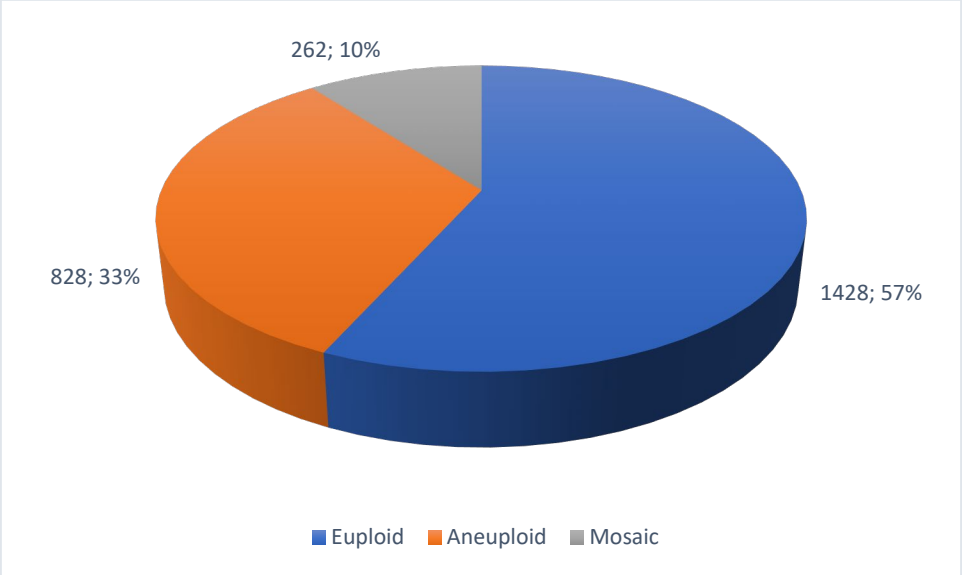


Figure 3-1. Preimplantation genetic testing for aneuploidy (PGT-A) results identified in blastocyst biopsies (n = 2518)
PGT-A result represented the number of observations and percentage of total results

3.2.1 **Euploid embryos**

The euploid embryos represented the most common chromosome status assigned to PGT-A tested embryos in this dataset. Of the 1428 euploid embryos, 53% (754/1428) were observed to be female (XX) and 47% (674/1428) were reported as male (XY). This study evaluated 747 euploid embryo transfers, the clinical outcomes of the transfers will be assessed in Section 3.3. Interestingly, 681 euploid embryos remain frozen for future transfer, these embryos represent ART cycles with more than one euploid embryo and those that did not perform an embryo transfer during the study period.

3.2.2 PGT-A results with chromosomal abnormalities

Table 3-2 details the 1120 chromosomal abnormalities found in this study. The 828 aneuploid results excluded the analysis of 61 complex abnormal embryos as the chromosomes involved were not specified on the PGT-A reports. The remaining aneuploid results were further classified as whole chromosome gains, whole chromosome losses and segmental aneuploidy. Triploidy (69, XXY) was observed in 17 embryo biopsies representing 1.5% (17/1120) of whole chromosome gains.

An embryo resulted as aneuploid which also included a mosaic event or an embryo with a double mosaic event represented 91 of the mosaic results recorded. Each mosaic event was analyzed individually, therefore 353 mosaic events were observed in this study. Chromosomal abnormalities affected all chromosomes, with 59% (657/1120) of aneuploid events represented by whole chromosome gains and losses.

Table 3-2. Summary of aneuploid and mosaic preimplantation embryos out of a total of 2518 embryo biopsies

	Count	**% of total**
Whole chromosome gain	264	24
Whole chromosome loss	393	35
Mosaicism	353	31
Segmental aneuploidy	110	10
Total	**1120**	**100%**

3.2.3 **Aneuploidy per chromosome**

The chromosomal abnormalities were further evaluated to determine the aneuploidy incidence per chromosome (Figure 3-2). The highest frequencies of aneuploidies and mosaic events per chromosome are highlighted in this section.

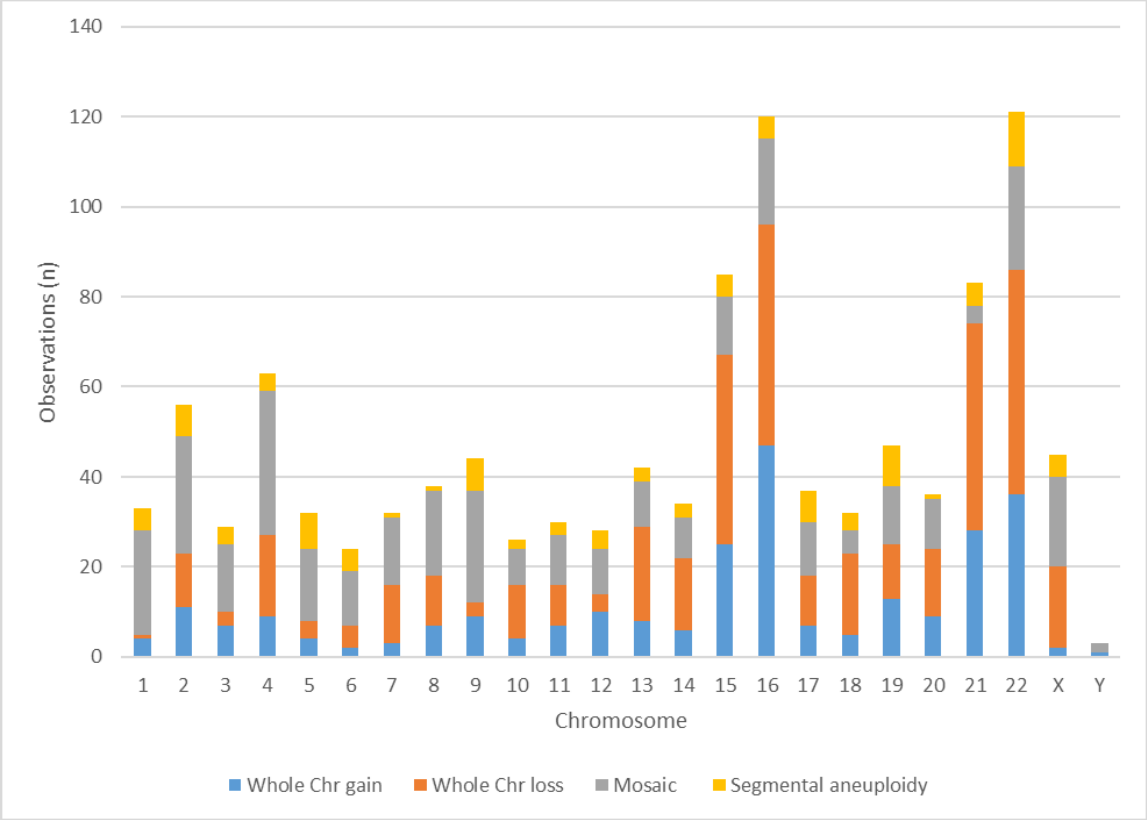


Figure 3-2. Aneuploidy events observed in the autosomal chromosomes (1-22) and sex chromosomes, X and Y (n = 1120).

Chromosomes 2, 4, 9, 13, 15, 16, 19, 21, 22 and X had the highest number of chromosomal abnormalities overall, the incidence of each abnormality is shown in Figure 3-3. Whole chromosome losses involving chromosome 22 constituted 41% (50/121) of the observed chromosome 22 abnormalities and were the most frequently observed aneuploidy in the dataset at 4.5% (50/1120) of all chromosomal abnormalities.

Trisomy 16 was the highest observed whole chromosome gain which represented 18% (47/264) of all whole chromosome gains, followed by chromosome 22 at 12% (36/264), chromosome 21 at 11% (28/264), and chromosome 15 at 9% (25/264). Trisomy's 21, 13 and 18 represented 3% (28/1120), 0.7% (8/1120) and 0.4% (5/1120) of all chromosomal abnormalities, respectively.

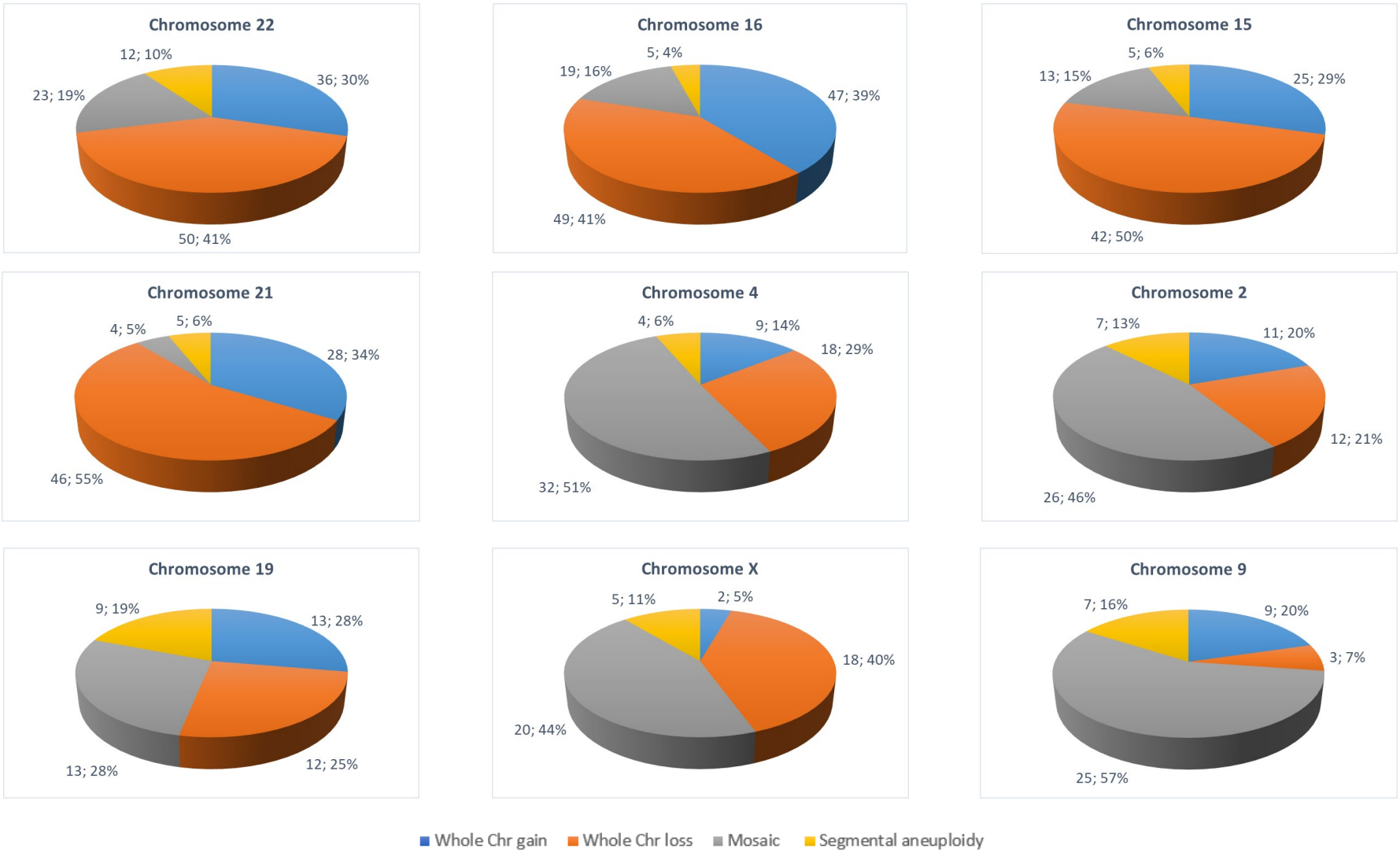


Figure 3-3. The number of observations and percentage of whole chromosome gains/losses, mosaicism and segmental aneuploidy found in the chromosomes with the highest incidences of aneuploidy events

Chromosomes 2, 4, 9 and X showed a higher incidence of mosaic events compared to whole chromosome gains, losses or segmental aneuploidies. Interestingly, larger chromosome 4 (32/353) and chromosome 2 (26/353) had the highest incidences of mosaicism. High-level mosaicism was observed in 60% (212/353) of PGT-A mosaic results and 40% (141/353) of mosaicism was recorded as low-level.

Chromosome 22 had the highest frequency of segmental abnormalities representing 11% (12/110). Notably, chromosome 19 and chromosome also showed a higher incidence of segmental aneuploidy representing 8% (9/110) and 7% (8/110) of all partial gains and losses, respectively.

Higher incidences of aneuploidy were recorded for the X chromosome (45/1120) compared to the Y chromosome (3/1120). Mosaicism of the X chromosome represented 44% (20/45). Complete loss of an X chromosome, termed Monosomy X, contributed to 40% (18/45) of X chromosome aneuploidies. The two cases with XXX results had no other aneuploidies found in the biopsy. The Y chromosome aneuploidies included one case of XYY and two low-level mosaic events.

3.2.4 Morphology in relation to ploidy

The embryos graded as excellent, good, average, and poor in the case groups were further evaluated to determine whether any correlation exists between morphology and chromosomal status (Figure. 3-4).

The excellent, good, average, and poor groups showed euploidy rates of 43% (621/1428), 28% (403/1428), 20% (289/1428) and 8% (115/1428), respectively. The excellent and good groups also had a high proportion of aneuploidies, 26% (212/828) and 25% (207/828), respectively.

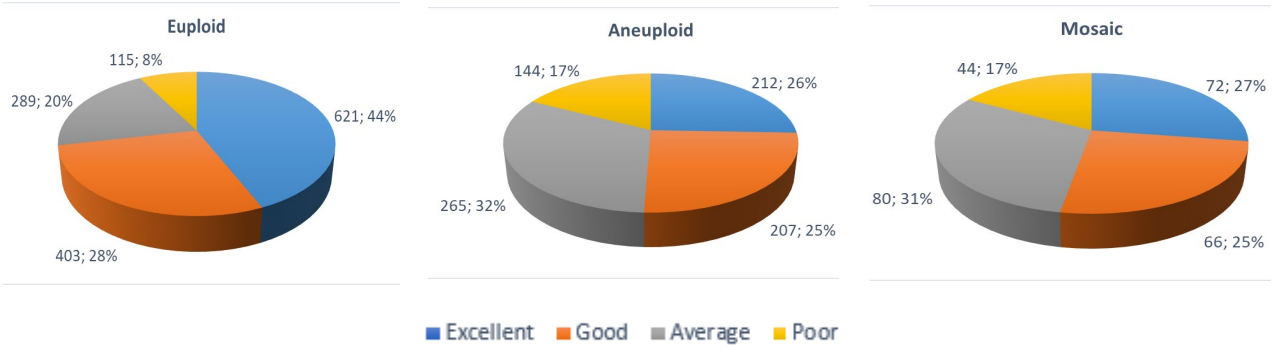


Figure 3-4. Morphological grading observed in euploid, aneuploid and mosaic embryos

A logistic regression analysis revealed that an excellent embryo was 2.75 (p <0.001) times more likely to be euploid, the odds of having a good embryo that was euploid was 1.80 (p <0.001) and an average embryo was equally likely to be euploid or aneuploid and a poor embryo is 0.79 times likely of being euploid (Table. 3-3).

Table 3-3. Logistic regression analysis of embryo morphology and the odds of being euploid

Risk Factor	**Odds Ratio**	**95% Confidence interval**	***P*-value**
Excellent	2.75	2.26, 3.36	<0.0001‡
Good	1.80	1.47, 2.21	<0.0001‡
Average	0.99	1.85, 0.54	0.3471
Poor	0.79	0.55, 0.80	<0.0001‡

‡ statistically significant p<0.05

3.3 The clinical effectiveness of PGT-A

The second objective of this study was to determine whether the selection of a euploid embryo improves clinical outcomes compared to the selection of embryos for transfer based on morphology alone.

The 854 PGT-A cases included in the study had 273 ART cycles where no embryo transfer was performed, of these 182 cases had no euploid embryos and 91 cases had at least one euploid embryo but performed no transfer within the duration of this study. Therefore, 581 PGT-A cases were included for analysis and evaluated in comparison to a control group which comprised 594 ART cycles.

3.3.1 **Transfer characteristics**

A total of 1175 ART cycles were included in the analysis with a total of 1765 embryos transferred. Most of the PGT-A cases had single embryo transfers while the control group revealed a higher number of double embryo transfers per ART cycle (Table 3-4). Multiple embryo transfers included ART cycles which had a single and double embryo transfer within the same cycle, the transfer of three embryos or two double embryo transfers. The case group had a significantly higher number of multiple transfers.

The case and control groups had 27 and 19 multiple embryo transfers, respectively. Both groups had 19 cycles with three embryos transferred on the same day. The case group had seven cycles with no established clinical pregnancy, one cycle with a triplet, three with a twin and eight with singleton clinical pregnancies. The control group had eight cycles that did not establish a clinical pregnancy, six cycles with a triplet, two with a twin and three with singleton clinical pregnancies. Eight PGT-A cases had two double embryo transfers on different days which resulted in two cycles that did not establish a clinical pregnancy from either transfer, four twin and two singleton clinical pregnancies.

Table 3-4. Embryo transfers performed in the case and control groups

	PGT-A	Controls	*P*-value
Treatment cycles with transfers	**581**	**594**	**0.6279[Z]**
Embryos transferred	747	1018	<0.0001[ǂ Z]
Single embryo transfer (% (n))	72% (418)	32% (189)	<0.0001[ǂ Z]
Double embryo transfer (% (n))	23% (136)	65% (386)	<0.0001[ǂ Z]
Multiple embryo transfers (% (n))	3% (27)	3% (19)	0.0011[ǂ Z]

[ǂ] statistically significant p<0.05
[Z]Chi-squared test

3.3.2 Clinical outcomes

Two proportion z-tests were used to determine the difference in the clinical outcomes of the PGT-A cases compared to control groups (Table 3-5). The first measure of transfer success is the detection of increased β-hCG levels which indicates a biochemical pregnancy. B-hCG was positive in 94% of ART cycles in the control group which was significantly higher than the 73% in the PGT-A case group (p = 0.0001).

Table 3-5. Clinical outcomes observed in ART cycles post-embryo transfer (n = 1175)

	PGT-A		*Controls*		*P-value*
	%	*n (N)*	*%*	*n (N)*	
Positive β-hCG	73	427 (581)	94	557 (594)	*0.0001*ǂ
*Implantation rate**	62	465 (747)	48	484 (1018)	*<0.0001*ǂ
*Fetal heartbeat**	60	447 (747)	45	453 (1018)	*<0.0001*ǂ
Singleton clinical pregnancy	65	310 (581)	21	127 (594)	*<0.0001*ǂ
Twin clinical pregnancy	11	66 (581)	25	147 (594)	*0.0002*ǂ
Triplet clinical pregnancy	0.2	1 (581)	6	6 (594)	*0.0131*ǂ
Overall clinical pregnancy	**65**	**377 (581)**	**47**	**280 (594)**	*0.0153*ǂ

ǂ statistically significant p<0.05
*No. of gestational sacs observed per embryo transfer
*No. of fetal heartbeats per embryo transfer

Implantation rate and clinical pregnancy rates are determined by counting the number of gestational sacs and fetal heartbeats per embryo transfer, respectively. These clinical outcomes were measured per embryo transferred as the number of embryos transferred directly correlates with the number of gestational sacs or fetal heartbeats observed. The implantation rate was 62% (465/747) in the PGT-A cases and significantly lower in the control group at 48% (484/1018) per embryo transfer (p = <0.0001). There were statistically higher pregnancy rates in the PGT-A cases compared to the controls, 60% (447/747) versus 45% (453/1018) (p<0.0001).

To measure the clinical effectiveness of PGT-A, this study focused on the ongoing clinical pregnancy outcome per ART cycle which were generally seen at the ART centre up to eight to twelve weeks of gestation. The clinical pregnancies were higher in the PGT-A group compared to the controls, 65% versus 47%, respectively (p = 0.0153), despite the higher β-hCG observed in the control group. The clinical pregnancy rates observed in the case [65%, 264/418)] and control [35% (66/189] group for single embryo transfers were also higher in the cases, further evaluations were performed on the overall clinical pregnancy rate. However, the control group had significantly higher twin and triplet clinical pregnancies compared to the PGT-A cases [25% versus 6% (p = 0.0002), 6% versus 0.2% (p = 0.0131), respectively].

A logistic regression analysis was used to determine the risk of a clinical pregnancy within the two populations. The results revealed that the ART cycles which included PGT-A were 2.07

more likely to establish a clinical pregnancy (p <0.0001) compared to the control group (Table 3-5).

3.4 Effect of maternal age on ART outcomes

The third objective of this study was to determine whether maternal age had any association with ploidy, morphology, and clinical outcomes.

3.4.1 **Maternal age in relation to PGT-A results**

The analysis of PGT-A results revealed 57% (1428/2518) euploid, 33% aneuploid (828/2518) and 10% (262/2518) mosaic results were observed in this dataset. These results were further evaluated per maternal age category (Figure 3-5).

The maternal ages were grouped into categories; the 30-34 year and 35–40-year age groups had the highest number of PGT-A results for analysis comprising of 46% (1149/2518) and 29% (736/2518), respectively (Figure 3-6). The ≥41-year age group consisted of 14% (357/2518) of the results, and the 26–30-year age group had a lower number of results for evaluation comprising of 10% (236/2518) of the population. One percent (40/2803) of the blastocysts were from the <25-year age category.

An aneuploidy rate of 25% (10/40) was observed in the <25-year age group. This was higher than the 22% (53/236) and 21% (155/736) observed in the 25-29 year and 30–34-year age categories, respectively (Figure 3-5). Importantly, 57% (204/357) of the embryos in the >40-year age category resulted as aneuploid, 34% (120/357) as euploid and 9% (33/357) as a mosaic. A chi-squared test was used to determine whether there was an association between maternal age and euploidy rate. The analysis revealed that euploid embryos decreased with advancing maternal age (p <0.0001). The incidence of mosaicism had no significant association with maternal age (p = 0.1221).

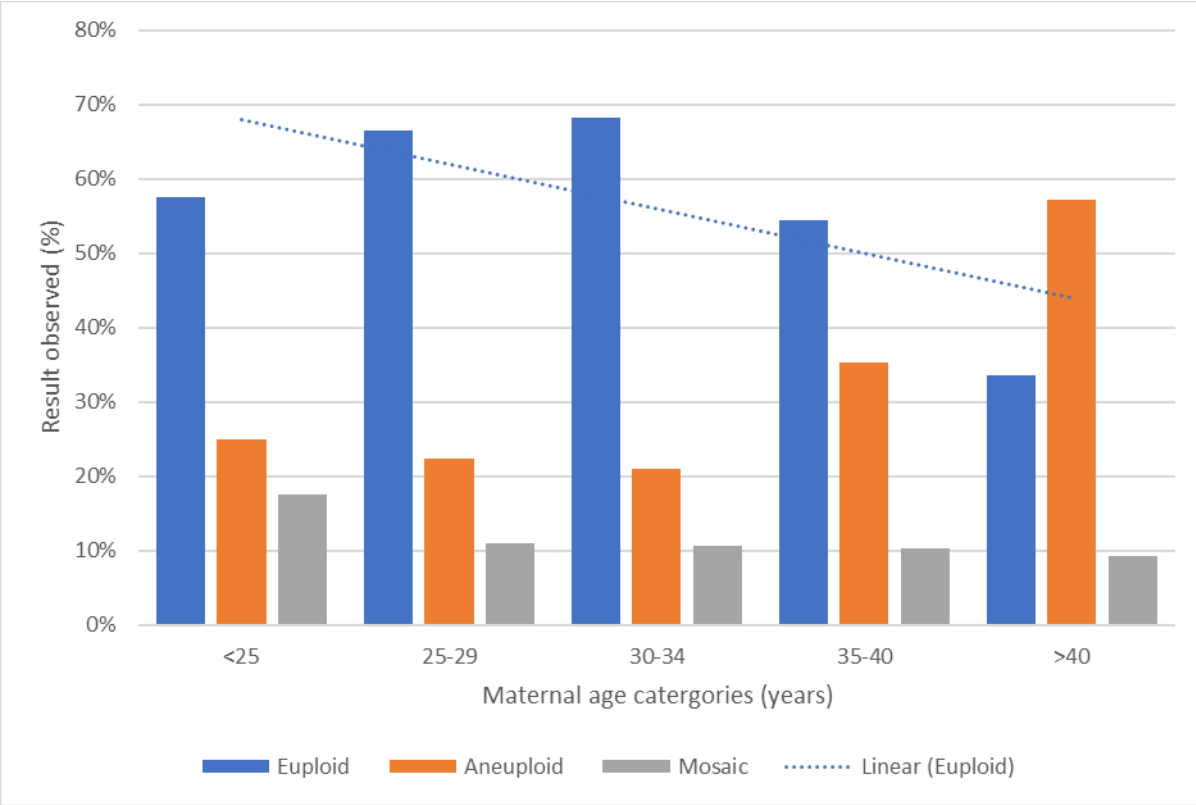


Figure 3-5. Euploid, aneuploid and mosaic PGT-A results observed per maternal age category (n = 2518)

Multinomial logistic regression analysis was performed using euploidy as the baseline outcome to estimate the relative risk of the other PGT-A results at the age of ART treatment (Table 3-6) The analysis revealed that the risk of having an aneuploidy embryo was higher relative to the risk of having a euploid embryo as maternal age increased (relative risk ratio (RRR) = 1.13, p <0.0001). There was no statistical significance with maternal age and the risk of having a mosaic embryo (RRR = 1.02).

Table 3.6 Multinominal logistic regression for risk factors associated with increasing maternal age using euploidy as the base outcome

Risk Factor	**RRR**	**Standard Error**	**95% Confidence interval**	***P*-value**
Aneuploid	1.13	0.012	0.92, 1.67	<0.0001[ǂ]
Mosaic	1.02	0.015	1.01, 1.16	0.1221
No result	0.99	0.035	0.98, 1.05	0.9062

[ǂ] statistically significant p<0.05

3.4.2 Morphology in relation to maternal age

The morphological assessment was evaluated in relation to maternal age (Figure 3-6). A total of 5695 case and control embryos were graded using the Gardner and Schoolcraft (1999) grading system, these gradings were then categorized as excellent, good, average, and poor.

The good category represented 41% (2308/5695) of the embryos followed by 35% (1991/5695) excellent, 16% (934/5695) average and 8% (462/5695) poor grade embryos. A chi-squared test was used to determine whether there was an association between maternal age and excellent embryos. The analysis revealed that the incidence of excellent embryos decreased with advancing maternal age (p = <0.0001).

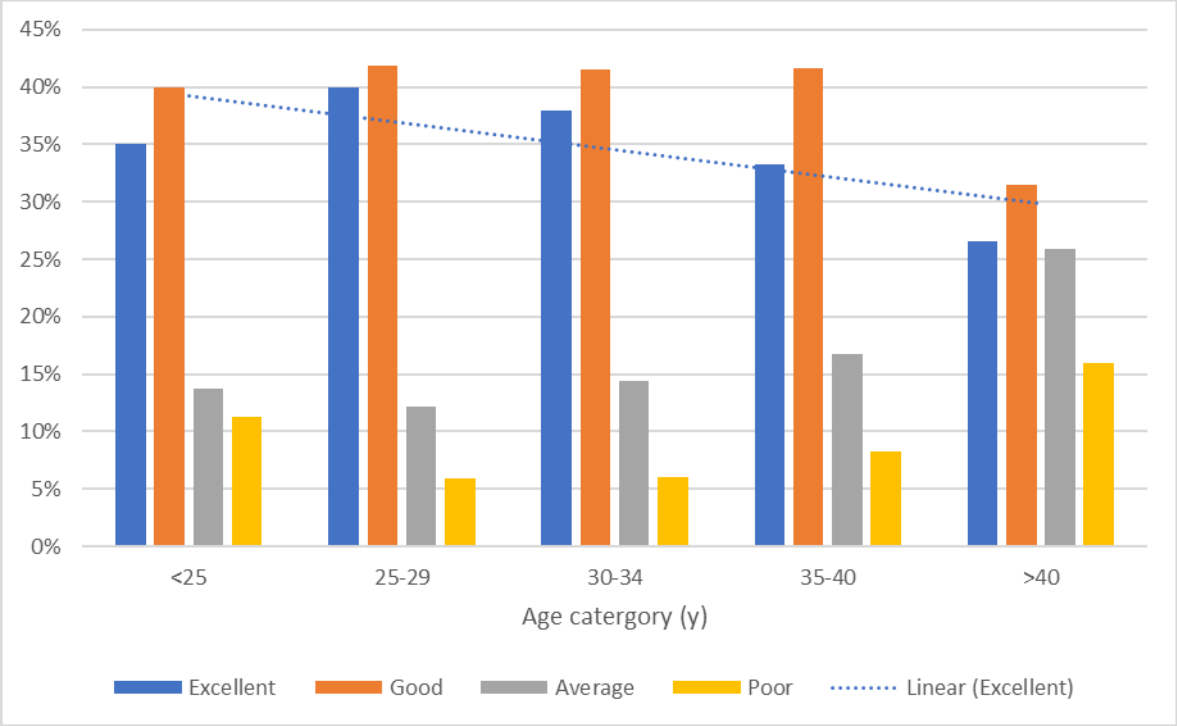


Figure 3-6. The incidence of excellent, good, average and poor embryos observed in the five maternal age categories in cases and controls (n = 5695)

3.4.3 Analysis of clinical pregnancy per maternal age category

A total of 657 clinical pregnancies were recorded in this study in 1176 ART cycles, 65% (337/581) of PGT-A cases achieved a clinical pregnancy and 47% (280/594) of the ART cycles had embryos selected based on morphology alone achieved a clinical pregnancy.

The rate of clinical pregnancy per ART cycle was then further evaluated per maternal age category. The 35–40-year age category represented 48% (567/1176) of the maternal age dataset. The 30–34-year age category had the second-highest number of ART cycles at 32% followed by >40-years at 12% and <25-years category at 0.4% of the population.

A multiple logistic regression analysis was used to determine whether PGT-A improves the likelihood of achieving a clinical pregnancy. The analysis revealed that PGT-A significantly improves the odds of achieving a clinical pregnancy in the 25-29, 30-34, 35-40 and >40-year age categories (Table 3-7). The <25-year age category did not have a sufficient number of ART cycles for statistical analysis.

Table 3-7. Multiple logistic regression analysis to determine the odds of achieving a clinical pregnancy per age category with the use of PGT-A

Age categories	*PGT-A cases**		*Controls**		*Odds ratio*	*95% CI*	*P-value*
	%	*n (N)*	*%*	*n (N)*			
<25	50	1 (2)	0	0 (3)			
25-29	74	37 (50)	53	26 (49)	2.52	1.08, 5.86	*0.0322*ǂ
30-34	68	120 (181)	47	90 (189)	2.16	1.42, 3.29	*0.0003*ǂ
35-40	65	182 (281)	51	145 (285)	1,77	1,27, 2,49	*0.0008*ǂ
>40	55	37 (67)	28	19 (68)	3.18	1.55, 6.51	*0.0015*ǂ

ǂ statistically significant p<0.05
*No. of clinical pregnancies observed per maternal age category

The odds of establishing a clinical pregnancy in the >40-year age category increased three times with the use of PGT-A compared to the control group (p = 0.0015). However, the 25–29-year age category showed higher odds of achieving a clinical pregnancy compared to the 35–40-year group (OR 2.52, p = 0.0322 vs OR 1.77, p = 0.0008) where aneuploidy rates were found to be higher.

Chapter 4

4 Discussion

This study provides an evaluation of PGT-A results and clinical outcomes of ART cycles at a single fertility centre. The key findings of the evaluation will be discussed in this chapter along with the overall conclusions, strengths, limitations, and recommendations for future studies.

The discussion that follows explores the implications of the findings. Firstly, the PGT-A results obtained in this study will be interpreted. Secondly, the evidence supporting the hypothesis that the selection of euploid embryos improves clinical outcomes will be discussed. Further to this, the findings on the maternal age effect on ART and clinical outcomes will also be explored.

4.1 ART outcomes

The maternal age of the overall study population had a mean age of 35 (±4.44 years), which is considered the beginning of advancing maternal age and when aneuploidy risk begins to increase exponentially (Franasiak et al., 2014a). The 35–40-year and 40-year-old age categories represented 60% of the dataset. Delayed childbearing is a fundamental societal change with more women pursuing higher education and careers, which has also resulted in increased demand for elective egg freezing and reproductive autonomy (Inhorn et al., 2018; Inhorn & Smith-Hefner, 2020). Thus, a higher frequency of over 35-year-old maternal age was expected in this study.

This study found increased use of egg donors in the control group. In the absence of aneuploidy screening, the use of donor eggs is an option to reduce the risk of chromosomal abnormalities for those opting for delayed parenthood (ASRM, 2013), which is supported by the findings of this study. Both groups had a mean donor age of 26 years (cases 26 ± 4.68; controls 26 ± 5.48). The mean donor age falls within the 25-29-year age category which included the analysis of 736 blastocysts and showed an aneuploidy rate of 22%. This finding indicates that, although the risk of aneuploidy in younger age groups is low, they are still at risk of adverse pregnancy outcomes. The finding is supported by Coates et al., (2017) who argue that PGT-A reduces aneuploidy risk in all age groups and allows for single embryo transfers. Based on the finding of the current study, the use of PGT-A in younger age groups is recommended.

4.2 Ploidy of preimplantation embryos

The current study detected aneuploidy across all 22 autosomal and sex chromosomes. FISH and qPCR only tested a subset of chromosomes, consequently, aneuploidies in untested chromosomes were undetected and embryos were categorized as euploid which contributed to the failure of PGT-A to show improvements in clinical outcomes (Lee et al., 2015). The new PGDIS guidelines have now disqualified other platforms besides NGS for aneuploidy screening of preimplantation embryos for aneuploidy (PGDIS, 2020). This study supports the statement of the PGDIS, as aneuploidies in untested chromosomes, mosaicism and segmental aneuploidies would have been missed if older technology was used for testing.

Of note, the results obtained through this study showed higher frequencies of whole chromosome losses compared to whole chromosome gains, this was unexpected as previous studies report similar rates of trisomies and monosomies (Alfarawati et al., 2011; Coates et al., 2017; Munné et al., 2019; Rechitsky, Simpson & Kuliev, 2020). Whole chromosome losses were demonstrated to be mainly caused by anaphase lag in human embryos (Vázquez-Diez & FitzHarris, 2018), a further investigation into the laboratory conditions could provide insight into this finding as laboratory conditions have the potential to influence replication mechanisms (Swain, 2019).

4.2.1 **Euploidy**

The current study identified 57% (1428/2518) euploidy in the PGT-A cases which is higher than previous reports; a large study performed by Babiriya et al., (2017) found a euploidy rate of 39% (518/1327) in blastocysts and 18% cleavage-stage embryos in a cohort of 264 patients with a mean maternal age of 38 (range 28-45 years), suggestive of selection against aneuploidy by increased euploidy rates seen in embryos surviving through development.

The STAR trial detected 43% (939/2178) euploid blastocyst by NGS in the PGT-A group (n = 330) with a mean maternal age of 33 (range 25-40 years). The euploidy rate observed in this study was higher than the reported euploidy rate of the STAR trial. The STAR trial noted a wide variation between euploidy rates between the 34 participant centres, the variation in PGT-A reporting could be the attributing factor to the differences in euploidy rates observed, as discussed in the following section.

4.2.2 **Chromosomal abnormalities**

The aneuploidy rate of 33% (828/2518) found in this study was much lower than the 54.2% of aneuploid embryos reported by Munne et al., (2019) in the PGT-A group of the STAR trial.

The authors noted that the various clinics in this study used different methods for the classification of euploid/aneuploid/mosaic embryos which may have contributed to the abnormalities reported. The study allowed for laboratories to use internal protocols, some based on internal validations, which created high variability in PGT-A reporting. Differences in reporting of PGT-A can influence the proportion of embryos resulted as euploid, aneuploid and mosaic which creates inconsistencies in multicentre studies.

Rechitsky, Simpson & Kuliev., (2020) reported an aneuploidy rate of approximately 46% in a large study including over 15 000 embryo biopsies subjected to PGT-A by NGS in a single unit, providing standardization in reporting protocols, which possibly contributed to the lower aneuploidy rates seen in the study, similar to the current study. They further noted that the chromosomes most involved in trisomy and monosomy events were 13, 15, 16, 18, 19, 21 and 22. These findings are similar to that of the current study.

4.2.2.1 Aneuploidies per chromosome

Chromosomes 22, 16, 15, 21, 4, 2, 19, X, 9 and 13 (incidence is reflected by the order), showed the highest number of chromosomal abnormalities observed in this study, similar to frequencies reported in previous studies (Alfarawati et al., 2011; Liang et al., 2013; Coates et al., 2017). Interestingly, chromosomes 4, 2 and 9 showed higher incidences of mosaicism, which will be discussed in Section 4.2.2.2.

Autosomal monosomies were most frequently found in chromosomes 16, 21 and 22, this finding is consistent with the study conducted by Zhang and colleagues who detected monosomy 16, 21 and 22 in blastocysts and chorionic villi from early pregnancy losses. The study reported that trisomy 22 is most frequently found at the blastocyst stage while aneuploidies involving chromosome 16 were shown to be the highest cause of early spontaneous abortions (Zhang et al., 2020). This shows that chromosomal abnormalities impact certain developmental stages of embryos however they do have the potential to implant and grow (Fragouli et al., 2013; McCallie et al., 2016).

Whole chromosome gains involving chromosomes 16, 21 and 22 occurred at the highest frequency in the current study, consistent with previous reports (Rubio et al., 2007; Fragouli et al., 2013; Fragouli, Munne & Wells, 2019; Zhang et al., 2020). While chromosomes 16 and 22 have been reported to result in pregnancy loss and stillbirth, trisomies involving chromosome 21 together with chromosomes 13 and 18 can result in a live birth. In South Africa, the prevalence of Down syndrome is estimated at approximately 2 per 1000 live births in both urban and rural populations.

Similar to Down syndrome, aneuploidies affecting sex chromosomes encompass an array of clinical implications and are difficult to predict severity at the blastocyst level. Interestingly, Turner syndrome in the full and mosaic form represented 3% (38/1120) of the chromosomal aneuploidies found in this study. Tuke et al., (2019) reported that Turner syndrome in the mosaic form has been shown to have a better prognosis in an adult population (54 ±7.77, range 42-69 years) compared to those with true Monosomy X. They further reported 45, X/46, XX mosaic females with normal birth rates and reproductive lifespan, with no reported cardiovascular impediments. However, 45, X/46, and XX mosaic embryos are not recommended for transfer in the absence of euploid embryos (Grati et al., 2018; PGDIS, 2020). Monosomy X has also shown to be the common cause of miscarriage with incidences of 10.7% found in 1000 products of the conception of first-trimester miscarriage (Pylyp et al., 2018).

Detection of all chromosomal aneuploidies and especially those involving chromosomes 13, 16, 18, 21, 22 and X as observed at high frequencies in this study, are important to identify before embryo transfer to prevent implantation failure, early pregnancy losses, spontaneous abortions and aneuploid pregnancies.

4.2.2.2 Mosaic blastocysts

Contrary to meiotic errors, mitotic errors found in this study proved to have no association with increasing maternal age (p = 0.1221), consistent with previous studies (Munné et al., 2002; Vera-Rodriguez & Rubio, 2017).

This study found that 10% (262/2518) of biopsies were classified as a mosaic which led to those embryos being unsuitable for transfer. The rates of mosaicism detected in preimplantation embryos are previously estimated to range between 4% - 30% (Capalbo et al., 2013; Popovic et al., 2018; Spinella et al., 2018; Munné et al., 2020). The findings of this study fall within previously reported ranges of mosaicism. While clinicians and patients were first understandably concerned with the transfer of mosaic embryos, several groups using NGS-based PGT-A have offered transfers of mosaic embryos to patients, when no euploid embryos were available, and have reported healthy pregnancies and babies (Grati et al., 2018; Victor et al., 2019; Munné et al., 2020).

Larger chromosomes 2, 4, and 9 showed a higher incidence of mosaic events compared to whole chromosome gains, losses or segmental aneuploidies. Mosaicism involving larger autosomal chromosomes (1-12) is preferred for transfer with the rationale that if the mosaicism is persistent then the pregnancy will not survive until a live birth (PGDIS, 2020; Rechitsky, Simpson & Kuliev, 2020). According to international guidelines, patients without euploid

embryos could consider mosaic embryos with chromosomes 2, 4 and 9 for transfer and not be discarded without consideration in the absence of euploid embryos.

A study conducted by Maxwell et al., (2016) found that mosaicism involving chromosomes 2, 3 and 7 are most frequently detected in CVS but rarely confirmed in amniocentesis. However, Kahraman et al., (2020) report one instance of trophectoderm mosaicism matching the results of amniocentesis. Interestingly, an embryo with a 35% loss of chromosome 2 was transferred, and chromosome analysis of peripheral blood found a 2% mosaic loss of chromosome 2 by FISH analysis and euploid epithelial cells from a buccal smear. No pathological features were noted during pregnancy and a healthy baby was born. Although the newborn presented without health complications, this is one of the early reports of mosaicism persisting from gestation until birth after the transfer of a mosaic embryo.

The ART facility in this study could implement mosaic transfers in the absence of euploid embryos, however, as highlighted above the need for evidence-based risk scores for mosaic embryo transfer, extensive genetic counselling, close pregnancy monitoring and prenatal testing must be utilized (Viotti et al., 2021). However, the accessibility of these services can be a challenge in low-income and resource settings.

4.2.2.3 Segmental aneuploidies

In the current study, 10% (110/1120) of all chromosomal aneuploidies were found to be partial gains and losses. Previous studies have reported an approximate range of 6% - 58% of embryos created through ART contain segmental aneuploidies. However, the large range is due to the lack of standardization in reporting and heterogeneity between the studies (Babariya et al., 2017; Treff & Franasiak, 2017; Escribà, Vendrell & Peinado, 2019; Girardi et al., 2020). The incidence of segmental aneuploidy found in this study falls within the previously reported ranges.

Segmental copy number variants can occur pre- or post- zygote formation, affecting all or a subset of cells, respectively. Girardi et al., (2020) performed multifocal biopsies, collecting an additional four TE and ICM biopsies for each blastocyst and found that 32% of segmental aneuploidies were of meiotic origin although the TE biopsy was only 50% predictive of the ICM genotype.

Segmental aneuploidies have shown reduced concordance with the ICM in studies conducted by Navratil et al., (2020), who only detected 42% (14/31) of segmental aneuploidies in the remaining embryo while Ou and colleagues showed 68% (43/63) concordance (Ou et al., 2020). Adding to reduced predictive power, cells in the S-phase of the cell cycle, when DNA is

replicated, could theoretically cause false-positive results, most likely resembling segmental gains (Van der Aa et al., 2013). Furthermore, the variation between laboratories in their criteria used to interpret segmental imbalances (Treff & Franasiak., 2017), coupled with the possible introduction of artifacts and technical noise by whole-genome amplification further complicates the specificity of NGS in detecting segmental aneuploidies (Capalbo et al., 2017).

Given the poor concordance and technical aspects surrounding the classification, segmental aneuploidies warrant a differentiation of these PGT-A results, from those reported to have whole chromosome abnormalities (Viotti., 2020). However, there are currently no guidelines on separating the two categories for the clinical management of embryos with segmental aneuploidies. Further studies are needed to determine the predictive power of trophectoderm biopsies in determining the true incidence of segmental aneuploidies in the entire embryo.

4.2.3 The relationship between ploidy and morphology

The present study also describes the correlation between morphology and euploidy rates. An interesting finding was that an excellent embryo was 2.75 (p <0.001) times more likely to be euploid. Furthermore, the excellent, good, average, and poor groups showed euploidy rates of 43% (621/1428), 28% (403/1428), 20% (289/1428) and 8% (115/1428), respectively, showing a correlation between morphology and ploidy, as previously reported (Alfarawati et al., 2011; Capalbo et al., 2014; Kim et al., 2019; Munné et al, 2019). All reports, including the current study, mention that although excellent and good-quality embryos have a higher probability of being euploid, they could also potentially be aneuploid, therefore morphology alone cannot be used to predict the genetic status of embryos (Alfarawati et al., 2011; Capalbo et al., 2014; Kim et al., 2019; Munné et al., 2019).

The findings of the current study suggest that in the absence of PGT-A, morphological evaluation is an appropriate tool for the selection of embryos for transfer, especially in developing countries with socio-economic challenges. However, morphology assessment together with PGT-A has shown positive outcomes in some patient groups, especially those of AMA with a higher percentage of aneuploid embryos, which is supported by the findings of the current study as discussed in Section 4.4 (Lee et al., 2019; Munné et al., 2019).

4.3 Clinical effectiveness of PGT-A

4.3.1 **Transfer characteristics**

In terms of clinical management, PGT-A was established as a tool early in the adoption of CCS technologies, to encourage single-embryo transfers. The reduced risk of the adverse events linked to multiple embryo transfers and multiple gestations are mitigated with SET (Derks et al., 2009; Yang et al., 2012; Forman et al., 2013).

It is interesting to note that the PGT-A group in this study had significantly higher single embryo transfers (72% versus 32%, p = <0.0001) whilst the control group had significantly higher double embryo transfers (65% versus 23%, p = <0.0001). A five-year trend analysis of ART practices in Africa revealed that multiple embryo transfers prevailed over single embryo transfers (Dyer et al., 2020). The authors noted that SET should be used for younger patients and egg donor cycles demonstrating that PGT-A should be used as a tool to promote single embryo transfers across all age groups.

4.3.2 **Clinical outcomes**

The control group showed significantly higher rates of positive β-hCG than the PGT-A cases (94% versus 73%, p = <0.0001). A biochemical pregnancy loss is usually only detected by levels of β-hCG as it occurs in the first few days of pregnancy. The incidence of biochemical pregnancy loss was estimated at 15–20% after frozen embryo transfer cycles (Kowalik et al., 1998; Al Mamari et al., 2019). The 94% of ART cycles in the control group with increased β-hCG drastically decreased to a 48% implantation rate after the presence of a gestational sac could not be confirmed by ultrasound, this indicates that 46% of biochemical pregnancy losses occurred in the control group which is higher compared to the rates reported above.

The PGT-A cases showed an implantation rate of 62%, indicating 11% biochemical pregnancy losses were observed, which is far less than the 46% reported in the control group. PGT-A has been reported to reduce biochemical pregnancy loss, especially in patients with a history of recurrent pregnancy loss and repeated implantation failure (Forman et al., 2014; Sato et al., 2019). This suggests that the biochemical pregnancy loss is mainly due to the transfer of chromosomally abnormal embryos which was supported by the findings of this study. The introduction of comprehensive chromosomal screening has shown that the implantation potential of embryos increases with the use of PGT-A compared to unscreened embryos (Schoolcraft et al., 2010; Scott et al., 2013; Greco et al., 2014; Rubio et al., 2017; Sacchi et al., 2019). Despite the heterogeneity of the studies in terms of design, size and patient

characteristics it is reasonable to conclude that PGT-A does improve implantation rates. Although β-hCG levels and implantation rates are good initial measures of ART success, the ultimate goal is to establish a clinical pregnancy and have a healthy live birth therefore these measures are important to include in studies assessing the clinical effectiveness of PGT-A.

The current study found that the PGT-A group had significantly higher clinical pregnancy outcomes per ART cycle compared to the control group. However, the control group had a significantly higher twin and triplet clinical pregnancies compared to the PGT-A cases which is directly related to the higher DET, and multiple embryo transfers practiced in this population. Since the introduction of PGT-A, single embryo transfers have become a routine practice as testing gives clinical confidence when transferring a genetically normal embryo which also reduces the risk of adverse events (Forman et al., 2014). The current study shows increased support for SET amongst those who opted for genetically screening their embryos before transfer.

The PGT-A group were 2.07 (p <0.0001) more likely to establish a clinical pregnancy following the transfer of a euploid embryo compared to the control group who used morphology alone. This result confirms the hypothesis of this study stating that the selection of euploid embryos improves clinical outcomes across all maternal age groups. The findings of this study are supported by the large retrospective study conducted by Sanders et al., (2021) on data collected by the Human Embryology and Fertilization Authority which showed improved clinical outcomes across all age groups in cycles that included PGT-A vs non-PGT-A cycles. However, these findings contradict the results of the STAR trial which failed to show improvements in ongoing pregnancy rates across all maternal age groups (range 25-40 years) (Munné., 2019). The multi-centre double-blinded RCT does hold the highest form of study however the lack of standardization in protocols limits the transfer of knowledge obtained from these studies from clinic to clinic (Sanders et al., 2021).

Although the retrospective nature of this study did not allow for the random allocation of a specific intervention before monitoring causing possible bias, the use of a logistic regression model adjusted for possible confounding factors was used to reduce the impact of sample bias and group heterogeneity. Therefore, the improved clinical pregnancy rates seen in the PGT-A group prove that PGT-A is a clinically effective tool that should be used for the selection of embryos.

The last reported average clinical pregnancy rate per oocyte aspiration was reported to be 31.7% in Africa in a study including 73 ART centres from 18 African countries (Dyer et al., 2020).

These ART cycles did not include PGT-A which may have increased the clinical pregnancy rates two-fold when considering the findings of this study. In settings, like South Africa, where childlessness has severe psychological, socio-cultural, and economic consequences, the option of PGT-A with ART treatment would be a welcomed option for patients struggling to establish a pregnancy, therefore the option should be presented with the appropriate counselling.

There was no statically significant difference in the miscarriage rates in the case and control groups. The last assessment performed by the ART centre is usually between seven and 12 weeks of clinical pregnancy, feedback on miscarriages occurring after this period relies upon the patient therefore this study might be missing miscarriage data due to lack of patient communication. In addition, live birth data is also only recorded if feedback is received from the patient, therefore this measure was excluded from the study as the information was not uniformly available as part of patient records.

4.4 Advanced maternal age

4.4.1 **PGT-A**

The 25-29 year and 30–34-year age categories showed the highest euploidy rates in this study. Of note, it was also found that euploid embryos decrease with advancing maternal age (p = <0.0001) which is related to the finding of aneuploid risk increasing with advanced maternal age (p = <0.0001). The increase in aneuploidy rate with advancing maternal age has been extensively demonstrated in previous studies (LeMaire-Adkins, Radke & Hunt, 1997; Uroz & Templado, 2012; Franasiak et al., 2014a). The current study supports the hypothesis that oocytes of older women are more prone to nondisjunction caused by meiotic errors (Wartosch et al., 2021).

4.4.2 **Morphology**

The current study also revealed that the incidence of excellent embryos decreases with advancing maternal age (p = <0.0001). The rate of euploidy has also been shown to decrease with advancing maternal age therefore, considering the association between ploidy and morphology, a decline in embryo grades was also expected with advancing maternal age.

As earlier demonstrated, excellent embryos have shown to be more likely to be euploid However, 8% of euploid embryos were found to be of poor quality in this study which may have been given lower priority for transfer. Viñals Gonzalez et al., (2019) found embryo transfer of poor-quality euploid embryos did not significantly affect implantation rates and live

birth rate per embryo transfer in AMA groups. Suggesting that lower morphology euploid embryos should not be overlooked as an option for transfer.

4.4.3 **Clinical outcomes in AMA patients**

The current study demonstrated that PGT-A improved the odds of achieving a clinical pregnancy in all maternal age categories. Notably, the >40-year age category showed the likelihood of achieving a clinical pregnancy increased by three times when transferring a euploid embryo ($p = 0.0015$). This is a significant finding that has been supported by previous randomized control trials (Rubio et al., 2017; Munné et al., 2019). Important to consider in the AMA category is the increased possibility of transferring an aneuploid embryo which can result in repeated implantation failure, or if implanted a distressing miscarriage and the associated medical risks. Taken together, the evidence supports the use of PGT-A in the AMA groups.

Although, as discussed in Section 4.1, albeit the low risk of aneuploidy in younger age groups, they are still at risk of adverse pregnancy outcomes. Coates and colleagues reported improved clinical outcomes with PGT-A in ART cycles using donor oocytes with a mean female age of 25 years (Coates et al., 2017). Although the retrospective study did not include a large number of ART cycles (398 cases and controls), it provided preliminary evidence that PGT-A benefits all age groups, however, the authors only reported improvements after double euploid embryo transfer and no significant difference in the implantation of live births in the groups that transferred single euploid embryos (Coates et al., 2017). Also, the ability for PGT-A to be used as a tool for promoting elective single embryo transfers cannot be understated.

Thus, the findings of this study support the use of PGT-A across all maternal age groups as the odds of achieving a clinical pregnancy with the transfer of a euploid embryo is significantly higher than in the group that used morphological assessment alone.

4.5 Clinical application

An important consideration is the cost of PGT-A testing which is generally expensive and an add-on cost to ART treatment, therefore, the clinical effectiveness must complement the cost-effectiveness. In South Africa, laboratory costs contribute between 35% - 48% of ART treatment at private centres as the majority of laboratory-related items are imported from various locations globally (Huyser & Boyd, 2013). The addition of PGT-A brings the addition of biopsy, freezing costs and the associated testing costs. Thus, the application of genetically screening embryos should be guided by evidence from high-quality studies which also include

investigations of developing countries that have unique challenges such as low local resources but also require high-quality and cost-effective ART treatment.

The results of this study show that the use of PGT-A shows significant improvements in clinical outcomes in the 40-year-old category, for the additional age categories the approximate doubled increased benefit must be assessed against the cost of ART treatment with morphological assessment alone. Limited data are available on the cost-effectiveness of PGT-A. Lee et al., (2019) found that PGT-A has the highest probability of being cost-effective compared to autologous ART strategies, social egg freezing and donor cycles in women aged 40 years following 6 to 12 months of infertility. Single-centre retrospective case-control studies, much like the current study, provide supportive evidence to rationalise future multi-centre meta-analyses randomized controlled clinical trials.

The STAR trial reported no difference in ART outcomes of populations randomized to either PGT-A or morphological assessment alone prior to single embryo transfer which contradicts the findings of this study. The authors of the STAR trial performed posthoc analysis for the age category 35–40 years which revealed significant improvements in the ongoing clinical pregnancy rate in this age group, supporting the use of PGT-A in this age category (Munné et al., 2019). However, the analysis was performed per embryo transfer, contrasting to the overall study being analysed with the reference point of ART cycle start (intent-to-treat), possibly influencing the statistics of the study.

Measuring outcomes per ART cycle, as applied in this study, gives a probability of success per initiated cycle. This assessment informs patients and clinicians of probable outcomes at the onset of treatment based on several factors. ART registries and some studies use factors such as maternal age and cause of infertility to guide treatment protocols in an intention-to-treat practice. This highlights the need for standardized clinical measures between studies that can allow for comparison (Lee, 2018).

While RCTs provide the highest level of evidence, single-centre studies, such as the present study, add significant value and may provide true efficacy of clinical interventions as it eliminates variation in treatment and PGT-A protocols. The reduction in time-to-pregnancy was highlighted by RCT performed by (Rubio et al., 2017) who used empirical data to perform theoretical calculations to show that the number of transfers needed to achieve a “healthy baby at home” was 1.8 in the PGT-A group while the group without PGT-A increased to 3.7 transfers, however, this study had a historic setting of aCGH and day-3 embryo biopsies. When considering the lengthy clinical journey that ART patients undertake, it is important to measure

the success from the patient's perspective who would possibly choose reduced treatment time if given the option.

Although evidence from observational studies and RCTs suggests offering PGT-A preferentially to patients with a poorer prognosis, such as those of AMA, recurrent implantation failure or recurrent pregnancy loss (Rubio et al., 2017; Lee, Wu, et al., 2019; Munné et al., 2019), the current study found clinical improvement in maternal populations that ranged from 21 to 48 years of age.

The International Federation of Fertility Societies performed a survey to assess the global trends in reproductive policy and practice; 97 countries responded to the survey, including South Africa and 10 other Sub-Saharan African countries. The survey reported the availability of PGT-A in 90 countries however only 48 countries reported that PGT-A is allowed by statutes, laws and guidelines (IFFS, 2019). As many countries move towards the use of PGT-A, the need for evidence-based medicine and appropriately designed studies are essential to aid clinicians, patients and medical committees to make informed decisions regarding ART strategies that include aneuploidy screening for embryo selection.

In light of the above, the trend toward single-embryo transfers is promoted by using PGT-A for embryo selection which should be presented to patients of all maternal ages. The findings of this study support the use of PGT-A across all maternal age groups however the cost-effectiveness should be considered in younger patients. The clinical benefit of PGT-A for AMA patients has been extensively proven in this and previous studies as the risk of aneuploidy increase with maternal age. Although much controversy surrounds the use of PGT-A, the increased demand coupled with the application of rapidly evolving technology warrants continuous investigation.

Chapter 5

5 Concluding remarks

5.1 Strengths

The strength of this study lies in the large sample size, a single ART centre setting and a genetic laboratory setting which allows for consistent embryo grading and PGT-A reporting, respectively. Furthermore, the study is novel in its contribution to the current body of PGT-A knowledge as it is the first evaluation of PGT-A conducted in South Africa. This study also draws the main findings per ART cycle which reduce patient bias.

The case-control study design provides strength in evaluating multiple factors influencing ART such as age, morphology, number of oocytes retrieved, and fertilization rates. These factors can provide further prognostic factors to guide treatment and management expectations of ART outcomes per maternal age group, a further evaluation of the current data is thus warranted.

5.2 Limitations

The retrospective nature of the study is limited to attempting to find correlations between past events and the current state but cannot establish causation as in the case in randomized control trials.

A limitation of this study was that the reason for seeking ART treatment was not assessed. This data would have provided information on whether primary or secondary infertility or other factors, such as social egg freezing, was the cause of seeking ART treatment. Data collection on the reasons for seeking ART treatment, especially in settings where studies are limited, could potentially influence clinical interventions.

Immediate success measures, implantation and ongoing clinical pregnancy rates do not offer the weighted measure that live births provide. Inadequate data on live births limit the impact of this study. Further evaluation of the live birth outcomes of the current cohort would also provide insightful data on the clinical effectiveness of PGT-A.

The clinical analysis of this study excluded 182 cycles without euploid embryos available for transfer possibly creating a patient bias for good prognosis patients. By excluding patients who do not produce euploid embryos a bias is automatically created for the inclusion of ART cycles with favourable outcomes. A reanalysis of the clinical outcomes to include the ART cycles without euploid embryos as part of the denominator to measure ART success could reveal comparable clinical outcomes.

5.3 Future studies

This study has mentioned several research areas within the current dataset that can be further evaluated. A systematic review of the evolution of PGT-A technologies and its clinical utility is needed to provide useful summarized evidence which can help guide clinical decisions.

The collection of demographic data such as medical history and reasons for seeking ART treatment could potentially influence clinical interventions. Live births and miscarriage data could potentially influence the outcomes of this study. Furthermore, the influence of PGT-A on time to pregnancy, with an analysis of previous ART cycles, and the financial implications could also be assessed to measure the clinical and cost-effectiveness of testing.

PGT-A traditionally had a binary reporting system for PGT-A, however, the introduction of NGS has changed reporting structures to include incidences of mosaicism and segmental aneuploidies which have caused a clinical conundrum for patients and clinicians to manage. Further studies are needed to rule out the reporting of true euploid embryos as mosaic or with segmental aneuploidy due to the biological, technical, and sampling challenges. A better understanding of these effects can aid in refining bioinformatics algorithms used for PGT-A analysis.

Further evaluation of the neonatal and postnatal outcomes of mosaic embryo transfers is needed to contribute to the current evidence-based risk assessment guidelines, which are currently used globally to make clinical decisions on the transfer of mosaic embryos. Similarly, given the poor ICM concordance and technical aspects surrounding the classification of segmental aneuploidies a differentiation of these PGT-A results is warranted.

Randomized clinical trials remain the gold standard however they are expensive, time-consuming and cannot be justified without high-quality retrospective analyses. It is recommended that future studies focus on strengthening the clinical utility of PGT-A with the assessment of clinical outcomes from well-designed studies, including those of retrospective

methodologies, which include standardization between ART outcome measures and PGT-A reporting without patient bias.

5.4 Conclusion

Over the past 40 years, ART practices have evolved and increased in use worldwide as infertility prevalence rise. Preimplantation genetic testing has become routine practice in some settings and evolved into an independent field of study. The findings of this study are relevant from an international perspective and are the first evaluation of PGT-A results and clinical outcomes in the South African setting.

The clinical pregnancy rate per ART cycle with the use of PGT-A was higher in all maternal age categories indicating that the transfer of euploid embryos improves clinical outcomes compared to cycles in which selected embryos are based on morphology alone.

This study also found that the risk of aneuploidy increases in patients of AMA which is associated with significant improvements in clinical outcomes with the use of PGT-A seen in the 40-year-old maternal age category. This indicates that there is compelling evidence supporting the use of PGT-A for AMA patients.

Smaller single-centre studies may provide more meaningful comparisons when evaluating PGT-A in an intent-to-treat analysis. This study shows that PGT-A is an effective tool for the selection of embryos prior to transfer which promotes single embryo transfers and improves clinical outcomes. Appropriate counselling and guidelines are needed to support the use of PGT-A in clinical practice and the cost-effectiveness should be weighed against the clinical effectiveness, especially in good prognosis patients.

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Appendix A: Letter of Permission

The Inner Circle, 159 Rivonia Road, Morningside, 2196 | P.O. Box 652837, Benmore, 2010
Tel: (011) 911 4700 | Fax: 086 510 0951
www.vitalab.com | info@vitalab.com

2019/02/21

From:
Dr Lawrence Gobetz
Medical Director – Vitalab Fertility Clinic

Re: Access to electronic patient records held at Vitalab Fertility Clinic

I, Dr Lawrence Gobetz, Medical Director of Vitalab Fertility Clinic, Sandton, Johannesburg hereby authorize MSc (Med) candidate Kajel Somaroo access to electronic patients records for those who underwent ART for the period of May 2017 to May 2019 for her study.

The following records will be accessed for participants and the control group:
1) ART treatment outcomes
2) PGT-A reports
3) ART clinical outcomes
4) Maternal ages

This data will be recorded and evaluated to meet the four objectives outlined in the study proposal. The restructuring of the protocol allows for the study objectives to be met; the data source will be changed.

Please do not hesitate to contact me for any further information

Yours sincerely

Dr L Gobetz
Medical Director – Vitalab Fertility Clinic
MBChB (Pret) F.C.O.G. (SA) with Reproduction Medicine

Reproductive Medicine Specialists
Dr. L. Gobetz, Dr. S. Volschenk, Dr. C. Venter, Dr. Y. Unterslak
Reg: 1997/000867/21 Vat: 4560163653 Pr No: 1606417

Dr L Gobetz M.B.,Ch.B., F.C.O.G.(S.A.)
Dr C Venter M.B. Ch.B . F.C.O.G.(S.A.) M.Med.(O & G)
Dr A Picton BSc (Wits), MBBCh (Wits), MMed O&G (UFS)
Dr S Volschenk M.B.,Ch.B., F.C.O.G.(S.A.) M.Med.(O & G) (cum laude)
Dr Y Unterslak M.B.,Ch.B. (Pret.), F.C.O.G.(S.A.), M.Med.(O & G)

Appendix B: Ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Ms Kajel Samaroo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M191015

NAME: (Principal Investigator)	Ms Kajel Somaroo
DEPARTMENT:	School of Pathology Next Biosciences (Pty) Ltd
PROJECT TITLE:	Preimplantation genetic testing for aneuploidy: An investigation of laboratory and clinical outcomes of 4500 embryo biopsies
DATE CONSIDERED:	25/10/2019
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Shelley Macaulay, Prof Amanda Krause and Dr Lawrence Gobetz
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	30/06/2020

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 Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. 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.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **October** and will therefore be due in the month of **October** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C: Participant data collection sheet

Patient Demographics	
Patient age	No. of years at time of ART
Date ART treatment received	YYYY-MM-DD
Donor Eggs	Yes/No
Donor Age	No. of years at time of donation
Donor Sperm	Yes/No
ART outcomes	
Harvested oocytes	Numerical
Fertilization	Numerical
Blastocysts	Numerical
Embryo ID	As recorded
Morphology	
Degree of expansion	1, 2, 3, 4
ICM grading	A, B, C
Trophectoderm grading	A, B, C
Clinical Outcomes	
Embryo Transfer	Yes/No
No Transfer	Yes/No
No. of embryos per transfer	Numerical
Biochemical pregnancy	Yes/No
Gestational Sac	Number of sacs present
Fetal Heartbeat	Number of heartbeats detected
Clinical Pregnancy	Number of pregnancies established
Miscarriages	Number of pregnancies losses recorded

Appendix D: PGT-A data collection sheet

Patient number	***Embryo 1 (Result Example)***
NGS Result	Euploid, Aneuploid, Mosaic, Aneuploid and Mosaic, No Result
Mosaicism Level	High- or Low- Level
Male embryo	Yes/No
Female embryo	Yes/No
Result (Detailed)	Interpretation of result
Chromosome 1-22 and sex chromosomes X and Y	Trisomy, Monosomy, Segmental gain or loss, Mosaic
Complex (3 or more aneuploidies)	Yes/No
Triploid (69,XXY)	Yes/No
Tetraploid (92,XXXY)	Yes/No