Optimizing IVF outcomes in the genomics era

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

Alison Coates

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This thesis encapsulates individual pieces of published or submitted manuscripts as follows:

Morphological and Kinetic Embryological Criteria and Association with Aneuploidy Rates: How Might they be Used to Choose the Best IVF Embryo for Transfer? **Coates A**, Coate B, Holmes L, Griffin DK. *Human Genet Embryol 2015, 5:3 (*Coates A, 2015)

Development and application of a novel strategy to explore blastocoel fluid and spent culture media as a source of embryonic DNA. Babariya, D, Manoharan, A Welch, C, Spaeth, K, Munne, S, **Coates, A**, Wells, D. *abstract submitted to ESHRE 2017*

The use of suboptimal sperm increases the risk of aneuploidy of the sex chromosomes in preimplantation blastocyst embryos. **Alison Coates**, John S. Hesla, Amanda Hurliman, Breanne Coate, Elizabeth Holmes, Rebecca Matthews, Emily L. Mounts, Kara J. Turner, Alan R. Thornhill, Darren K. Griffin, *Fertility and Sterility® Vol. 104, No. 4, October 2015 (Coates et al., 2015)*

Differences in pregnancy outcomes in donor egg frozen embryo transfer (FET) cycles following preimplantation genetic screening (PGS): a single center retrospective study. **Alison Coates**, Brandon J. Bankowski, Allen Kung, Darren K. Griffin, Santiago Munne. *Journal of assisted reproduction and genetics, November 2016 (Coates et al., 2016a)*

Elective frozen-thawed vs fresh transfer of euploid embryos identified by PGD using next generation sequencing; A randomized controlled trial. **A.Coates,** A. Kung, J.S. Hesla, B.J.Bankowski, E.A.Barbieri[1], E. Mounts, B. Ata, J.Cohen, S. Munné, *Fertility and Sterility, Vol. 107, No. 3, 723-730 March 2017 (Coates et al., 2017)*

Mosaicism rates in embryos resulting in live birth or miscarriage

Authors: Tormasi, S, Capaldi, R. Gouw, F. Welch, C. Munne, S. **Coates, A.**

Poster Abstract PGDIS 2016 (Tormasi, 2016)

Planning for the future: how many oocytes do patients need to harvest to achieve their fertility goals? **Alison Coates** Emily Mounts, Brandon Bankowski, Santiago Munne *Abstract poster ASRM annual meeting 2016 (Coates et al., 2016b)*

Other publications generated during the timeframe of production of this thesis are listed below. They have not been included as part of the general thesis but have been referenced if appropriate. My contribution to each body of work is noted here.

Validation of next-generation sequencing for preimplantation genetic screening of embryos. Kung, A. Munne, S. Bankowski, B. **Coates, A**. Wells, D. *Reprod Biomed Online. 2015 Dec; 31(6):760-9 (Kung et al., 2015)*

Personal contribution to the manuscript: I biopsied all embryos for use in the validation study.

No diagnosis after day 3 biopsy: indicative of embryo prognosis or biopsy error

O-147 ASRM annual meeting (Yeboah et al.)

Personal contribution to the manuscript: I advised the author and edited the text.

High Variability in chromosome abnormality rates in embryos from young infertile women. S. S. Sawarkar, J. Zhang, D. L. Hill, J. S. Hesla, **A. Coates**, L. Ribustello, S. Ghadir, S. Munne.*P-102 Tuesday, October 18, 2016 (Sawarkar et al.)*

Personal contribution to the manuscript: Chromosome data from blastocysts biopsied in my own laboratory was used as part of a multicenter retrospective study by the reference genetics laboratory (Reprogenetics) to illustrate variability of aneuploidy rates within populations of young infertile women

The four published manuscripts where I was first author have been inserted as chapters mainly as they appeared in print. The methods sections for stimulation, culture and DNA testing protocols for each paper have been consolidated into one general methods section at the end of the specific aims section and referenced in each chapter to prevent repetition. Parts of this thesis consist of unpublished work using data generated from IVF cycles in my own laboratory.

Table of contents **page**

List of abbreviations

aCGH	array Comparative Genomic Hybridization
ART	Assisted Reproductive Technology
BPA	Bisphenol A
CC	Clomiphene Citrate
CDC	Center for Disease Control
CPM	Confined Placental Mosaicism
CVS	Chorionic Villus Sampling
DNA	Deoxyribonucleic Acid
ET	Embryo Transfer
FET	Frozen Embryo Transfer
FISH	Florescent In-Situ Hybridization
FSH	Follicle Stimulating Hormone
GC	Gestational Carrier
GnRH	Gonadotropin Releasing Hormone
hCG	Human Chorionic Gonadotropin
hMG	Human Menopausal Gonadotropin
HTF	Human Tubal Fluid
ICI	Intra-Cervical Insemination
ICM	Inner Cell Mass
ICSI	Intra-Cytoplasmic Sperm Injection
IP	Intended Parent
IUI	Intra-Uterine Insemination
IVF	*In-Vitro* Fertilization
LH	Luteinizing Hormone
MDA	Multiple Displacement Amplification
MK	Morphokinetic
MtDNA	Mitochondrial Deoxyribonucleic Acid
NGF	Non-Growing Follicles
NGS	Next-Generation Sequencing
NICS	Non-invasive Chromosome Screening
ORM	Oregon Reproductive Medicine
PGDIS	Pre-Implantation Genetic Diagnosis International Society
PEP	Primer Extension Pre-amplification
PGS	Pre-Implantation Genetic Screening
POC	Products of Conception
PSSC	Premature Separation of Sister Chromatids
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
RPL	Repeated Pregnancy Loss
SAB	Spontaneous Abortion
SNP	Single Nucleotide Polymorphism
TE	Trophectoderm
WGA	Whole Genome Amplification
WOI	Window Of Implantation

Thesis abstract:

In order to optimize pregnancy rates during IVF cycle, we have to grow embryos in such a way to allow them to reach their full potential *in-vitro*. As IVF has evolved since the first live birth in 1978, culture conditions have improved and we have reached a stage where embryos can thrive to the blastocyst stage *in-vitro* if programmed to do so. IVF cycles typically produce multiple embryos during one cycle. Establishing which embryo has a higher potential to result in a live birth than its sibling embryos has been attempted over the last 30 years by using non-invasive and invasive techniques. Methods to choose which embryo to transfer range from basic morphology to establishing ploidy status of each embryo by biopsy.

Aneuploidy is the most common cause of implantation failure and miscarriage in human reproduction and increases with maternal age, however all maternal ages exhibit varying degrees of embryonic aneuploidy. While some non-invasive techniques have shown promise in predicting which embryos have the highest implantation potential, the only way currently to establish ploidy status of embryos in the embryology lab is to biopsy, then assay embryonic cells before transfer.

To improve IVF success, original studies derived from retrospective analysis of clinic data, or prospectively designed studies are essential and a number of fundamental biological questions pertaining to chromosome abnormalities and their relationship to IVF embryo development remain unanswered. The overall aim of this thesis was thus to provide further insight into the cytogenetic basis of early human development by pursuit of the following specific aims:

(1) To test the hypothesis that we can predict aneuploidy levels in human IVF embryos without embryo biopsy for PGS by analysis of basic morphokinetic criteria and spent media from cell free embryonic DNA, (2) to test the hypothesis that the ICSI technique may create aneuploidy in embryos, (3) to establish novel patient populations that may benefit from the use of PGS, specifically male factor infertility patients and young oocyte donors, (4) conducting a randomized controlled trial (RCT) to establish the optimal transfer strategy (fresh vs frozen) for euploid embryos in patients using their own oocytes,(5) to test the hypothesis that identifying mosaic embryos among a cohort of embryos could increase live birth rates and reduce miscarriage rates by avoiding these embryos for transfer and (6) to use the data generated from PGS/IVF cycles to provide a framework for creating realistic expectations for patients planning for their fertility future.

The conclusions of each aim were as follows: I demonstrated that poorer quality embryos showed an increased rate of aneuploidy but not large enough to predict aneuploidy for each individual embryo. Analysis of cell free DNA in spent culture media is at its early stages of development, but the study presented in this chapter using a novel WGA technique, showed that there is potential for its future use as a non–invasive PGS method.

I found that aneuploidy rates were similar in embryos generated from normal sperm whether they were created using ICSI or standard insemination using a donor oocyte model to minimize the maternal age effect (aneuploidy rates of 21% for standard IVF vs 23% for ICSI. P=>0.05 NS) concluding that the ICSI technique does not create embryonic aneuploidy.

Donor oocyte recipients (average age of donor 25) benefited from PGS in cryopreserved embryo transfer cycles by significantly increasing live birth rates per embryo from 36% with no PGS to 59% per PGS screened embryo (p=0.0008). Male factor infertility patients presenting with oligozoospermia, were shown to exhibit a significantly higher incidence of sex chromosome abnormalities in pre-implantation embryos compared to patients with normal sperm using ICSI (6.1% for oligozoospermic samples vs 1.6% for normal semen samples. P=0.0007). Both of these patient groups could benefit from offering PGS as part of their IVF cycle.

The RCT showed that freeze all cycles had higher live birth rates than fresh cycles (77% of frozen embryo transfers vs 59% of fresh embryo transfers. P=0.04).

When comparing transfer of embryos screened by NGS with those screened by aCGH, the conclusion in the relatively small subset of patients was that live birth rates for embryos screened with aCGH and NGS appear to be similar, with a 2% trend in favor of NGS (61% aCGH vs 63% NGS live born offspring per embryo transferred. P=>0.05 NS).

Lastly, the retrospective analysis of data using PGS cycles to calculate how many oocytes are required to create one euploid blastocyst depending on maternal age, resulted in a useful tool to advise patients on how many cycles of IVF they may need to complete their family.

Taken together therefore, this thesis provides fundamental insight into the chromosomal basis of early human development, introduces new referral categories for PGS and informs the practical use of IVF/PGS in the future.

List of tables **page**

List of figures **page**

1 General Introduction

1.1 Infertility and IVF

1.1.1 Definition and incidence

Infertility is defined by the World Health Organization as "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (Zegers-Hochschild *et al*., 2009). The ability to have a child is a basic desire for the majority of humans. We have a built-in biological urge to reproduce and if we are denied this by our inability to conceive readily or even at all then the psychological and social ramifications are far reaching and life changing (Poddar et al., 2014). Baker and Robert wrote in their book on Healing the Infertile Family that "*parenting is the bond that seals the generation together and the opportunity to pass along the life experiences to the next generation is what, for many of us, gives life its meaning*" (Baker LA, 1993). The occurrence of Infertility or subfertility according to the NIH is around 15% of couples trying to conceive. https://medlineplus.gov/infertility.html

1.1.2 Etiology and treatments for infertility

The reasons for infertility or sub-fertility can be tubal factor, uterine factors, male factor infertility, endometriosis, ovulatory disorders, luteal phase defects, repeat pregnancy loss, unexplained, or advanced maternal age. Data compiled by the Center for Disease Control and

Prevention (CDC) in the United States of America (USA) for 2014 shows there is a broad distribution of infertility etiologies of patients presenting for help with conception using Assisted Reproductive Technology (ART). ART includes all treatments where oocytes and embryos are handled outside of the body (Table 1).

Diagnosis	Tubal factors	Uterine factors	Ovulatory dysfunction	Diminished ovarian reserve	Endo-meiosis	Male factor	other factors	Unknown factors	Multiple female factors	Multiple male factors
% of reported cycles	13%	6.2%	14.6%	31.7%	8.9%	33%	15.7%	12.7%	12.1%	17.3%

Table 1: showing characteristics of 169,602 patients presenting for ART in 2014 (CDC, 2014)

Treatments to alleviate infertility range from simple interventions such as timing intercourse to occur at the time of ovulation, to In-Vitro Fertilization (IVF).

More simple assisted conception treatments only apply to patients presenting with patent fallopian tubes, motile sperm available, normal hormone profiles and a functional uterus. These treatments would include, timed intercourse around the time of ovulation, clomiphene citrate (CC) administration during the follicular phase of the cycle to mildly stimulate follicle growth, hCG administration mid cycle to trigger ovulation, gonadotropin administration to encourage multiple follicle growth along with intra-cervical or intra-uterine insemination (ICI or IUI) of prepared sperm at the time of ovulation. These methods to assist conception have variable success rates which are also dependent on maternal age and sperm quality.

There have been studies comparing timed intercourse with stimulated and unstimulated IUI and expectant management but the overall conclusion seems to be that for these patients who have largely unexplained infertility, no method has benefit over another (Bahadur *et al*., 2017; Gunn and Bates, 2016; Veltman-Verhulst *et al*., 2016).

In one of the attempts to prove efficacy of each of these treatments, Bhattacharya *et al* performed a randomized controlled trial (RCT) comparing expectant management, oral clomiphene citrate (CC) and unstimulated intrauterine insemination (IUI) (Bhattacharya *et al*., 2008). They randomized patients less than 35, with unexplained infertility with patent fallopian tubes and normal sperm to these 3 groups for 6 months of treatment. They showed cumulative live birth rates for each treatment regimen over the 6 months treatment period, was 17%, 14% and 23% for each group respectively. Ongoing pregnancy rates with stimulated IUI is reported as being around 16% per cycle dependent on maternal age and quality of sperm (Merviel *et al*., 2010). Patients who have patent fallopian tubes and motile sperm will usually begin their infertility treatment plan with these less invasive methods to assist conception and if unsuccessful may move onto IVF. Patients who have non-patent fallopian tubes or severe sperm defects only have the option of attempting IVF to achieve conception.

1.1.3 *In-vitro* Fertilization (IVF)

A cycle of IVF involves hyper-stimulating the ovaries resulting in the recruitment of multiple follicles and subsequently the retrieval of an average of 10-20 oocytes per cycle (own unpublished data). Sperm are either inseminated or injected into each mature oocyte on the day of oocyte retrieval and the resulting embryos remain in culture until the blastocyst stage of development. The availability of multiple oocytes in one cycle increases the chance of finding a euploid oocyte to fertilize.

One cycle of IVF represents multiple cycles of attempting pregnancy without intervention depending on the number of oocytes retrieved and maternal age. Live birth rates of IVF per cycle vary between centers. The national live birth rates per cycle published by the center for disease control (CDC) in the USA for 2014, according to maternal age are shown in table 2.

Maternal age	<35	35-37	38-40	>40
Live birth rate	40.3%	20.5%	18.1%	21%

Table 2: CDC national US data on live births per cycle for 2014 (208,604 cycles)(CDC, 2014)

The number and type of cycles carried out in the USA in 2014 is shown in table 3.

Cycle type	Fresh embryos non donor eggs	Frozen embryos non donor eggs	Fresh or frozen embryos donor eggs
# cycles	92,942	56,308	20,522
% of all cycles	54.8%	33.2%	12.1%

Table 3: CDC national US data showing the distribution of ART cycle types in the USA for 2014 (CDC, 2014)

1.1.4 Evolution of IVF

The first live birth from an IVF cycle in 1978 gave hope to infertile patients that they may be able to have their own genetic offspring (Steptoe and Edwards, 1978). Since this first IVF birth, the percentage of positive outcomes per cycle have increased due to the evolution of methodologies at each stage of the process, including ovarian superovulation protocols, culture methods, micromanipulation techniques (Intra-cytoplasmic sperm injection (ICSI) and embryo biopsy).

IVF Live birth rates per embryo transfer (ET) in 1982 were around 23% (Edwards and Steptoe, 1983; Wang and Sauer, 2006) compared to the best centers in 2015 achieving live birth rates of over 60% per transfer in certain categories of patient (Oregon Reproductive Medicine 2015 published CDC data (www.cdc.gov) 35-37 own oocytes, fresh ET 65%/ET).

Ovarian superovulation was originally carried out using urinary derived follicle stimulating Hormone (FSH) and human menopausal gonadotropin (hMG) (Muasher *et al*., 1985). Oocyte retrievals were performed using laparoscopy at all times of the day and night due to naturally occurring uncontrolled LH surges. Gonadotropin Releasing Hormone (GnRH) agonists were used in the mid-1980s for poor responder patients to prevent premature luteinization and ovulation (Shaw *et al*., 1987) but by the 1990s this protocol was used worldwide for the majority of patient cycles, providing more manageable workflows for clinics and preventing premature ovulation occurring before retrieval. The basic stimulation protocol of artificial suppression followed by artificially stimulating multiple follicle growth by the use of gonadotropins has been modified to

suit poor and good responders so that cycles can be optimized for each patient (Schoolcraft *et al*., 1997; Surrey *et al*., 1998).

Patrick Quinn's group (Quinn *et al*., 1985) developed more physiologically normal culture media based on what was already known about human tubal fluid based on studies by Lippes (Lippes *et al*., 1972). Henry Leese's group performed elegant human tube perfusion experiments (Tay *et al*., 1997) to establish the composition of human tubal fluid *in-vivo*. This led to further refinements after David Gardner's group (Gardner, 1998) observed differences in nutrient requirements of embryos beyond the early cleavage stage of development. This led to the development of sequential media to support optimal embryo growth for the entire pre-implantation period *in-vitro* (Gardner and Lane, 1998; Gardner and Schoolcraft, 1998).

The use of low oxygen tension has been shown to be beneficial to embryo culture (Bavister, 2004; Karagenc *et al*., 2004) and is therefore now standard of care in many IVF labs throughout the world. Low oxygen and high CO2 is either supplied in a pre-mixed gas concentration (5% CO2, 5% O2 balance N2) or special tri-gas incubators are supplied with 100% CO2 gas to achieve the goal of 5-6% CO2 and 100% Nitrogen gas to displace atmospheric oxygen to 5%.

When embryos are growing outside of the body, they have very little protection from potential toxic compounds in the surrounding air. Therefore embryology labs have attempted to minimize exposure to embryos in their care by using low Volatile Organic Compound (VOC) emitting

materials during construction and using carbon and HEPA filtration systems to scrub the air coming into contact with the embryos *in-vitro*. Studies have shown increases in pregnancy rates after the introduction of filtration and air handling units to IVF lab systems (Carre *et al*., 2016; Khoudja *et al*., 2013; Merton *et al*., 2007; Morbeck, 2015).

Optimized culture conditions and stimulation regimens have combined to create an environment in which embryos can develop to their full potential *in-vitro*. Extended culture of embryos to the blastocyst stage allowed for better embryo selection and a reduction in the number of embryos transferred resulting in a reduction in the numbers of multiple births.

The development and implementation of Intracytoplasmic Sperm Injection (ICSI) in the early 1990s (Palermo *et al*., 1992) permitted severe male factor infertility patients to conceive their own genetically related offspring which prior to the advent of ICSI had been virtually impossible. ICSI involves the introduction of a single sperm across the oolemma into the ooplasm of a mature oocyte using a fine glass injection needle (pipette) (figure 1)

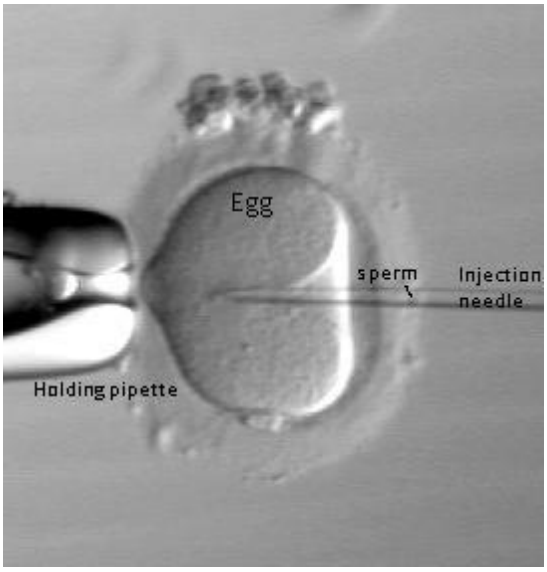


Figure 1: Illustrating the ICSI process. A holding pipette steadies the oocyte during the injection (own image)

Improvements in cryopreservation methods have resulted in better survival rates of embryos. Slow

freezing resulted in low embryo survival rates post thaw due to the formation of ice crystals during the freezing and/or thawing process, which translated to lower pregnancy rates post transfer. The introduction of vitrification (Kuwayama, 2007) resulted in an increase of survival of between 20-30% post thaw, from 60% with slow freezing to over 90% with vitrification (Fasano *et al*., 2014; Rienzi *et al*., 2016).

Vitrification relies on brief exposure to high concentrations of cryo-protectants followed by ultra-rapid cooling to achieve a glass like state of intracellular fluid to avoid ice crystal formation. Much improved survival has resulted in higher pregnancy rates when transferring these vitrified/warmed embryos compared to slow frozen embryos (Richter *et al*., 2016; Rienzi *et al*., 2016).

1.2 Factors contributing to the success or failure of a cycle of IVF

Although IVF has evolved over the last 30 years to the point where live birth rates can be as high as 60% per embryo transferred, there are still factors that contribute to implantation failure and miscarriage that we have yet to discover and remedy. Embryonic competency plays a large part in the success or failure of a cycle of IVF but if the uterine environment is suboptimal then a competent embryo is unable to implant and give rise to a live born infant (Katzorke *et al*., 2016; Teh *et al*., 2016).

1.2.1 Factors affecting uterine receptivity

For implantation to occur, endometrial development has to be in synchrony with embryo growth. Implantation occurs around 6-10 days post ovulation *in-vivo* (Wilcox *et al*., 1999). *In-vitro* cultured embryos have to be in the uterus, ready to implant at the time the uterus is receptive. This is termed the window of implantation (WOI). The uterus responds to either endogenous or exogenous hormonal control (Groenewoud *et al*., 2012). In order to discuss the importance of the endometrium as part of the IVF process, I have described in the next section how the natural cycle creates an endometrium suitable to receive an embryo.

1.2.1.1 Preparation of the uterus for conception in the natural cycle

On day 1 of the natural menstrual cycle, estrogen and progesterone are low. Low levels of estrogen signal the hypothalamus to release Gonadotropin Releasing Hormone (GnRH) which signals the anterior pituitary to produce Follicle Stimulating Hormone (FSH) and Luteinizing

Hormone (LH). These hormones stimulate follicular development. As the follicular phase of the menstrual cycle progresses, rising estrogen produced by the growing follicle(s), proliferates the endometrium in preparation for implantation of the embryo. As estrogen reaches a peak, the LH surge is triggered and this causes the oocyte to mature in the follicle and then the follicle to rupture releasing the oocyte. A corpus luteum is created from the now empty follicle which produces progesterone to maintain the endometrium until implantation occurs. As progesterone rises the endometrium thickens and vascularizes further into the luteal phase of the cycle. If implantation occurs, the embryo will actively produce hCG which continues to maintain the endometrium. If the oocyte is not fertilized or implantation fails, then around 14 days after ovulation, progesterone levels will drop and the endometrium will be shed as menstruation commences.

1.2.1.2 Preparation of the uterus in a stimulated IVF cycle for transfer

During an IVF cycle to harvest oocytes, the pituitary gland may be downregulated by the use of GnRH agonist prior to cycle start so that endogenous hormones are suppressed. Alternatively the use of antagonist might be administered mid-stimulation to help prevent a premature LH surge. Exogenous administration of FSH and/or HMG results in multiple follicular recruitment and development. As these follicles increase in size they each produce estrogen which proliferates the endometrium. Once the follicles reach around 17mm in diameter, an artificial LH surge is created by administering human Chorionic Gonadotropin (hCG) or combination of high dose agonist (Lupron, AbbVie inc, Chicago, IL) /low dose hCG which has the same effect as LH on

oocyte maturation and subsequent ovulation. As the timing of hCG administration is controlled, IVF clinics are able to time procedures to harvest oocytes before the patient ovulates and loses the oocytes to the abdominal cavity. If an embryo transfer is to occur in the stimulation cycle then exogenous progesterone is administered post oocyte retrieval to maintain the endometrium. Embryo transfer occurs on day 5 of embryo growth therefore 6 days post oocyte retrieval.

1.2.1.3 Preparation of the uterus for a frozen embryo transfer

Frozen thawed embryos can be transferred in a natural or artificial cycle. A recently published randomized non inferiority study showed no differences in outcomes between natural and programmed cycles during FET cycles (Groenewoud *et al*., 2016). Retrospective studies are unable to accurately decide which method gives the optimal outcome as patients who have regular cycles and can therefore achieve a natural cycle transfer are a different population to those who can only achieve an adequate lining using medication.

In the natural transfer cycle, transfer is timed 7 days post hCG trigger or 6 days post LH surge allowing endogenous hormones to control endometrial development as described previously in this chapter. An artificial medicated cycle can involve downregulation of the pituitary gland and administration of exogenous estrogen to build the endometrium. Once the thickness and pattern of the endometrium are appropriate, progesterone administration will commence. The transfer is scheduled 7 days post progesterone commencement.

1.2.1.4 Uterine receptivity

Implantation is a complex process involving both the embryo and the endometrium. The glandular endometrium responds to hormonal signals during the menstrual cycle. Gene expression changes throughout the cycle and at the most receptive phase a myriad combination of compounds including growth factors, cytokines and glycoproteins are secreted into the uterine cavity, along with immunosuppressive agents to prevent rejection of the embryo (Melford *et al*., 2014).

The most receptive phase of the cycle for implantation has been termed the "window of Implantation" (WOI) and this appears to be around 5-7 days post ovulation in humans (Lessey, 2000). Timing of embryo transfer during an IVF cycle aims to capture that window of implantation by placing embryos in the uterus around that time. The WOI may not however be the same for all women (Mahajan, 2015; Ruiz-Alonso *et al*., 2013). The endometrial receptivity array (ERA) was developed by analyzing an array of 238 genes found to be expressed at different stages of the menstrual cycle. An algorithm was devised to predict a personalized WOI for patients with repeated implantation failure (Ruiz-Alonso *et al*., 2012) so that embryo transfer could be delayed or moved forwards depending on the optimal day for implantation defined by the gene expression profile. Gomez *et al* (Gomez *et al*., 2015) states that the ERA has shown that one in four patients with repeated implantation failure have a displaced WOI. High circulating estradiol levels during the stimulated cycle have been reported to disrupt the cells of the endometrium

around the time of implantation resulting in lower implantation rates than patients with lower estradiol levels (Basir *et al*., 2001).

1.2.2 Factors affecting embryo development in-vitro

Environmental factors influencing embryo growth *in-vitro* and the intrinsic viability of each embryo are interdependent and both influence the outcome of an IVF cycle. Growing embryos *in-vitro* exposes them to potential hazards otherwise not encountered if growing *in-vivo*. *In-vivo*, embryos are protected from environmental toxins by the maternal milieu and they are provided an ideal combination of nutrients, physiological pH, consistent temperature and oxygen tension to allow for optimal growth potential. The challenge over the last 30 years in the IVF laboratory has been to achieve a stress free environment for growth so that physiological equivalency is met (Swain, 2010; Swain *et al*., 2016).

1.2.2.1 Culture media

There is well documented evidence that nutrition in early gestation both in animal and human models can have long reaching effects on health into adulthood. Roseboom *et al* (Roseboom *et al*., 2006) studied over 2000 victims of the Dutch famine who were *in-utero* during that time period. Effects of maternal undernutrition on offspring in early gestation resulted in more coronary heart disease, a more atherogenic lipid profile, disturbed blood coagulation, increased stress responsiveness and more obesity. Women exposed to famine in early gestation also had an increased risk of breast cancer. Exposure to famine during any stage of gestation was

associated with glucose intolerance. Low birth weight for gestational age has been associated with higher incidence of coronary heart disease (Barker, 1995, 2007) and Barker revealed that this was largely due to poor nutrition during pregnancy resulting in epigenetic changes in embryo development. As there is strong evidence to support negative effects of suboptimal nutrition on fetal development *in-vivo*, culturing embryos for the first 5 -6 days post conception *in-vitro* in suboptimal culture media has to have similar effects (Khosla *et al*., 2001; Kleijkers *et al*., 2016).

Culture media in use today has been modified over the years but still is based loosely on mouse embryo culture media (Chronopoulou and Harper, 2015). As we are unable to test batches of culture media on human embryos, the culture media manufacturers have to rely on the readily available supply of mouse embryos to pass or fail each batch of media as it comes off the production line (Quinn and Horstman, 1998). Mouse embryos and human embryos however are not equivalent in their needs and sensitivity (Ackerman *et al*., 1985; Ackerman *et al*., 1984). The assurance to the end user by testing media on a mouse model is only that the media has no obvious toxins and that the media is able to support growth of mouse embryos to the blastocyst stage.

Composition of culture media today is still basically a balanced salt solution including various concentrations of glucose, pyruvate, and lactate to permit embryo development past traditional developmental blocks (Gardner and Lane, 1996). The evolution of more advanced formulations has included the addition of amino acids, chelating agents (EDTA) and growth factors. Media

manufacturers are reluctant to share exact compositions of their commercially made media for fear of losing market share if their "secret" ingredients are revealed.

A review of the literature published in 2013 (Mantikou *et al*., 2013) of comparative studies of different culture media concluded that there is no evidence to show one media is better than another in human clinical IVF but there are some individual studies which do show differences from one media to another. A recent randomized controlled trial (RCT) by Keijkers *et al* (Kleijkers *et al*., 2016) showed an increase in the incidence of low birthweight offspring using G5 media (G-series, Vitrolife) compared to Human Tubal Fluid (HTF) (Irvine Scientific, Irvine, CA).

The ESHRE working group on culture media recently carried out a review of the literature (Sunde *et al*., 2016) and concluded that based on background literature from animal models, human culture media may have the same potential to change the phenotype of offspring. They also recommended that there should be more transparency of media composition from media manufacturers so that clinical studies can be interpreted.

1.2.2.2 Temperature

Maintaining constant and accurate temperature *in-vitro* is crucial to a successful culture system. In 1990, Sue Pickering and Peter Braude (Pickering *et al*., 1990) performed an experiment looking at the effect of oocyte cooling on the spindle apparatus. Cooling for 10 minutes resulted in damage to 50% of the oocytes' spindles and cooling for 30 minutes resulted in all of the oocytes

having damaged spindle apparatus. A study by Danadova and colleagues concurred with this observation and showed higher rates of chromosomal defects post cooling in mouse oocytes (Danadova *et al*., 2017).

Incubators are typically maintained at 37^{o}C but "normal" body temperature naturally fluctuates between 36.1^{o}C to 37.2^{o}C (NIH, 2017). With this in mind Hong *et al* (Hong *et al*., 2014) cultured 805 sibling oocytes in two groups: one group at 37^{o}C and one group at 36^{o}C. The group cultured at 37^{o}C resulted in a higher rate of blastocyst formation than the group at 36^{o}C (48% vs 41%) but equivalent fertilization, aneuploidy and sustained implantation rates between the two temperatures.

1.2.2.3 pH

In order that intracellular pH of oocytes and embryos is maintained *in-vitro*, extracellular pH ideally should be maintained at a constant physiologically equivalent value so that stress to oocytes and embryos is minimized. However, the ideal pH of media used to culture human embryos is difficult to establish. Yedwab and colleagues (Yedwab *et al*., 1976) in 1976 performed experiments to test the intrauterine temperature, oxygen tension and pH of 66 women during their cycle. A pH on average of 7.2 and 7.1 was observed. As pH is not a stand-alone measurement and depends on other solutes in the media, differing media compositions may require different optimal pH values (Swain, 2012; Swain *et al*., 2016). Most culture media used for human IVF are buffered with bicarbonate and require equilibration in a 5-6% CO_2 environment to reach a pH of

7.2-7.3 but again these recommended upper and lower limits of pH in any given media are somewhat dependent on media composition.

1.2.2.4 Oxygen tension

Atmospheric oxygen is 21% but *in-vivo* oxygen saturation is much lower than this and is variable depending on which organ is under consideration. Ottosen *et al* (Ottosen *et al*., 2006) and Yedwab *et al* (Yedwab *et al*., 1976) found that human uterine oxygen tension was around 11%. Studies have been carried out comparing atmospheric and reduced oxygen tension in which to grow human embryos *in-vitro* (Kasterstein *et al*., 2013; Meintjes *et al*., 2009). In these studies, blastocyst formation and live birth rates were increased in low oxygen tension environments compared to room air. Many laboratories have adopted the practice of culturing in 5% oxygen in their incubators contributing to improved rates of blastocyst formation (Guo *et al*., 2014; Meintjes *et al*., 2009). Although the exact method of improvement in embryo growth is unknown, there seems to be only a benefit to lowering oxygen tension in the growing environment (Swain *et al*., 2016).

1.3 Determining embryo viability

Over the past 30 years many attempts have been and are still being made to define the implantation potential and viability of each embryo. As culture conditions have been optimized, the ability to culture embryos beyond day 3 has allowed for better selection of embryos for transfer.

As our goal is to minimize stress to the embryo *in-vitro*, the ideal way to test an embryo's implantation potential *in-vitro* would be to use a non-invasive rather than an invasive method. If a non-invasive method showed definitively the ploidy status of any given embryo then this would be our choosing tool of choice. Non-invasive testing methods are attractive because they do not detract from the embryo itself. In this section I present a summary of non-invasive methods that are currently used or have been used in the past, novel methods that have been proposed and how effective they are in relation to choosing that elusive baby making embryo.

1.3.1 Embryo Morphology

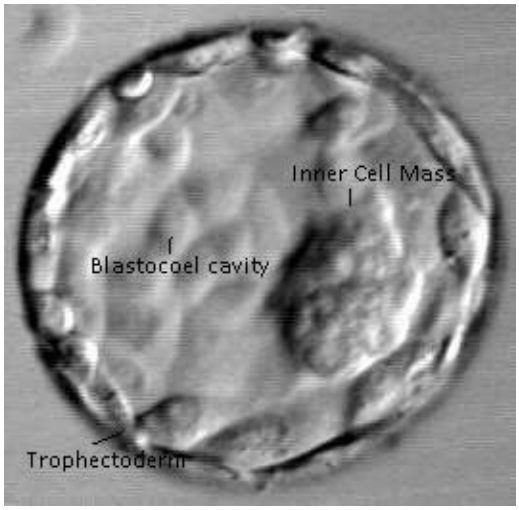
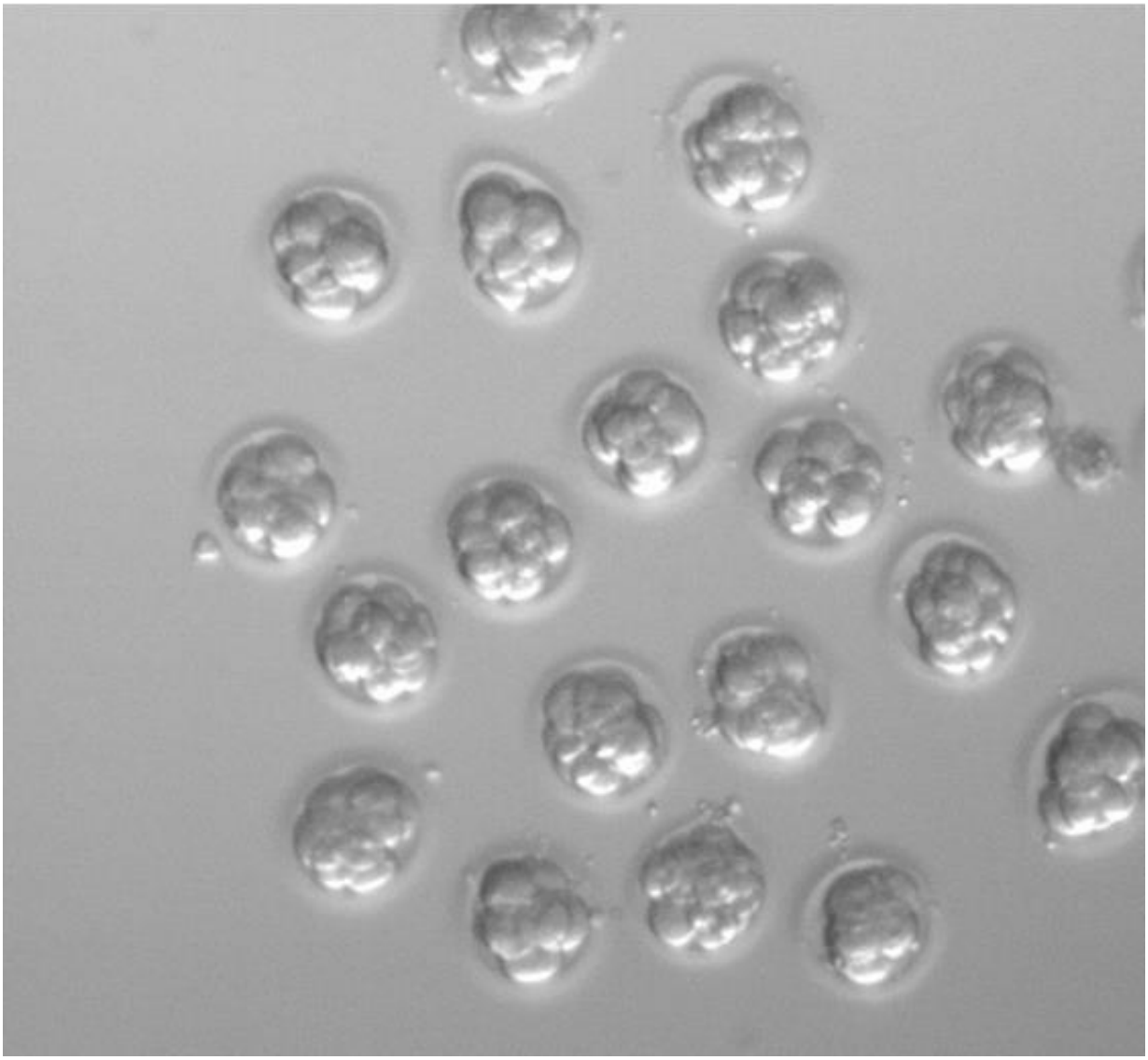


Figure 2: Embryo day 5 of development at the blastocyst stage. Discrete inner cell mass and multi-celled well expanded trophectoderm cell layer indicates a morphologically good quality embryo with good implantation potential (own image).

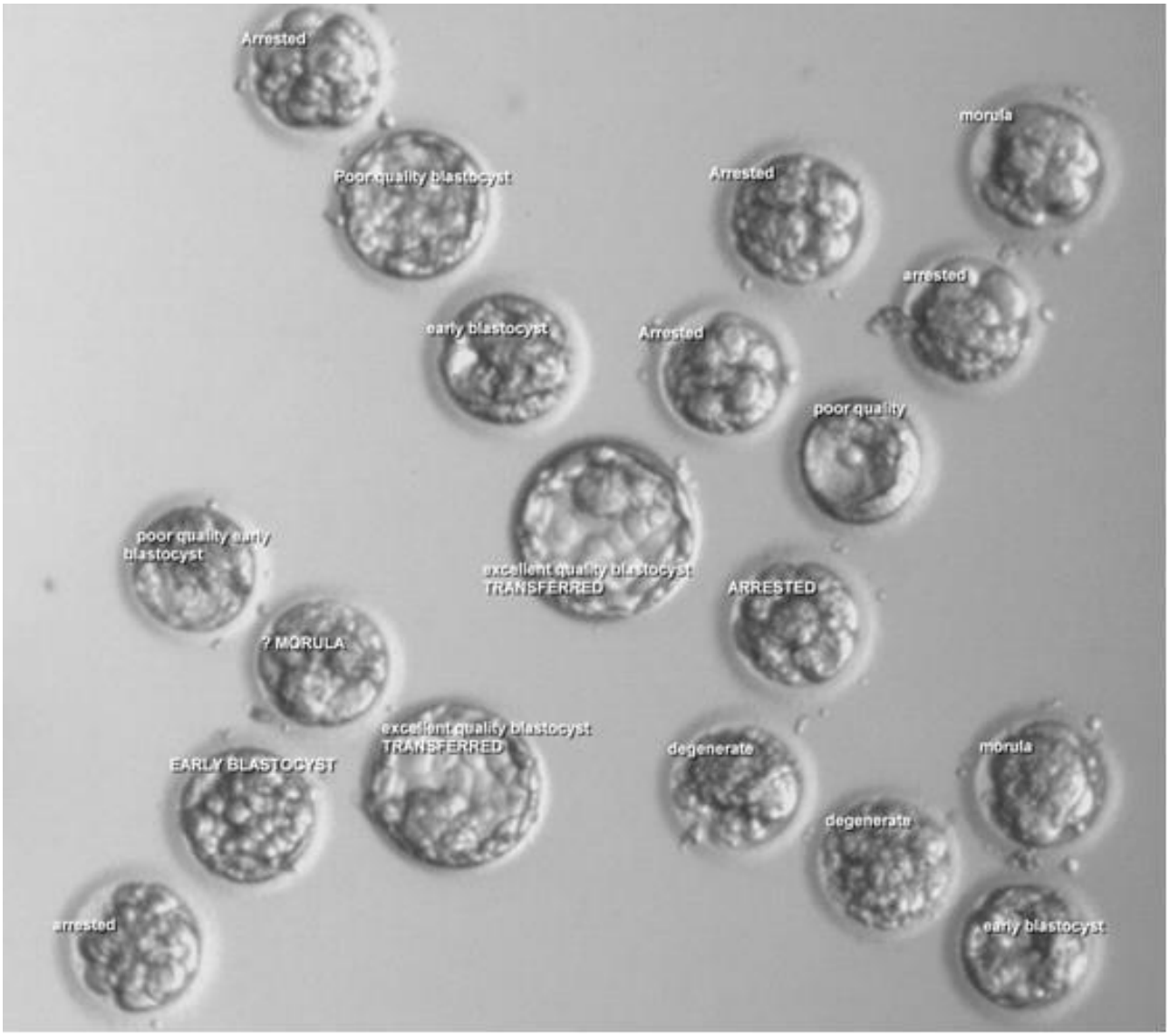
This is the simplest tool used by embryologists to choose within a group of embryos. Historically the best available embryo is chosen from a cohort on day 2, 3 or 5 based on morphology alone. Leaving embryos in culture until day 5 enables better embryo choice for transfer as on average 50% of all embryos stop growing after day 3 (own data).

In figure 3, we have a picture of day 3 embryos from one patient grown in our laboratory. All the embryos look quite similar and some of these will continue to grow to form blastocysts and some will arrest at this early cleavage stage. We can only guess which will arrest and which will continue to develop by assessing morphology on day 3. If we were to replace one or two embryos on day 3 we would be much less likely to choose an embryo that is chromosomally normal than if we give the embryos chance to declare themselves by growing for another 2 -3

days. At this point around half of the embryos will stop growing and the choice for transfer is narrowed down as illustrated in the next picture of the same cohort on day 5.



(a) Day 3 cohort of embryos



(b) Day 5 cohort of embryos

Figure 3: Illustrating how embryo growth begins to be asynchronous after day 3(a). The best quality embryos are usually chosen for transfer to the uterus and the remainder that develop to the blastocyst stage (b)can be frozen if good quality. There are 2 clear leaders in this cohort above therefore the choice is relatively easy.

If an embryo has successfully developed beyond day 3 at which stage the embryonic genome is activated (Niakan *et al*., 2012) and has managed to grow until day 5 or 6 it has at least a chance of implanting. If an embryo is chosen on day 3 we are still guessing which have continued to grow and which have already arrested. However, if an embryo grows to the blastocyst stage and has good morphological appearance it still does not tell us the whole picture about the developmental potential of an embryo.

Blastocyst morphology and growth rate correlates to some extent with ploidy status but is not predictive definitively of aneuploidy. Some embryos with the best morphologic appearance are aneuploid while those with poorer appearance are found to be euploid (Alfarawati *et al*., 2011; Davies *et al*., 2012; Richter *et al*., 2001). Aneuploidy is discussed in section 1.4

1.3.2 Pronuclear position and nucleoli pattern

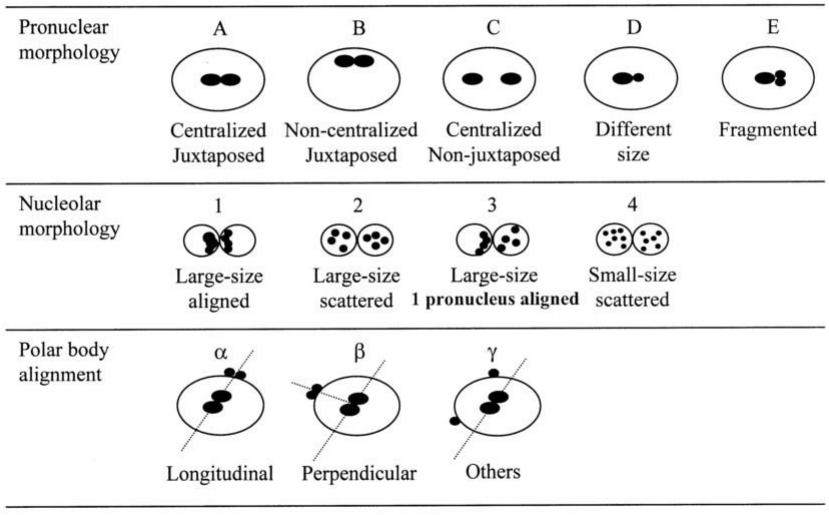
Many groups have studied pronuclear morphology and attempted to correlate this with blastocyst formation potential, implantation potential and ploidy status (Gianaroli *et al*., 2003; Kattera and Chen, 2004; Scott *et al*., 2000; Zollner *et al*., 2002).

Richard Scott's group (Scott *et al*., 2000), used only nucleoli alignment as a secondary selection tool to morphology. Even numbers of nucleoli in each pro-nucleus distributed at the area that the pro-nuclei meet seemed to predict blastocyst formation with some accuracy and positively correlated with implantation. Zollner and colleagues (Zollner *et al*., 2002) looked at nucleoli alignment in conjunction with pro-nuclei placement to predict blastocyst formation which it did in IVF embryos but not in ICSI derived embryos. Arroyo's group on the other hand (Arroyo *et al*.,

2010) used only pronuclear morphology as a secondary selection tool and correlated patterns with ploidy status of the embryo. They showed no association with ploidy status of embryos and pronuclear morphology.

With the scoring system at its most complex, Gianaroli's group took into account the position of pronuclei in the ooplasm, the pattern of nucleoli in each pronucleus and the angle of the pronuclei from the polar bodies (figure 4). In this study FISH was used for ploidy determination and a association was only found between complex aneuploidies and the more chaotic arrangements of pronuclei and nucleoli. Euploidy could not be accurately predicted (Gianaroli *et al*., 2003).

Diagram showing the different configurations that identify pronuclear morphology, nucleolar morphology, and polar body alignment with respect to the longitudinal axis of pronuclei. Each configuration is conventionally given a capital letter in the case of pronuclear morphology (A–E), a number for nucleolar morphology (1–4), and a symbol from the Greek alphabet (α–γ) to identify the polar body alignment.



Gianaroli. Pronuclear morphology and chromosomal status. Fertil Steril 2003.

Figure 4: pronuclear morphology and chromosomal status by permission of Gianoroli (Gianaroli et al., 2003).

Interpreting nucleoli patterns is a time consuming practice and if an analysis is carried out in real time this may mean that zygotes remain outside the incubator for longer than they need to be resulting in stress to the developing embryos. Images could be captured to examine once the embryos have returned to the incubator but the 3 dimensional nature of pro-nuclei requires movement of the image or embryo to capture fully the pattern of nucleoli and the position of the pronuclei in the ooplasm. Time lapse microscopes are able to capture in real time the formation of pronuclei and would be able to allow captured data to be used to choose early cleavage embryos for transfer if required.

If growing embryos to the blastocyst stage is not possible for whatever reason and if Pre-Implantation Genetic Diagnosis (PGS) is not possible in labs around the world, gross pronuclear morphology may be a relatively low cost, non-invasive adjunct to add to basic morphology to allow for better selection of early cleavage embryos for transfer.

1.3.3 Morphokinetics

Embryos have a particular pattern of growth which can be monitored and correlated to some extent with embryo viability. The observation of morphokinetic events to use them as an indicator of embryonic competency began with monitoring early cleavage of day 2 embryos. Several groups have associated early embryo cleavage with increased implantation potential (Fenwick *et al*., 2002; Lundin *et al*., 2001). Embryos that underwent the first cleavage division 25-27 hours post insemination had a higher implantation rate than embryos cleaving later. ICSI derived embryos showed a stronger association with higher implantation potential probably due to more accurate timing of the sperm actually entering the oocyte.

The timing of morphokinetic (MK) events has been taken to the next level by actually watching the whole developmental process from zygote to blastocyst by using time lapse photography. This has been achieved by installing small microscopes into existing incubators (Eeva, Auxogyn) and the development of a benchtop incubator that has the camera as part of the incubator (Embryoscope, Vitrolife, Sweden). These time lapse camera systems utilize dishes that have individual wells for identification purposes.

Preliminary data suggests that observing the timing of MK events is unable to predict euploidy with any reliability but will predict good quality blastocyst formation (Kramer *et al*., 2014; Motato *et al*., 2015; Rienzi *et al*., 2015; Storr *et al*., 2015). Campbell and colleagues (Campbell *et al*., 2013) noted that the timing from the first signs of cavitation to complete expansion had the highest positive prediction of ploidy but not conclusively. Chavez *et al* (Chavez *et al*., 2012) analyzed MK

events up to the 4 cell stage and demonstrated that only 30% of aneuploid embryos exhibit parameter values within normal timing windows. They also analyzed fragmentation formation and its reflection of aneuploidy and demonstrated that fragments sometimes hold chromosomal material which is then re-absorbed into the cells concluding that fragment removal could be detrimental to embryo development.

Growth rates in embryos vary from lab to lab based on variations in culture conditions and stimulation regimen. Therefore implementation of time lapse incubation in any lab with any real effect in predicting embryo viability would have to involve each lab establishing its own acceptable time frames for each morphokinetic event. Establishing standardized times to events is difficult if not impossible due to clinic to clinic variations.

1.3.4 Combining Morphokinetics with spent media analysis

Many of the non-invasive techniques described earlier have some positive predictive potential for implantation so to combine some of those techniques has been proposed. Dominguez' group in 2015 (Dominguez *et al*., 2015) combined morphokinetic analysis with analysis of the proteome of the spent media surrounding the embryo. They found that their implantation rates if the second cell cycle division (the time from division to a 2 cell embryo to a 3 cell embryo) was within a certain time range and if Interleukin-6 were present in the spent media (figure 5). They also found a association with Stem Cell Factor presence in the media and with the absence of Interferon.

1.3.5 Analyzing spent culture media

Embryos are grown in droplets of culture media. These droplets can be analyzed for depletion from or addition to the media of compounds during the time that the embryo is in culture.

1.3.5.1 Metabolomics

Metabolomics is the analysis of metabolites produced or utilized in the surrounding media by the embryo as it develops. Establishing which metabolic markers can be used reliably as an indicator of embryo health *in-vitro* is a challenge that has been attempted by many groups over the last 20 years. Conaghan *et al* (Conaghan *et al*., 1993) carried out the first clinical studies analyzing pyruvate uptake levels from culture media as a metabolic evaluation of embryo health. The studies only looked at a snapshot of time in culture from day 2 to 3, and found some association with pyruvate uptake and embryonic potential but not conclusively and concluded that morphology still remained the best indicator of implantation potential. Hardarson *et al* looked at Near Infrared (NIR) spectroscopy as a tool for identifying embryos with increased viability rates (Hardarson *et al*., 2012). The NIR spectral profile of spent media was analyzed during IVF cycles. Two groups were compared, the control group with embryos chosen for transfer using morphology alone and the test group utilizing the NIR profile to choose embryos for transfer. There was no difference in pregnancy outcome between the control group and the test group.

Henry Leese (Leese *et al*., 2008), proposed his “quiet embryo hypothesis” which describes the principle that an embryo that has a lower level of metabolic activity is less stressed in its

environment. So an overall low production of waste products and uptake of nutrients is a sign of a healthy embryo. His work with glucose and pyruvate uptake and lactate production in spent media (Butcher *et al*., 1998; Conaghan *et al*., 1998; Leese, 2002) has shown some association with embryo health but does not categorically show which embryo to choose based on metabolomics alone.

1.3.5.2 Proteomics

Proteomics is the analysis of amino acid turnover by pre-implantation embryos from spent culture media. The turn-over of amino acids varies at different stages of development.

Picton *et al* (Picton *et al*., 2010) showed that aneuploid embryos have a different pattern of uptake and excretion to that of euploid embryos but not consistently so therefore turnover rates are probably unable to predict implantation potential alone. Dominguez *et al* (Dominguez *et al*., 2008) showed a significant difference in protein profiles from implanted and non-implanted blastocysts.

Brison *et al* (Brison *et al*., 2004) used reverse phase HPLC to analyze the change in concentration of 18 amino acids in spent culture media over a 24 hour period. Embryos were cultured singly in 4ul drops from day 1 to day 2 at which point 2 embryos were transferred to the patient's uterus. The spent media drops were frozen and sent for analysis and the results were retrospectively correlated with implantation and live birth rates.

A previous study by Houghton *et al* (Houghton *et al*., 2002) had studied amino acid turnover as a prediction of blastocyst formation. In that study the amino acids whose turnover predicted blastocyst formation *in-vitro* were not the same as those that predicted pregnancy and live birth in the Brison 2004 study following transfer. Blastocyst formation from early cleavage human embryos developing *in-vitro* was predicted by Alanine (Ala), Arginine (Arg), Glycine (Gln), Methionine (Met) and Asparagine (Asn) turnover between days 2 and 3 (Houghton *et al*., 2002) whereas pregnancy was predicted by Asn, Gly and Leucine(Leu) between days 1 and 2 (Brison *et al*., 2004). This profile could be used as an adjunct to identify blastocysts which are actually not viable.

Botros *et al* in 2008 (Botros *et al*., 2008) reviewed the literature from research carried out over the last 20 years and concluded that overall embryos with higher implantation potential altered their environment differently to those embryos that had a lower implantation potential.

1.3.6 Cumulus gene expression

As cumulus complexes are in close contact with the oocyte during its' development and play a huge role in the maturation of the oocyte, Fragouli *et al* (Fragouli *et al*., 2012) looked at the follicular environment as a possible indicator of embryo health. The hypothesis was that cumulus complexes regulate oocyte maturation *in-vivo* and the close communication between the oocyte and cumulus cells may show some specific gene expression that correlates with the ploidy status of an oocyte. The expression of 96 genes was analyzed and 2 specific genes showed a significant

difference in their expression in the cumulus cells of euploid and aneuploid oocytes. One of these genes is involved in intracellular signaling and homeostasis, and the other regulates carbohydrate metabolism and apoptosis.

Oocytes from older women have been shown to have a different cumulus cell gene expression profile compared to cumulus cells from younger women (McReynolds *et al*., 2012). We now know that cumulus cells and the oocyte communicate with each other and the genes expressed correspond with oocyte quality (Fragouli *et al*., 2014). Further research may show this to be a reasonable non-invasive method to quantify implantation potential in the oocyte.

1.3.7 MicroRNA in follicular fluid

A study by Machtinger *et al* (Machtinger *et al*., 2017), proposed that profiles of extracellular Micro RNAs (exmiRNAs) in follicular fluid may correlate with embryo viability. They analyzed the exmiRNA profiles of follicular fluid of oocytes from 40 patients and did find association with oocytes that failed to fertilize and those that fertilized normally. They also found four exmiRNAs that were preferentially expressed when a good quality day 3 embryo developed versus a poor quality day 3 embryo. This area of research has potential for future advancement but is at the very earliest stages of investigation.

1.3.8 Mitochondrial DNA quantification

Mitochondria are the primary energy manufacturing organelles for all cells. The number of mitochondria in a mature oocyte is by far the highest of any cell at around 2 x 10^6. This is at least twice the copy number found in somatic cells such as muscle or nerve, which also have a high energy requirement (St John *et al*., 2010). Embryonic mitochondria are inherited from the oocyte. Paternal derived mitochondria introduced via the sperm at fertilization are doomed for destruction by ubiquitin-dependent proteolysis as described first by Schatten's group in 1999 (Sutovsky *et al*., 1999; Sutovsky *et al*., 2004). During oogenesis mitochondrial replication ensues resulting in the highest concentration of mitochondria per cell once the oocyte matures. Once fertilization has taken place, mitochondrial replication pauses resulting in fewer mitochondria per cell with each cell division. Once blastulation commences (around day 4-5 in humans), the cells forming the trophectoderm begin to replicate mtDNA and the concentration per cell increases. The inner cell mass remains at a constant density of mtDNA. The high concentration of mitochondria in the trophectoderm serves as an energy source for the Na+ pump transportation system bringing nutrients to the relatively quiescent inner cell mass (Hewitson and Leese, 1993; Houghton, 2006).

It has been proposed that embryos that are aneuploid, derived from oocytes of advanced maternal age or are growing in a suboptimal environment generate a higher number of energy producing mitochondria per cell in response to those stresses. This corresponds with the idea of the "quiet embryo hypothesis" postulated by Henry Leese (Leese, 2002), which states that an

embryo in a physiologically ideal state needs less energy to develop than an embryo in suboptimal conditions. With this in mind, the hypothesis that a high number of mitochondria per cell in a pre-implantation embryo might equal a reduced chance of conception if transferred was put forth.

As mitochondria each have between 1-10 copies of their own DNA (mtDNA) this has been explored as a way to measure the number of mitochondria per cell in human embryos and correlate that with implantation potential in a clinical setting. Several genetics companies have begun to offer the test as an adjunct to whole chromosome analysis with names such as "Mitoscore" (Igenomix) and "Mitograde" (Reprogenetics-Cooper Surgical).

Trophectoderm biopsy involves removing between 3-10 cells from the blastocyst. To establish how many cells are present in the biopsy tube, the total amount of nuclear DNA is determined which can then be divided by the known amount of nuclear DNA in one euploid human cell to arrive at the number of cells in the biopsy. The total quantity of mtDNA in the sample tube is measured, then the total quantity of mtDNA can then be divided by the number of cells to arrive at the amount of mtDNA per cell.

Dagan Wells' group (Fragouli *et al*., 2015b) examined day 3 early cleavage embryos and blastocysts that had been biopsied from several IVF laboratories. The ploidy status for each embryo was established and the amplified DNA was then analyzed for the quantity of mtDNA per

cell using qPCR or NGS. In this study, euploid embryos exhibiting a high level of mtDNA above a threshold never implanted whereas embryos with levels below this threshold implanted and gave rise to viable pregnancies in 59% of the cases. They also concluded that embryos from patients of advanced maternal age exhibited higher levels of mtDNA per cell compared to embryos from younger women. In another study by Diez-Juan *et al* (Diez-Juan *et al*., 2015) the association between increasing maternal age and corresponding increases in levels of mtDNA were not seen.

The most recent publication by Victor and colleagues (Victor *et al*., 2016) is a large single center study analyzing data from a total of 1,396 embryos derived from 259 patients. They established ploidy status and mtDNA quantification by NGS and qPCR. In order to accurately establish mtDNA amounts in any one cell the group developed a calculation to correct for embryo ploidy and gender. Previous studies have used a standard male euploid genome as a known template to establish sample size (Diez-Juan *et al*., 2015; Fragouli *et al*., 2015b). This group observed that as nuclear genomes are not all equal in length (diploid female human genome is composed of 6,072,607,692 base pairs, whereas the male counterpart is 5,976,710,698 base pairs long, a difference of 1.58%) then without correcting for embryo gender the calculation of mtDNA quantity per cell would be inflated by 1.58% in male embryos. The same can also be said for aneuploid embryos which would all have different sized genomes depending on the chromosome error or errors. With the correction factor applied this group found no difference between the amount of mtDNA per cell in euploid or aneuploid embryos, of embryos generated from

advanced or young maternal age and they also found no association between mtDNA levels in embryos that implanted and those that did not.

The other issue with establishing the number of mitochondrial organelles by using mtDNA quantification is that each mitochondria has a variable mtDNA copy number of between 1-10 per organelle (Satoh and Kuroiwa, 1991). Therefore the copy number of mtDNA probably does not correlate well with the number of mitochondria per cell.

1.3.9 Summary of factors contributing to the success or failure of an IVF cycle

In order to achieve a successful conception, embryos have to be grown with the minimal amount of stress *in-vitro*, the embryo with the most potential has to be chosen by the embryologist for transfer and the uterus has to be receptive. While there may be other factors to consider when choosing an embryo for transfer, the main reason for implantation failure is aneuploidy (Harton *et al*., 2013) which is discussed in section 1.4.

1.4 Aneuploidy

Aneuploidy is the main cause of implantation failure and non-viability in human embryos (Spandorfer *et al*., 2004; van den Berg *et al*., 2012). An aneuploid cell has extra or missing chromosomes. Aneuploidy of embryos occurs in all humans to a greater or lesser degree depending primarily on maternal age but can be affected by other factors. In this chapter I discuss the mechanisms of aneuploidy and how the detection of aneuploidy in human pre-implantation embryos has contributed to improvements in IVF outcomes (Dahdouh *et al*., 2015b; Forman *et al*., 2013b; Forman *et al*., 2012b; Schoolcraft *et al*., 2010; Scott *et al*., 2012; Tan *et al*., 2014).

1.4.1 Mechanisms of chromosomal mal-segregation

Chromosomal mal-segregation can occur during meiotic or mitotic division. Paired chromosomes that become dissociated from one another too early or too late in the cell cycle, or fail to separate during the correct sequence of meiotic or mitotic events, result in aneuploid cells with extra or missing chromosomes (Angell, 1997; Cupisti *et al*., 2003).

There are numerous mechanisms by which chromosomal mal-segregation occurs however the primary ones in this context are:

- Non disjunction (at meiosis I or II, or mitosis (cleavage))
- Anaphase lag
- Precocious separation of sister chromatids

1.4.1.1 Non-disjunction

Either paired chromosomes fail to separate during meiotic anaphase 1 or sister chromatids fail to separate at meiosis 1 or meiosis 2 resulting in a whole chromosome moving to the same pole as its' homologue. Non disjunction results in missing or extra chromosomes in the resulting oocytes or sperm and therefore monosomy or trisomy in the resulting embryo (Angell, 1997; Cupisti *et al*., 2003) (figure 5).

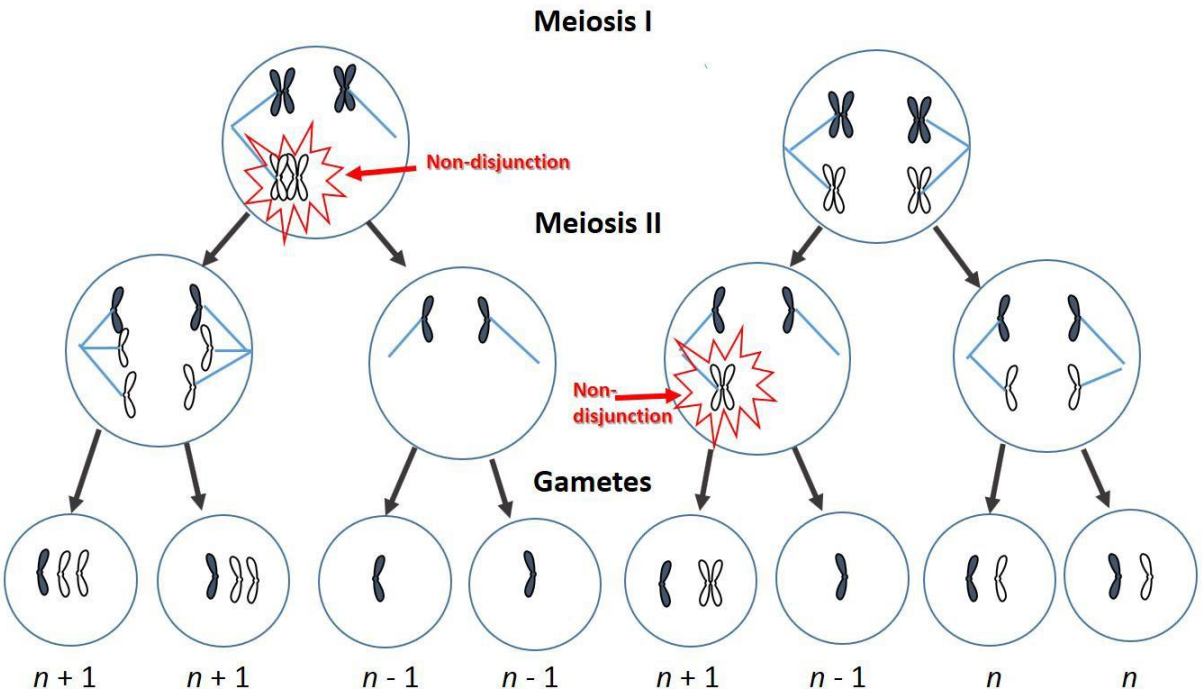


Figure 5: showing non-disjunction at both meiosis I and II, resulting in the formation of aneuploid and euploid gametes

1.4.1.2 Anaphase lag

If a chromatid is delayed in its' movement during anaphase and fails to attach to the spindle with it's' chromosome group, it may lag behind the other chromosomes and sometimes gets lost. The rogue chromosome or chromatid becomes incorporated into a micronucleus and eventually degrades (Coonen *et al*., 2004). Anaphase lag results in a monosomy only (figure 6).

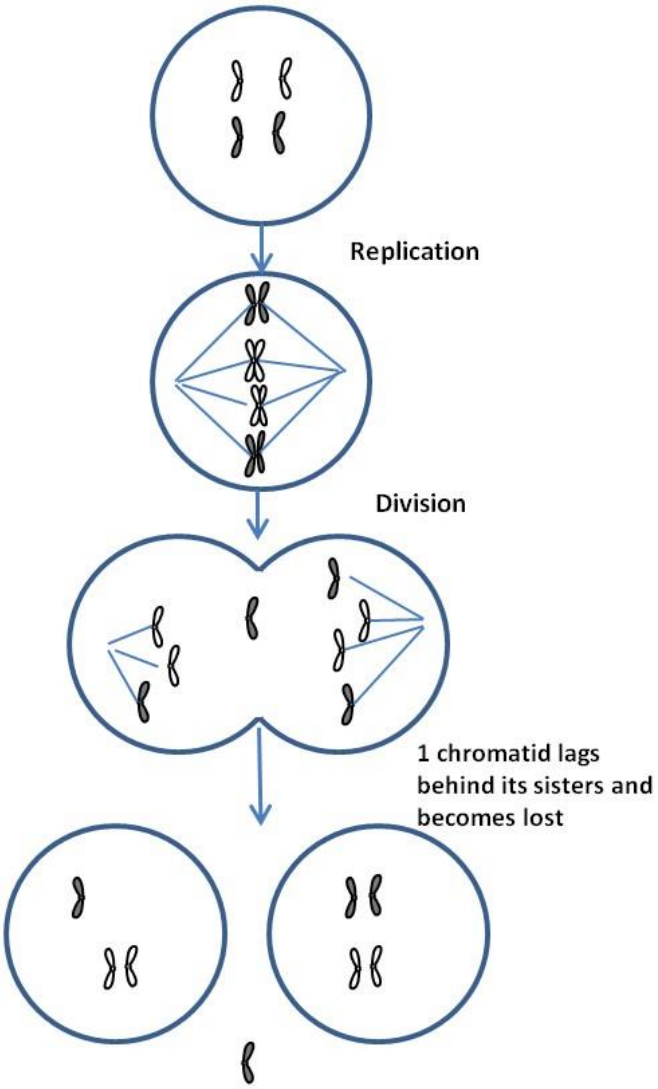


Figure 6: Anaphase lag: showing the loss of one chromosome as it fails to attach to the spindle and is then lost during cell division.

1.4.1.3 Precocious separation of sister chromatids (PSSC)

Precocious separation of chromatids is when the chromatids separate at the wrong point in the cell cycle (figure 7).

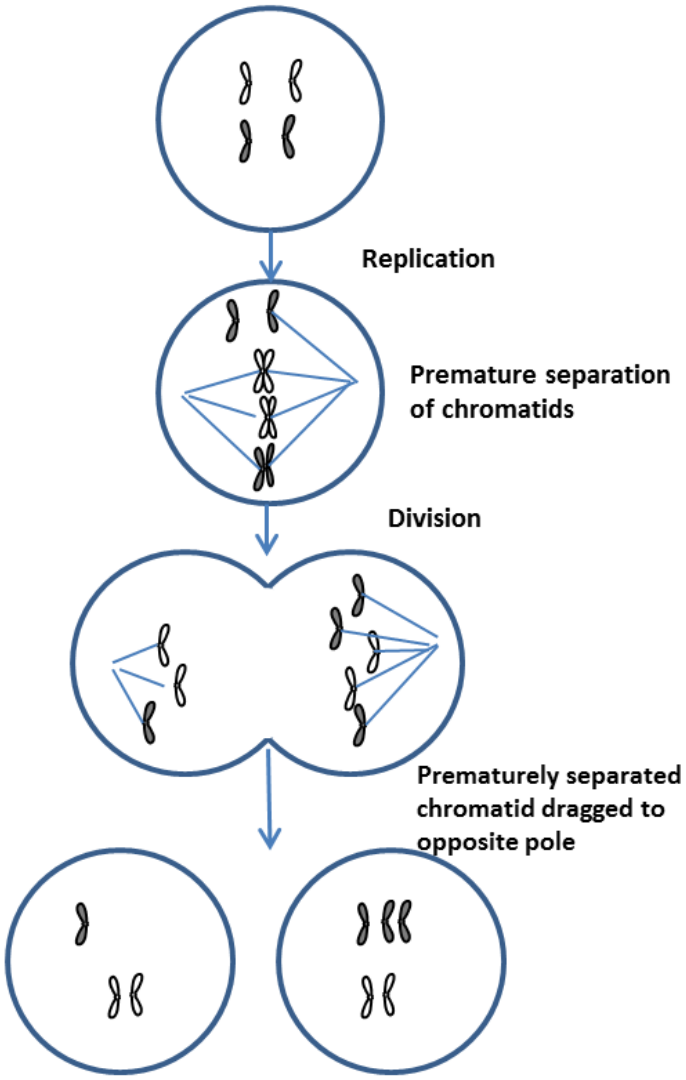


Figure 7: Example of PSSC resulting in a monosomic and trisomic cell

Precocious separation of sister chromatids appears to account for 11 times more chromosomal defects in oocytes than non-disjunction (Gabriel *et al*., 2011). PSSC occurs primarily because

cohesin proteins which are responsible for keeping sister chromatids together begin to degrade to some extent over time (Revenkova and Jessberger, 2005). Degrading cohesin proteins result in sister chromatids falling apart before their allotted time in the meiotic process leading to random segregation and age related aneuploidy (Yun *et al*., 2014). There may also be an increase in defects of other parts of meiosis occurring with advanced maternal age including the formation and stabilization of chiasmata and the spindle assembly checkpoint (Handyside, 2012).

1.4.2 Oocyte aneuploidy

Oocytes are formed during the fetal phase of development beginning around week 10. The process of primary oocyte formation resulting in several million primordial follicles is complete by around the 5th month of pregnancy (Wallace and Kelsey, 2010). There is then a prolonged resting phase in oogenesis which ends shortly before ovulation. Oocytes in the form of primordial follicles can remain in the ovary for up to 50 years. No other cell in the human body remains viable for so long. The average maximum population at 18-22 weeks post conception of primordial follicles is around 300,000, dropping to 180,000 at 13 years old, 65,000 at aged 25, 16,000 at age 35 and at menopause (average age 49) there may only remain around 1000 primordial follicles in the ovaries.

1.4.2.1 Advanced maternal age and its effect on oocyte aneuploidy

The maternal effect on oocyte aneuploidy is well documented. As maternal age increases so does the aneuploidy rate in preimplantation embryos (Franasiak *et al*., 2014b; Harton *et al*., 2013;

Hassold *et al*., 1980; Munne *et al*., 2007a). As described in the section above, the cell that is a precursor to a mature oocyte is formed *in-utero* and has remained dormant from 11 to 50 years prior to each primordial follicle being recruited to produce a mature oocyte. Therefore, a likely explanation of the increase in aneuploidy with maternal age is due to the degradation of bonds between chromatids over time and therefore the disruption of the cell cycle (Duncan *et al*., 2012).

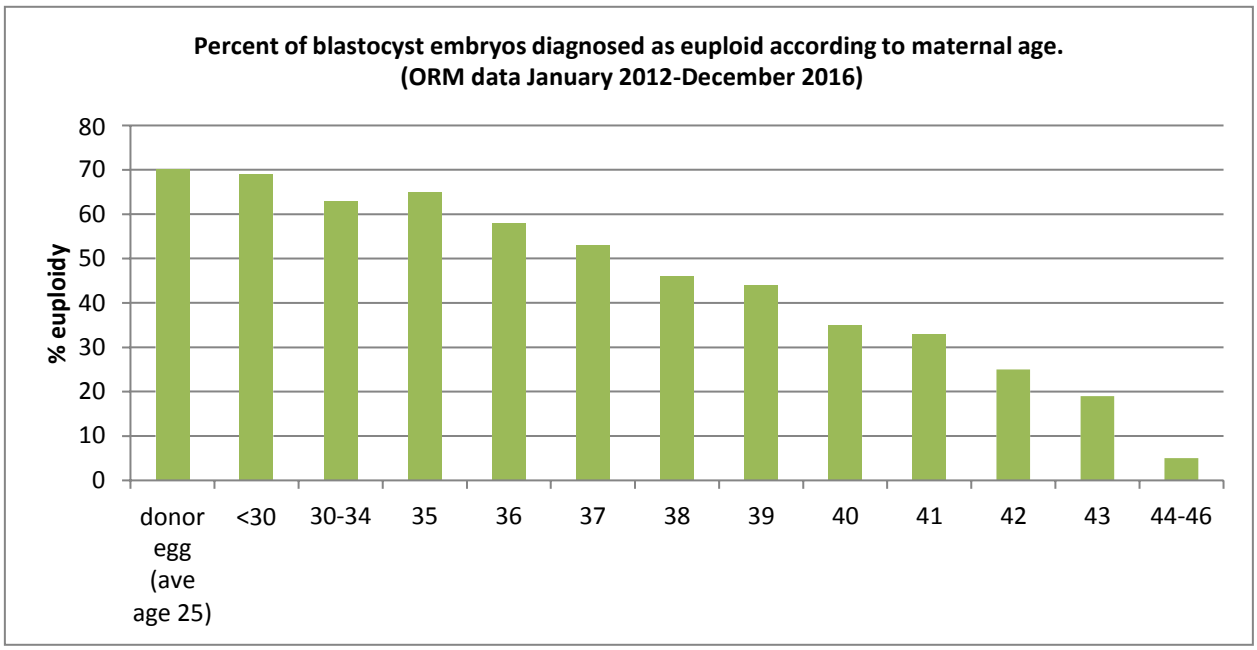


Figure 8: Graph showing the effect of advancing maternal age on the percent of embryonic aneuploidy (own data)

1.4.2.2 Environmental effects on oocyte aneuploidy

Aneuploidy can be initiated by environmental influences on the developing oocyte. Patricia Hunt's group (Hunt *et al*., 2003), discovered an increase in meiotic aneuploidy in murine oocytes after inadvertent exposure to 2,2-(4,4-dihydroxy-diphenol)propane or Bisphenol A (BPA) which

is an estrogen mimicking compound used in the manufacture of poly-carbonate plastic products and resins, such as those used to line cans containing food and beverages and also used in dental sealants. This compound disrupts meiosis by promoting non-disjunction resulting in a dramatic increase in aneuploidy. Hunts' group postulated that the same impact could be seen in human reproduction and this work contributed to the current awareness of the damage that BPA has the potential to do with extended exposure. Many plastics are now manufactured without BPA because of this work.

A study in 2012 (Ehrlich *et al*., 2012), investigated serum BPA levels in patients undergoing IVF and found that higher levels of serum BPA correlated directly with reduced numbers of developing oocytes and suboptimal embryonic development. Other estrogenic imitators or carcinogenic compounds in the environment have the potential to disrupt meiotic or mitotic division in similar ways to BPA (Mandrioli *et al*., 2016).

1.4.3 Sperm aneuploidy

Sperm are produced in the coiled seminiferous tubules of the testis. They are constantly generated from precursor cells (germ/stem cells) which line the tubules. Approximately 120 million sperm are produced per day (equivalent to making about 1200 sperm per heartbeat). This process of spermatogenesis has been shown by Turek *et al* to take approximately 64 days in humans. http://theturekclinic.com/services/male-fertility-infertility-doctor-treatments-issues-

zero-sperm-count-male-doctors/spermatogenesis-production/. A review by Amman in 2008, showed that spermatogenesis reaches completion within 64-80 days (Amann, 2008).

1.4.3.1 Advanced paternal age and its effect on sperm aneuploidy

Because sperm cells are created constantly, increasing paternal age does not have the same dramatic effect on sperm ploidy status as advanced maternal age has on the chromosome complement of oocytes, however a study by Griffin *et al* in 1995 (Griffin *et al*., 1995) did show a direct association with increased sperm disomy for the sex chromosomes and increasing paternal age. Precocious separation of chromatids is not common in sperm due to the constant renewal of sperm cells, however it has been observed but not as frequently as within aging oocytes (Uroz *et al*., 2008).

A 2011 review of the literature on the paternal age effect on sperm aneuploidy by Fonseka and Griffin (Fonseka and Griffin, 2011) had conflicting conclusions from many studies reviewed. They concluded that there is some evidence to support the existence of a paternal age effect on sperm disomies of chromosome 1, 9, 18, 21, X and Y, although other chromosomes (3, 6–8, 10–14, 17) do not seem to have age-related patterns. A more recent review of the literature by Sagi-Dain in 2015, concluded that advanced paternal age is not associated with a reduction in pregnancy rates. The review considered only studies using donor oocytes to limit the well documented maternal age effect (Sagi-Dain *et al*., 2015).

Data generated from blastocyst biopsy cases in our own lab concur with the conclusions of the Sagi-Dain data and figure 9 shows the difference quite dramatically when analyzing overall euploidy rates of embryos using only young donor oocyte cases to illustrate the effect of paternal age compared to embryos created with sperm from men less than 40 years old with various maternal ages.

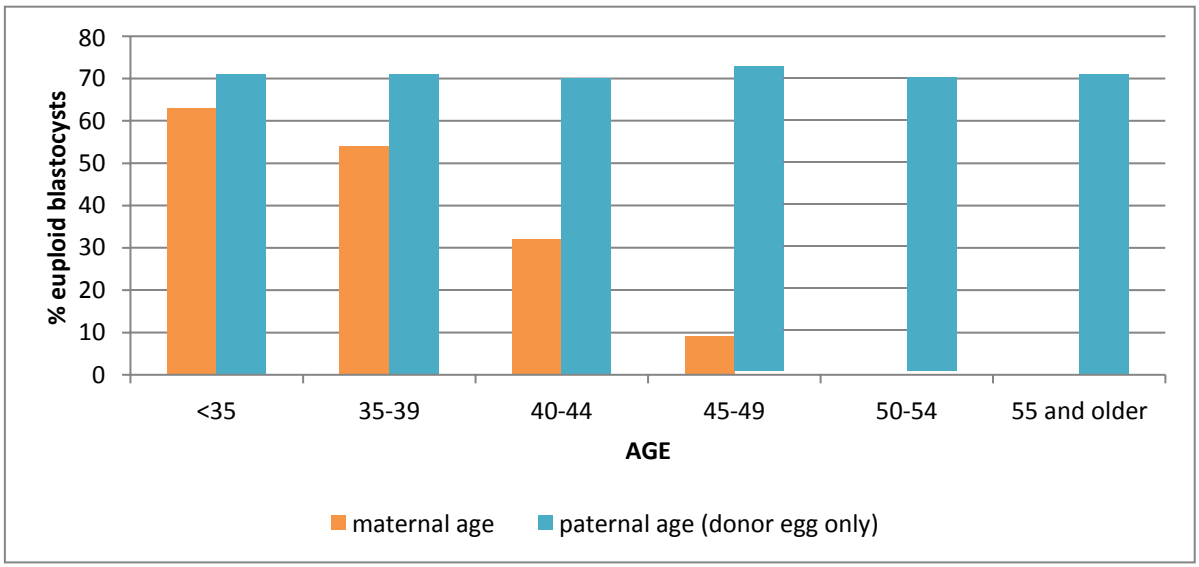


Figure 9: ORM own data showing the difference in paternal age effect on embryonic aneuploidy compared to maternal age effect

1.4.3.2 Sperm quality and sperm aneuploidy

The incidence of aneuploidy in sperm of male factor infertility patients however has been well documented (Bernardini *et al*., 2005; Griffin and Finch, 2005; Ioannou and Griffin, 2011; Tempest and Griffin, 2004). Patients with poor semen parameters are more likely to have a higher ratio of aneuploid to euploid sperm in their ejaculates than patients with normal semen parameters. The mechanism of sperm aneuploidy is the same as oocyte aneuploidy, with non-disjunction,

anaphase lag or precocious separation of chromatids leading to missing or extra chromosomes in the gamete cell.

1.4.3.3 Environmental effects on sperm aneuploidy

Exposure to known estrogenic imitators or carcinogens such as Bisphenol-A (BPA) has been shown to have a negative effect on ploidy status of sperm. As BPA is used in the manufacture of thermal paper used in receipts, cashiers were targeted as a group to study to see if dermal transfer during receipt handling increased levels of urinary BPA (Lv *et al*., 2017). Male cashiers were studied and were found to have 3 times the pre exposure levels of urinary BPA after handling receipts compared to urinalysis prior to exposure. As BPA has been previously shown to be a meiotic disruptor (Hunt *et al*., 2003) resulting in non-disjunction errors in gametes it has been assumed that sperm aneuploidy rates could be affected by BPA exposure. Other compounds such as pyrethroids (Radwan *et al*., 2015) used in agriculture are associated with increased sperm aneuploidy and phthalates, used as plasticizers in many products in have been shown to affect sperm quality (Pant *et al*., 2011).

1.4.4 Embryonic (post zygotic) aneuploidy

Most embryonic aneuploidy originates from the combination of genetic material from aneuploid gametes, aneuploidy oocytes, sperm or both. However, an embryo can originate from a euploid sperm and euploid oocyte creating a euploid zygote and then become aneuploid during subsequent mitotic division. Non disjunction or anaphase lag (section 1.4.1) can occur post fusion of gametes in individual cells during mitosis, creating embryos that have multiple different cell lines. These different cell lines can be a combination of euploid/aneuploid or aneuploid/aneuploid mosaics. With euploid/aneuploid mosaics there is potential for the aneuploid or euploid cells to dominate embryonic development and for the developing fetus to have one or both cell lines. The earlier the error occurs post fertilization, the higher the percentage of the embryo that will be abnormal and in fact, if only the aneuploid cells proliferate, in theory the embryo could present as 100% aneuploid.

1.4.4.1 Advanced maternal age and its effect on embryonic aneuploidy

As maternal age increases so does the rate of occurrence of full aneuploidy (Harton *et al*., 2013; Munne *et al*., 2007a) caused by errors during meiotic divisions during oocyte formation (section 1.4). Embryonic (post zygotic) aneuploidy which gives rise to mosaicism does not increase with advancing maternal age as illustrated in figure 10, using data from 6690 blastocysts from 955 PGS cycles – January 2016-March 2017 (own unpublished data).

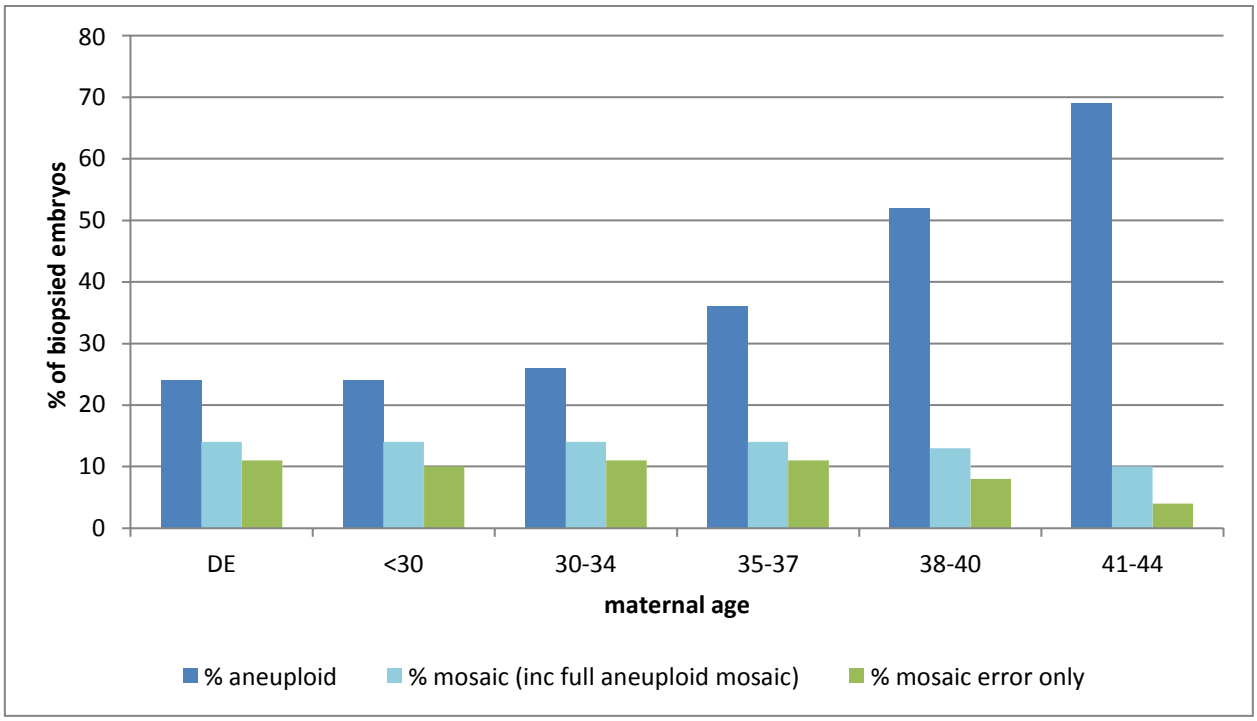


Figure 10: showing how full (meiotic) aneuploidy rates increase with advancing maternal age but mitotic mosaic aneuploidy does not.

1.4.4.2 Environmental effects on embryonic aneuploidy

It has been postulated that post zygotic aneuploidy could be caused by suboptimal environmental conditions during *in-vitro* culture. Potential environmental *in-vitro* stresses include high or low pH of media, temperature or osmolarity, high oxygen concentration, toxins in culture media or oil, presence of volatile organic compounds in the lab air, embryo toxic plastic ware, toxins in gas supply to incubators.

Terry Hassold's group in 2002 (Bean *et al*., 2002) noted that mosaicism due to non-disjunction or anaphase lag occurring during mitotic division, increased in mouse embryos cultured in 5% CO_2

and room air. When O2 levels were decreased to 5% the mosaic rate decreased to *in-vivo* levels. This study illustrated that changes to culture parameters can affect ploidy status of embryonic cells and could be extrapolated to the human model.

Significantly variable rates of embryonic mosaicism were found in 4 different IVF centers in a study by Munne *et al* (Munne *et al*., 1997). They examined early cleavage frozen-thawed embryos donated for research. In one clinic the mosaic rate increased in embryos created after 1991 which could have correlated with a change in culture methods in the lab or an introduction of a new micromanipulation technique creating non- disjunction or anaphase lag during mitosis. Any stress placed on the embryo during its *in-vitro* development has the potential to disrupt mitosis and create aneuploidy in those cells (Swain, 2010; Swain *et al*., 2016). Hickman *et al*, performed a sibling oocyte study where half of each patients embryos were grown in one culture media (group 1) and the other sibling embryos were grown in a different culture media (group 2) (Hickman C. , 2016). Embryos grown in group 2 reached the blastocyst stage quicker than in group 1 and the rate of euploid embryos was double in group 2 (29%) compared to group 1(16%) (p=0.02). This study illustrates beautifully that variations in culture conditions has the potential to affect embryonic (post zygotic) aneuploidy.

1.5 How do we detect aneuploidy in pre-Implantation embryos?

In order to screen for aneuploidy embryonic DNA has to be obtained and analyzed. Embryo biopsy involves removing whole cells from an embryo to analyze nuclear DNA however a novel hypothesis is currently under investigation to establish if cell free DNA expressed by embryos *in-vitro* can be detected and reliably represent the embryo proper. This cell free DNA can be found in the blastocoel cavity and spent culture media.

1.5.1 Non-Invasive PGS detecting cell free DNA in spent culture medium

A proof of concept study by Shamonki *et al* (Shamonki *et al*., 2016) was published in 2016 to investigate if cell free DNA could be detected in spent culture medium and if so did this correlate with the ploidy status of the corresponding embryo. They cultured 57 embryos hatched on day 3, in 15ul drops from day 3 -5/6 and then removed the embryos, biopsied and froze the embryos and attempted to amplify any cell free DNA that had been released by the embryo into the media. 55/57 of the samples had detectable DNA levels from 2-642ng/ul. 6 of the samples that had the highest level of DNA (from 52-642 ng/ul) were tested with aCGH. All 6 gave a result but only the embryo with the highest amount of DNA at 642ng/ul gave a reliable result. This corresponded with the trophectoderm biopsy of 45XY -13. Another of the samples did correlate with the trophectoderm biopsy but the result could not be classed as reliable. The amplification method used in this study was Repli-G single cell kit (Qaigen) which is an MDA platform, and the products were analyzed with aCGH.

Xu *et al* (Xu *et al*., 2016) performed a study analyzing spent media for cell free DNA and completed the first clinical transfers of embryos to patients based on the spent media DNA analysis alone. They termed the method Non-Invasive Chromosome Screening or NICS. They carried out an initial study establishing concordance between biopsy results and NICS. To validate their results, 43 embryos were cultured from day 3 to day 5 and the spent media drops and biopsy samples were analyzed concurrently. 21 embryos diagnosed as euploid from the biopsy had a concurrent result from the NICS.16 embryos diagnosed as aneuploid from the biopsy had an aneuploid result from the NICS (although only 6/16 had the exact same losses or gains). There were 2 false negative results where the NICS was euploid and the biopsy was aneuploid and 4 false positives where the NICS was aneuploid and the biopsy was euploid. 86% of the embryos therefore had a concurrent result between the biopsy and the NICS and 14% of embryos had a non-concurrent result overall. Their clinical application involved 7 patients and 9 embryo transfers replacing embryos solely diagnosed as euploid by NICS. The 9 transfers resulted in 6 live born allegedly chromosomally normal babies (authors stated that a karyotype had been performed). The DNA amplification method used in this study was the MALBAC (Multiple Annealing and Looping Based Amplification Cycles (Zong *et al*., 2012)) WGA method and the products were analyzed on an Illumina HiSeq NGS platform.

Wga

A recent study by Feichtinger *et al* (Feichtinger *et al*., 2017) biopsied both polar bodies post fertilization of 22 embryos reaching the blastocyst stage. They analyzed the pooled PB DNA with

aCGH and grew the resulting embryos in single 25ul drops, to day 5-6 in a single step medium. They sampled 5ul of spent media at the blastocyst stage. Eighteen of 22 samples amplified and achieved concurrent aneuploidy/euploid results in 13 of 18 samples (72%). Cell free DNA is fragmented in segments of around 200bp in size (Fan *et al*., 2010). Traditional amplification methods may not be as accurate in amplifying these smaller fragments and may be the cause of incongruous results between nuclear DNA and cell free DNA. An alternative modified MDA based amplification method is currently being investigated and preliminary results from our own laboratory are described in section 2.1.2 of this thesis.

1.5.2 Blastocoel fluid biopsy ("Blastocentesis")

As an embryo progresses beyond the multicellular stage and 2 separate cell lines begin to form (inner cell mass and trophectoderm) the embryo begins to blastulate. The proliferation of the trophectoderm cell layer is directly correlated with an increase in the production of intracellular ATP which powers sodium ion pumps in the trophectoderm layer (Houghton *et al*., 2003). An increase of Na ion concentration on the inside of the trophectoderm layer instigates the intake of water by osmosis across the membranes leading to an expansion of the blastocoel cavity.

Artificial blastocoel collapse before cryopreservation has been shown to improve survival of blastocysts post thaw by reducing the formation of ice crystals during freezing/vitrifying (Darwish and Magdi, 2016; Van Landuyt *et al*., 2015). Because of this evidence, many IVF labs have adopted this process into routine practice either by the use of an ICSI pipette aspirating fluid from the

cavity or by a laser pulse applied to the trophectoderm layer. As blastocoel fluid is being routinely removed, it was proposed that this fluid could be collected and analyzed for the presence of cell-free DNA in the hope that this could be used to determine ploidy status of the blastocyst.

Several groups have attempted to aspirate blastocoel fluid to assess the DNA complement and potentially correlate that with the karyotype of the embryo (Gianaroli *et al*., 2014; Magli *et al*., 2016; Tobler *et al*., 2015). Tobler *et al* (Tobler *et al*., 2015) analyzed the blastocoel fluid from 96 donated embryos to compare cell-free DNA in the fluid with the ploidy status of the ICM or trophectoderm. They were only able to read the DNA complement in 68% of the embryos and with the ones that resulted in a read they found a high level of discordance between the fluid composition and nuclear DNA. Sureplex amplification was used with aCGH for all of these studies, the combination of which has been postulated to be suboptimal for cell free DNA analysis due to the small sized fragments of DNA (see cell-free DNA section 1.5.1). Discordant results between the fluid and trophectoderm biopsy may be due to errors in amplification of the smaller cell free DNA segments than nuclear DNA from a biopsy. The issue of suboptimal amplification of these small sections may be overcome by alternative amplification methods currently under investigation (Wells *et al* 2017 unpublished). The blastocoel cavity often contains small fragments or whole cells that are not incorporated into the embryo itself (figure 11).

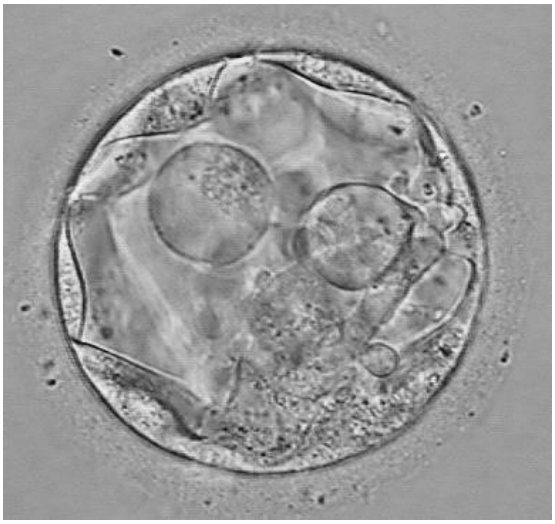


Figure 11: Blastocyst with free cells in the blastocoel cavity, unincorporated in the blastocyst proper (own observation).

If these are aspirated with the fluid and analyzed this may be a reason for discordant results between the fluid and biopsy. For this reason and the technically challenging nature of the process in a clinical setting, blastocentesis may not be a viable alternative to trophectoderm biopsy to determine ploidy status of an embryo.

1.5.3 Embryo Biopsy

Biopsy is possible at different pre-implantation developmental stages as a means of determining the aneuploidy status of the embryo but also for preimplantation genetic diagnosis (PGD) of single gene disorders.

- Polar body biopsy
- Blastomere biopsy
- Trophectoderm biopsy

Each stage has its advantages and disadvantages and these are summarized below.

1.5.3.1 Polar body biopsy

Polar bodies (PB) are created during meiosis and are extruded from the oocyte is it matures (PB1) and is subsequently fertilized (PB2). Removal of these packets of DNA and analyzing the DNA complement is a relatively non-invasive technique for establishing ploidy status as the oocyte or zygote itself remains intact. In a proof of principle study by Geraedts (Geraedts *et al*., 2011) both polar bodies were biopsied from 226 zygotes and their chromosome complements were analyzed using aCGH. Predictive ploidy status was established in 86% of these zygotes.

Polar bodies are not always discrete in structure and it is not uncommon to find a fragmented polar body which may or may not contain all of the chromosomal material that has been extruded from the oocyte. Biopsy of the first polar body alone has been shown to be poorly predictive of

ploidy status of the resulting biopsy but predictive of the oocytes potential to reach the blastocyst stage (Grifo *et al*., 2015a).

Polar body biopsy is useful in countries which allow limited numbers of embryos to be grown past the zygote stage such as Germany. The advantage of PB biopsy is that it is relatively non-invasive compared to day 3 or blastocyst biopsy.

1.5.3.2 Day 3 (blastomere) biopsy

One or 2 cells are removed on day 3 of development and analyzed for chromosome complement. In theory, the whole embryo should be represented by one cell, unless the embryo is mosaic. Hanson *et al* (Hanson *et al*., 2009) reanalyzed 199 embryos diagnosed using FISH as chromosomally abnormal or with no result post day 3 biopsy 34% of the embryos diagnosed as aneuploid had at least one cell that had the same original diagnosis. 96% of the embryos diagnosed as aneuploid were confirmed as being aneuploid but not concurrently with the original diagnosis.

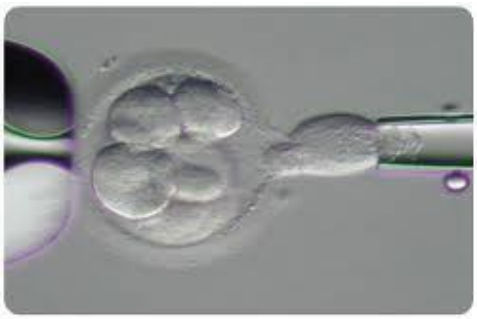


Figure 12: biopsy of single blastomere from day 3 embryo

All cells on day 3 are not necessarily incorporated into the blastocyst itself. Frequently rogue cells are seen on day 5 and 6 outside of the blastocyst itself inside the *zona pellucida*. These cells are not part of the embryo and remain behind in the *zona pellucida* as the embryo hatches out (figure 16).

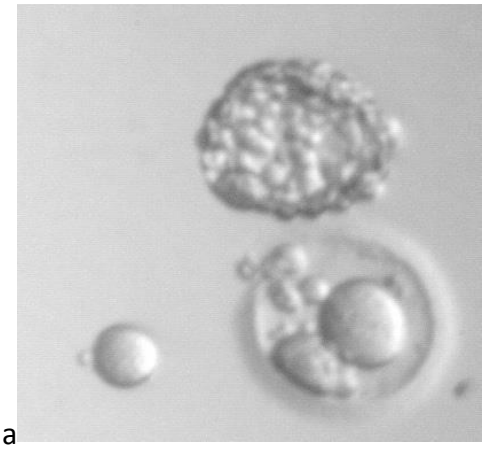
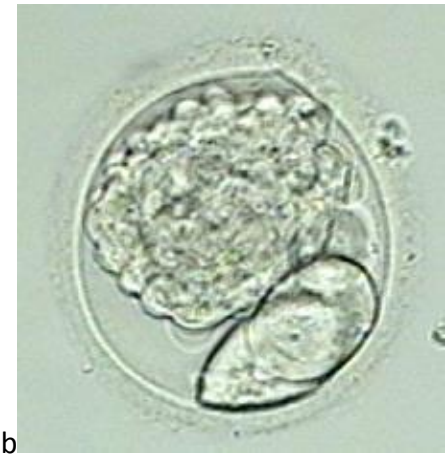
a  b 

Figure 13: examples of cells not incorporated into the blastocyst proper which could be sampled on day 3 and may not represent the embryo. a) Completely hatched embryo showing cells not incorporated into the blastocyst left behind in the discarded zona post hatching
b) Thawed embryo still in zona with large cell outside of the blastocyst.

To remove cells on day 3 may mean testing cells that would never have become part of the final embryo. To use information from these cells to assess the chromosomal complement of the embryo can give rise to a false diagnosis. Barbash-Hazan *et al* (Barbash-Hazan *et al*., 2009) showed 18% of embryos diagnosed on day 3 as aneuploid were mosaic and by day 5 10% of the original cohort had self–corrected. Northrop *et al* (Northrop *et al*., 2010) re-analyzed 50 blastocysts previously diagnosed as aneuploid on day 3 using FISH, and found that that 58% of these embryos were diagnosed as euploid. They concluded that cleavage-stage FISH technology is poorly predictive of aneuploidy in morphologically normal blastocysts.

Disadvantages of day 3 biopsy are that removal of one or two cells on day 3 has been shown to be detrimental to embryo growth and development. The rate of growth is slowed post biopsy (Kirkegaard *et al*., 2012; Tarin *et al*., 1992) the dynamic sequence of events is disrupted by blastomere biopsy therefore reducing implantation and pregnancy rates (Bar-El *et al*., 2016; Scott *et al*., 2013c).

The only perceived advantage of day 3 biopsy are that embryos can be grown out to day 5 post biopsy and transferred fresh to the uterus. Fresh transfer in a stimulated uterus however may not be an optimal environment for embryos to thrive (Bhattacharya, 2016; Maheshwari and Bhattacharya, 2013; Roque *et al*., 2015; Shapiro *et al*., 2011).

1.5.3.3 Trophectoderm biopsy

Around half of the oocytes that fertilize typically arrest after day 3. An embryo that develops to the blastocyst stage has begun to differentiate into 2 distinct types of cells; trophectoderm (placenta and membrane precursor cells) and inner cell mass (ICM) (fetal precursor cells).

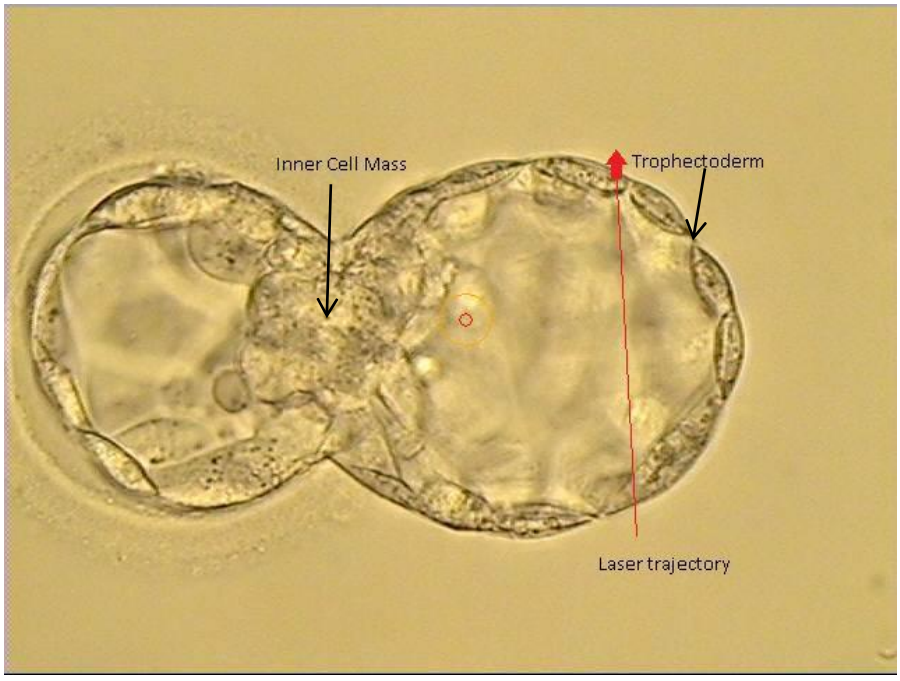


Figure 14: Blastocyst hatched on day 3 to allow trophectoderm cells to extrude, cells removed utilizing a laser on day 5.

The trophectoderm layer can be biopsied and tested for ploidy status avoiding the inner cell mass. The trophectoderm has to be sufficiently expanded to allow the removal of several cells (approximately 3-10) for analysis. Cells for testing are removed using laser pulses to separate the cell junctions (figure 17). The excised piece of tissue is placed in a microfuge tube until DNA amplification and the embryo is placed back in culture to recover before transfer or freezing.

Embryos seem to recover very well from the biopsy procedure itself, self-sealing the trophectoderm layer and re-expanding the cavity within an hour (personal experience). Long term effects of trophectoderm biopsy on offspring have not been studied. Embryos that have been biopsied implant at high rates when transferred to the uterus. Ongoing pregnancy rates are also high and therefore the short term disruption of the trophectoderm layer during the biopsy seems to have no detrimental effect on pregnancy rates.

A study in 2012 (Olcha *et al*.), showed lower initial BhCG levels from singleton pregnancies resulting from embryos that had been biopsied at the blastocyst stage compared to singleton pregnancies resulting from un-biopsied embryos. The study demonstrated that while the initial levels were low they eventually caught up with the non-biopsied pregnancies and birth weights and gestational age at birth were the same for the 2 groups. This is not a surprising finding as we are removing a part of the trophectoderm for testing.

Disrupting the cell layer in theory could have a negative effect on the embryo's polarity during implantation. Placentation may be affected resulting in a higher rate of abnormal placenta placement or cord insertion which is already observed at a higher rate in IVF pregnancies compared to naturally conceived pregnancies (Englert *et al*., 1987; Healy *et al*., 2010). This supposition has yet to be studied, however this was outside the scope of this thesis.

Trophectoderm biopsy has become a widely used technique in clinical IVF laboratories for the detection of aneuploidy, single gene defects and chromosomal imbalances (Capalbo *et al*., 2016a; Chen *et al*., 2011; Kokkali *et al*., 2005).

1.6 The evolution of PGS

The original purpose of PGD was to identify embryos affected with a particular single gene defect from families with a known heritable disease. An unaffected embryo would be selected and transferred to the uterus resulting in a child without the particular disease.

Research began in the UK in the 1980s into methods to successfully diagnose single gene defects in pre-implantation embryos (Handyside *et al*., 1989; Penketh *et al*., 1989; Winston *et al*., 1989). Couples who carry or are affected by diseases caused by specific genetic mutations have previously had the option of choosing to terminate an affected fetus or giving birth to an affected child after positive identification of the defect at amniocentesis or CVS months into the pregnancy. Researchers used donated spare embryos left over from IVF cycles to establish the techniques of removing one-two cells from day 3 embryos, amplifying the DNA and using PCR to identify the mutation.

The first applications were to avoid X-linked disease by sexing embryos and only transfer female embryos to the uterus. Sexing of human embryos was successfully carried out in 1989 identifying the Y chromosome via Florescent In-situ Hybridization (FISH) (Griffin *et al*., 1991; Griffin *et al*., 1992; Handyside *et al*., 1989; Penketh *et al*., 1989). The first clinical PGD cycles were performed to sex select embryos in 7 couples with X-linked mutations. One couple carried a mutation for adrenoleukodystrophy, one couple X-linked mental retardation and the other 5 couples were all at risk of transmitting other X-linked diseases to male offspring. Seven cycles of

IVF/PGD resulted in 5 live birth events; 4 sets of female twins and one singleton female (Handyside *et al*., 1990; Hardy and Handyside, 1992).

The ESHRE PGD consortium began collecting valuable follow up data on PGD and PGS cycles in 1997. The first data collection rounds I-III, collated data from 787 cycles which were all day 3 biopsies. PGS was mainly limited to patients of advanced maternal age at this time. During the first 10 years of data collection summarized in a paper by Harper and Harton (Harper et al., 2012) data had been collected from 89,373 biopsied embryos and 21,543 transferred embryos. Both day 3 and day 5 biopsies were included in the data sets over this time period but the majority of cycles considered were day 3 biopsy and FISH or aCGH. In 2017 many laboratories at least in the USA, are using blastocyst biopsy and NGS for ploidy determination, therefore the conclusions of the data collections by the ESHRE consortium are not applicable to the latest methodology in use today.

1.6.1 Specific single gene mutation detection

Identification of a specific single gene defect in embryos created from parents with known family histories of a particular inherited genetic mutation was successfully carried out in 1992 (Handyside *et al*., 1992). Day 3 embryos were biopsied and the single gene mutation, p.Phe508del (delta F508), responsible for cystic fibrosis, was identified in time for a transfer of a non-affected embryo to the patient's uterus on day 5 of development. Three couples where both parties were carriers for the mutation underwent IVF to create multiple embryos for testing. Embryos from each couple were biopsied on day 3 with 1-2 cells being removed from a slit in the *zona pellucida*.

The cells were lysed and the DNA amplified and the mutation was identified using PCR. Unaffected or carrier embryos were transferred to the uterus of 2 of the patients resulting in a live birth of one single unaffected child.

Single gene mutation identification has been revolutionized in the last few years by the development of a SNP array based chip technology called Karyomapping (Handyside *et al*., 2010). This technology does not require extensive test preparation to establish linkage for each mutation but is an off the shelf chip that comes with over 30,000 SNPs specifically chosen as they are near known mutation sites. Parental DNA is required to establish linkage and the location of the mutation has to be known.

1.6.2 Aneuploidy detection

Aneuploidy detection was borne out of sex selecting embryos for preventing sex linked disease and the number of chromosomes identified has increased with the use of advancing genomic technology.

1.6.2.1 Fluorescence *In-situ* Hybridization (FISH)

Aneuploidy detection in preimplantation embryos was initially limited to a restricted number of chromosomes utilizing FISH. FISH was first applied to preimplantation embryos to detect the sex chromosomes (Griffin *et al*., 1991). Overtime, several improvements increased the efficiency of the test, such as scoring criteria (Munne *et al*., 1998b) and fixation techniques (Velilla *et al*.,

2002); however, the most beneficial improvement and was the increase in number of chromosomes screened. This was achieved in 1993 (Schrurs *et al*., 1993) by autosomal chromosome specific detection in embryos using one probe to detect chromosome 18 and then multiple probe FISH labeling chromosomes 18, X and Y by Munne *et al* (Munne *et al*., 1993). The number of chromosomes that were able to be identified from a single cell increased up to a limit of 12 chromosomes (Munne *et al*., 1998a). However, it still remained that FISH was limited to screening less than 24 chromosomes. The highly skilled nature of the technique to fix cells and the advancement of multi-celled sampling at the blastocyst stage, contributed to the decline in FISH usage in clinical IVF cases.

1.6.2. 2 Quantitative-Polymerase Chain Reaction (qPCR)

This technique involves a direct PCR amplification on the sample, rather than whole genome amplification of the DNA sample, therefore reducing the chance of allele dropout. The turnaround time of the assay is only 4 hours making it an attractive method for clinical use (Treff *et al*., 2012). The number of data signals is sufficient to count whole chromosomes; however, segmental aneuploidies from translocations require additional targeted assays, which itself involves time, and additional validations. The test is offered by several clinical groups resulting in improved clinical outcomes compared to using embryos with unknown ploidy status (Forman *et al*., 2013a; Forman *et al*., 2012a; Forman *et al*., 2012b; Scott *et al*., 2012).

1.6.2.3 Whole Genome Amplification

Sampling single (day 3 biopsy) or between 3-10 cells (blastocyst biopsy) from an embryo is challenging because of the small amount of original DNA available for analysis. Some platforms for pre-implantation genetic screening (PGS) (aCGH, CGH, NGS and SNP arrays) require further amplification of the DNA before analysis. There are two commonly used WGA methodologies for PGS:

PCR based amplification methods: Primer Extension Pre-amplification (PEP) and Degenerate Oligonucleotide PCR (DOP-PCR). PEP is now most commonly used for PGS for aneuploidy (Sureplex, Illumina). These techniques have been found to exhibit incomplete genome coverage and amplification bias when a sequence is overrepresented in the amplified DNA due to preferential binding of the primers to specific regions but the technique works well for ploidy determination.

Multiple Displacement amplification (MDA) (Repli-g,Qiagen). MDA provides a more uniform amplification across the whole genome and reduces amplification bias compared to PEP PCR. This method is more commonly used for single gene mutation detection rather than ploidy determination.

Novel Amplification method (NAM). This method is a modified version of MDA developed for the capture and amplification of short sections of cell free DNA. It has been used for the spent media study in chapter 2.2.2. The method is currently awaiting patent approval.

1.6.2.4 Comparative Genomic Hybridization (CGH)

Comparative genomic hybridization (CGH) was the first 24-chromosome screening method applied to pre-implantation embryos, polar bodies, and oocytes, carried out by Wells *et al* (Wells *et al*., 2002; Wells *et al*., 1999). WGA was carried out using DOP-PCR. CGH allows the comparison of two DNA samples from 2 different sources, known euploid control and test sample, to establish if they have gains or losses of whole or parts of chromosomes. The method involves isolating DNA from 2 sources, one a known euploid sample, the other the test sample. Each sample is labelled with fluorescent molecules of different colors (usually red and green). The DNA is denatured so it becomes single strands and then is hybridized to a euploid metaphase spread of chromosomes. A fluorescence microscope and computer software are used to compare the colored fluorescent signals emitting from the DNA. If red and green fluorescent molecules are used to label the 2 DNA samples then the presence of both chromosomes will display as yellow. If there is a loss or gain then the color will be red or green. The whole procedure requires around 3-5 days to complete testing making it a clinically challenging time consuming process.

1.6.2.5 Micro-Array Comparative Genomic Hybridization (aCGH)

Micro-Array CGH works on the same principle as CGH, but at a lower cost and a faster processing set up. WGA is again used to create more DNA product for testing. However, where CGH uses a metaphase spread of control DNA for hybridization, aCGH uses an array of spots of DNA attached to a slide each which constitutes around 150,000 bases of unique sequences from a specific chromosome. The amplified sample is hybridized to these small pieces of DNA and the

fluorescent color product indicates loses or gains in each chromosome. A validation study comparing aCGH with PCR and FISH (Gutierrez-Mateo *et al*., 2011) concluded that this method has the same accuracy as m-CGH but with a faster turnaround time. The first clinical utilization of aCGH for PGS was used in 2010 to establish ploidy status in combination with blastocyst biopsy. Illumina's aCGH method uses two sets of DNA (sample/reference) that are uniquely labeled with fluorescent dyes (cy3 and cy5) and subsequently hybridized to bacterial artificial chromosomes (BACs-which are set mapped sequences). The ratio of the intensities of each fluorochrome is computed, and compared to the reference. This is not a direct copy number computation, and therefore mosaicism cannot be interpreted with aCGH without extra bioinformatics.

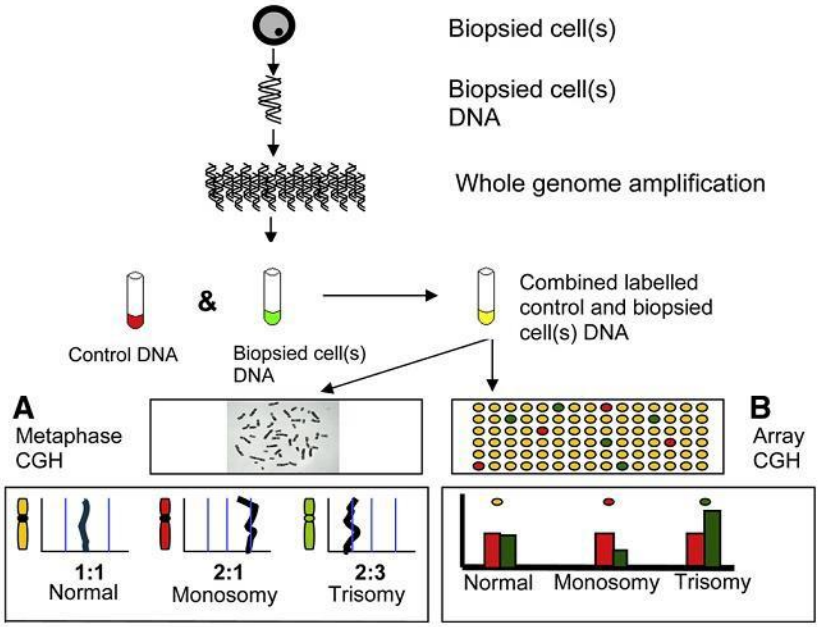


Figure 15: showing the principle of aCGH. by permission of (Harper and Harton, 2010).

1.6.2.6 Single Nucleotide Polymorphism (SNP) arrays

Individual variations contained within the genome, are termed Single Nucleotide Polymorphisms (SNPs). SNPs occur most commonly in non-coding regions of the genome. SNPs found in coding regions of the genome are responsible for traits such as height and eye color or inherited disease causing mutations. In order to find a trait SNP or disease SNP on the genome a comparison has to be made between a genome without the disease or trait causing SNP.

The use of SNP arrays for whole chromosome copy number relies on parental DNA to create SNP markers for each chromosome in the specific region determined for identification of each chromosome during analysis. WGA is required for this technique (Xiong *et al*., 2014). Hetero-zygosity and quantification of the SNP alleles allows for the identification of losses or gains based on parental DNA. SNPs are also able to determine uniparental isodisomy (Handyside, 2011; Treff *et al*., 2011). As of 2010 over six million SNPs had been identified in the human genome (Javed and Mukesh, 2010). SNP array technology for whole chromosome copy number determination, relies heavily on proprietary algorithms to determine probability of losses and gains of individual chromosomes.

1.6.2.7 Next Generation Sequencing (NGS)

The newest method for PGS utilizes a low-pass whole-genome sequencing approach using the Ion Torrent Personal Genome Machine (PGM) (Life technologies)(Kung *et al*., 2015), the MiSeq, NexSeq and HiSeq (Illumina)(Fiorentino *et al*., 2014; Victor *et al*., 2016; Wang *et al*., 2014). The core concepts of next generation sequencing (NGS) is the ability to massively parallel sequence

small DNA fragments, until an appropriate depth of coverage is achieved per sample. This method requires whole genome amplification. An aliquot from the amplified DNA sample is removed for library preparation where the amplified DNA strands are fragmented into small pieces (36-200 bps). Each sample is then ligated with a 5' and 3' unique adapter, with subsequent purification and size selection steps in preparation for sequencing. The principal advantages of NGS is the ability to detect copy number variation allowing for the detection of mosaicism (Munne and Wells, 2017), simultaneous testing of samples reducing the cost per sample, and the potential for future clinical applications, such as simultaneous detection of aneuploidy and monogenic diseases (Wells *et al*., 2014).

1.6.2.8 Summary of available aneuploidy detection techniques

The following table summarizes all the techniques used for counting chromosomes with the number of data signals and details of what can be detected with each method. Each technology has its advantages and disadvantages, and there is currently much debate in the IVF community about which technology is optimal for the determination of aneuploidy and if the high resolution methods used are producing too much information for the purpose of counting chromosomes. The biggest disadvantage that all of these technologies have in common is that the sample size of the biopsy is very small with between 3-10 cells available to analyze.

	FISH	q-PCR	aCGH	SNP array	Ion-Torrent NGS	Veriseq NGS
Total # independant data signals	11	96	2,700	32,000	100,000	700,000
Full chromosome analysis?	No	Yes	Yes	Yes	Yes	Yes
Deletions/Duplications	No	No	Yes	Yes	Yes	Yes
Unbalanced translocations	No	No	Yes	Yes	No	Yes
Parental DNA required	No	No	No	Yes	No	No

Figure 16: Summary of PGS technology evolution.

1.7 Is aneuploidy always unequivocal?

As the number of data signals or reads has increased per chromosome with the evolution of genomic technology, the ability to detect partial deletions and duplications, translocations and mosaic cell lines in the trophectoderm sample is now possible. The detection of mosaicism means that there are now 3 classifications of ploidy status; Euploid, aneuploid and mosaic.

1.7.1 What is a mosaic embryo?

If one or more cells in an embryo at any stage of growth has become aneuploid during mitosis due to PSSC or non-disjunction, there may develop 2 or more distinct cell lines resulting in a mosaic embryo (figure 17).

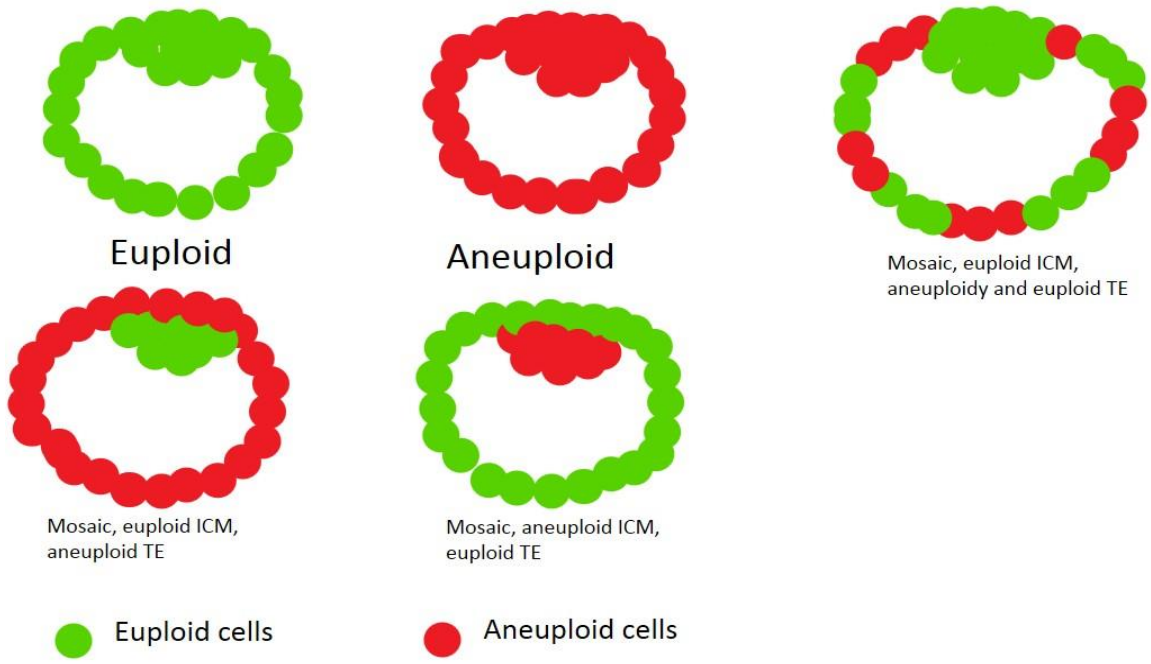


Figure 17: Possible combinations of cell- lines in mosaic and non-mosaic blastocysts (ICM = inner cell mass, TE= Trophectoderm).

1.7.2 Detecting mosaicism in blastocysts

It is not possible to detect mosaicism for PGS in a day 3 embryo which is to be used for transfer as only 1 cell is removed for testing. If this cell is aneuploid or euploid then this will be the assumed diagnosis for the whole embryo. Mosaicism does exist in early cleavage embryos as has been shown by dis-aggregating donated embryos and analyzing all cells (Chow *et al*., 2014). The most important question to ask when we sample cells from the trophectoderm of a blastocyst is are we removing cells that accurately represent the embryo as a whole? A pre-implantation blastocyst embryo consists of approximately 120 cells, around 30 of which are discrete inner cell mass cells and the remainder form the trophectoderm layer (Hardy *et al*., 1989). A blastocyst

biopsy involves taking between 3-10 cells from the trophectoderm layer of the embryo which in theory should be a better representation of the whole embryo than a single cell from a day 3 embryo. However, it is only possible to detect mosaicism in a blastocyst if the biopsy is removed at the junction of euploid and aneuploid cells within the trophectoderm.

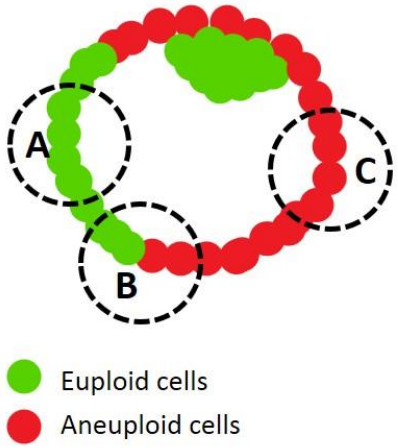


Figure 18: = *possible areas to be biopsied on the trophectoderm layer. This diagram shows that depending on where the biopsy is taken from, this embryo could be classified as euploid (A), mosaic (B) or aneuploid(C).*

Abnormal cells can be distributed through the trophectoderm either in nested groups where the abnormal cells form a discrete area of aneuploidy or a more generalized distribution where aneuploid cells are dispersed throughout the trophectoderm layer (Scott and Galliano). If the aneuploid cells are nested then the likelihood of sampling those cells is more haphazard. If the aneuploidy is more generalized then the sampling may be more representative of the whole embryo.

As the only way to truly realize the extent of mosaicism in a blastocyst is to completely dissect the whole embryo (Taylor *et al*., 2016) which results in the embryos demise, the result from the small biopsy sample has to be accepted.

1.7.3 Rate of mosaicism in blastocysts and in pregnancy

Reported rates of mosaicism in pre-implantation blastocysts vary depending on which platform and assay is used. When Array CGH was used for PGS, mosaicism was observed but not reported and results were classed as aneuploid (over 50% mosaic aneuploidy) or euploid (less than 50% mosaic aneuploidy). If the NGS result profile is clean and artefacts have not been introduced during sequencing or amplification (see figure 28) then a true mosaic result is easy to categorize but if the results are noisy then a mosaic result is more difficult to call and could be attributed to DNA degradation or sample mishandling. Critics of mosaic results argue that there is not enough evidence that these results firstly are fact or artifact and if they are fact what effect these results have on pregnancy outcome and the phenotype and genotype of live born offspring. Mosaic thresholds above and below which an embryo will be classified as mosaic rather than aneuploid or euploid have been established within the PGS community. PGDIS mosaic reporting guidelines (PGDIS 2016), were based on the Illumina platforms (MiSeq, NexSeq) with VeriSeq assay and these thresholds had little clinical data to support their determination. Other NGS platforms such as the Ion Torrent PGM platform will also detect mosaicism but the threshold for classifying a mosaic result is higher than with the Illumina platform. In our own lab, the Ion Torrent platform resulted in isolated mosaic rates of around 10% of all blastocysts screened for aneuploidy compared to 20% with the MiSeq Illumina platform. Both of these rates are higher than the rate seen in ongoing pregnancies. Fetal-placental mosaicism in pregnancy exists and is found during confirmation studies of Chorionic Villus Sampling (CVS) used as a diagnostic screen for aneuploidy at 9-11 weeks of pregnancy. CVS involves 2 stages, direct preparation and long term culture. 1.3%

of CVS cases are discordant from the direct to the long term preparation. The most common discordance being aneuploid placenta (direct preparation) and euploid fetus (confirmed in the long term preparation) (Malvestiti *et al*., 2015; McGowan and Blakemore, 1991).

According to a study of 4000 CVS samples by Stetten *et al*, (Stetten *et al*., 2004) confined placental mosaicism where only part of the placenta is chromosomally different to the fetus, occurs in 0.7% of pregnancies. There are many permutations of placental mosaicism that can exist:

- Mosaicism only in the placenta which does not affect placental function,
- Mosaicism only in the placenta which affects placental function
- Mosaicism in the placenta and the fetus
- Mosaicism only in the fetus but not in the placenta
- Complete fetal/placental discordance with aneuploid fetus, euploid placenta or *vice versa*

1.7.4 Where do the cells of the trophectoderm originate from?

Trophectoderm in a blastocyst gives rise to chorionic ectoderm (Bianchi *et al*., 1993). These cells are not derived from any part of the inner cell mass. A trophectoderm biopsy to screen for aneuploidy only analyzes the precursor cells that give rise to the cells of the placenta.

The Chorionic Villus consists of chorionic ectoderm (derived from trophectoderm) and chorionic mesoderm (derived from the inner cell mass). Once a CVS biopsy is received in the cytogenetics lab, the maternal cells are removed, and the fast dividing cells of the chorionic ectoderm are spread and examined. As these cells are in such a rapid growth phase, many of them will be in metaphase and therefore their chromosomes can immediately easily be seen (Mhairi G.

MacDonald). As these cells are not derived from the inner cell mass they do not necessarily represent the developing fetus. Subsequent long term culture of a CVS biopsy allows multiplication of the slower dividing mesenchymal core cells which are derived from the inner cell mass. Long term culture of the CVS biopsy therefore is a more reliable form of analysis of the fetus itself. As we are limited to sampling trophectoderm from blastocysts, there is the potential for similar misdiagnosis rates if mosaicism is present, as with a direct preparation with CVS.

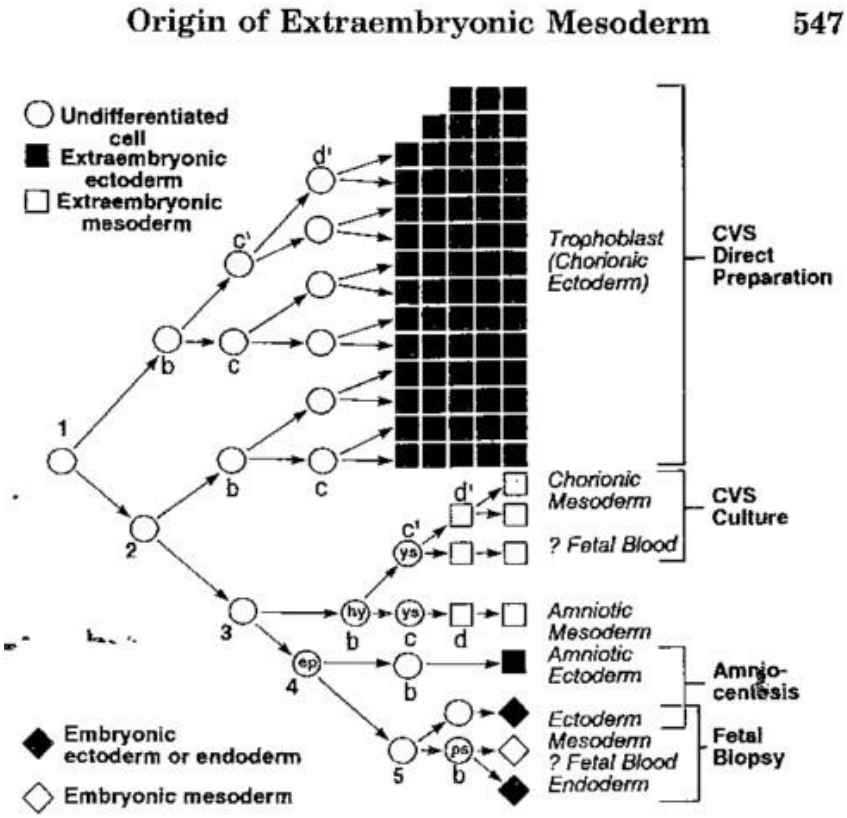


Figure 19: origin of extra-embryonic mesoderm: used by permission of (Bianchi et al., 1993))
(1)= fertilized egg gives rise to (1b) trophoblast precursor (trophectoderm) and (2) totipotent stem cell. (1b) trophectoderm gives rise to trophoblast (chorionic ectoderm) = CVS direct prep
(2) gives rise to(2b) second trophoblast cell line and (3) stem cell inner cell mass
(3) gives rise to (3b) hypoblast (CVS culture) and (4) epiblast (amniocentesis)

1.7.4.1 Risk of discordant cell lines in the ICM versus trophectoderm

Capalbo (Capalbo *et al*., 2013) reanalyzed cells from 66 embryos previously screened for aneuploidy by aCGH. By isolating ICM cells from the trophectoderm and analyzing their individual ploidy status using FISH, they were able to conclude that 79% were concordant and 21% were discordant for both cells lines. Only 3% of the embryos had a euploid/aneuploid discordance between the ICM and trophectoderm.

In contrast, when mosaic aneuploidy was detected in trophectoderm, it was only indicative of aneuploidy in the corresponding ICM in 58% of embryos, although for complex mosaics this increased to 83% (Munne and Wells, 2017).

1.7.5 Does the ratio of euploid to aneuploid cells matter to the "severity" of the mosaic diagnosis?

The Preimplantation Genetic Diagnosis International Society (PGDIS) position statement on chromosomal mosaicism, published in 2016 (PGDIS, 2016), stated that less than 20% mosaic aneuploidy should be considered euploid, greater than 80% mosaic aneuploidy should be considered aneuploid and between 20-80% mosaicism should be classified as a mosaic result.

The proportion of aneuploid cells in any mosaic sample may or may not reflect the extent of mosaicism in the whole embryo. If 10 cells are biopsied and one/ten is aneuploid, the percent of mosaicism would be stated as 10%. If 5/10 of the cells are aneuploid then the percent of

mosaicism observed would be 50%. However this could have been the same embryo and the biopsy could have been taken at different places on the trophectoderm (figure 18).

In a 2015 communication published by Greco *et al* (Greco *et al*., 2015), they described transferring known mosaic embryos to patients after appropriate consent. The percentage of mosaic aneuploid cells seen in each biopsy sample was stated. Eighteen embryos were transferred and 6 gave rise to live born offspring. The percentage of mosaicism ranged from 35%-50% in the live born group and the remaining 12 embryos which did not result in a viable pregnancy ranged from 30-50%.

To consider the degree of mosaicism in a sample when deciding whether to preferably transfer an embryo diagnosed as "mildly" mosaic or "highly" may not be helpful in avoiding possible fully aneuploid or mosaic aneuploid conceptions from these embryos.

1.7.6 Should patients transfer mosaic embryos and, if so, does the affected chromosome matter?

Some IVF clinics have elected to transfer embryos diagnosed as mosaic with patient consent and extensive counselling. The aforementioned PGDIS position statement on mosaicism (PGDIS, 2016), stated that mosaic designated embryos should only be considered for transfer if no other embryos exist for a particular patient. Greco's previously mentioned New England Journal of Medicine communication (Greco *et al*., 2015) reported that 18 embryos classified as mosaic were transferred to consenting patients and 6 of these resulted in a live birth with a normal karyotype.

This alerted the IVF community to the possibility that if we discard mosaic embryos we may be discarding embryos that could give rise to an apparently normal fetus with an abnormal placenta (confined placental mosaicism CPM) or that the abnormal cells may not proliferate past the early pre-implantation stage resulting in a euploid conception overall.

Munne *et al* (in press 2017) recently analyzed data from multiple centers that have elected to transfer known mosaic embryos to patients. The data was split into the following categories: mosaic monosomy, mosaic trisomy, complex mosaic (i.e. multiple chromosomes involved) and partial mosaic (table 4).

	Mosaic monosomy	Mosaic trisomy	Complex mosaic	Mosaic segmental *	All mosaics together
# embryos transferred	34	20	50	39	143
# with negative pregnancy test	12	7	34	16	69
# miscarriages	6	1	3	8	18
# ongoing pregs %/embryo	16 (47%)	12 (60%)	13 (26%)	15 (38%)	56 (39%)

Table 4: Showing outcomes of transferring known mosaic embryos to patients. *Munne et al in press 2017. *Mosaic segmental= part of the chromosome not the whole chromosome in some of the cells is affected*

The PGDIS guidelines (PGDIS, 2016) stated that mosaic results of certain chromosomes should be considered for transfer in preference to other chromosomes based on their ability to give rise to a live born offspring based on the phenotype of a full aneuploid result for a particular chromosome. The logic behind this reasoning is to assume that if the fetus were to become a full aneuploid for a particular chromosome deletion or duplication detected in the biopsy, then

embryos should only be transferred that are not capable of live born viability with a full aneuploidy. For example, trisomy 21, 18 and 13 mosaics would not be recommended for transfer as they give rise to viable offspring in the full aneuploid state. The guidelines also state that a monosomy mosaic is of lower risk of giving rise to a live born compared to a trisomy mosaic embryo due to the fact that no full monosomy aneuploids give rise to viable live borns apart from monosomy X, however as is shown in table 5, mosaic forms of monosomy can be compatible with life.

The prioritization of mosaic embryos for transfer based on their affected chromosome may not be useful in a clinical setting, as mosaicism in viable offspring has been demonstrated to exist for almost every chromosome (monosomy and trisomy) with varying degrees of phenotypic expression. Even if a live born infant with a normal peripheral karyotype results from a mosaic embryo they may harbor confined mosaicism in an organ or tissue which may have an adverse effect on the long term health of the child itself or the child's subsequent offspring (Campbell *et al*., 2014b). Examples of mosaicism existing in live born offspring are summarized in table 5.

Chromosome Affected	Outcomes
1	Live born mosaic trisomy 1 severe developmental delays, facial dysmorphism (Lo *et al*., 2016)
2	Complete trisomy 0.16% of first trimester losses. Mosaics if CPM survive well, maybe IUGR, if detected in fetus have poor survival.(Chen *et al*., 2013; Tug *et al*., 2017)
3	Complete trisomy lethal. Mosaics if CPM survive well, mosaic trisomy 3 offspring have multiple anomalies, mental retardation etc. (Kekis *et al*., 2016)
4	Complete trisomy 4 responsible for 2-3% of SABs. Mosaics result in multiple anomalies in live borns. May be tissue limited.(Brady *et al*., 2005)
5	Complete trisomy lethal. Mosaics if CPM survive well. Mosaics result in multiple anomalies in live borns. May be tissue limited (Sciorra *et al*., 1992)
6	Complete trisomy rare and lethal. Case of mosaic trisomy 6 with heart defect (Cockwell *et al*., 2006).
7	Live born complete trisomy 7 has never been observed. Mosaic trisomy 7 in the fetus associated with multiple defects and developmental delay. CPM associated with normal infants. Mosaicism found in fibroblasts of skin cells. Mosaic monosomy 7 characteristics: syndrome with hyperplasia of the bone marrow, genetic males may have some degree of feminization of the external genitalia, may have dysmorphic features, may have adrenal hypoplasia(Flori *et al*., 2005)
8	Complete trisomy 8 occurs in 0.8% of SABs. Monosomy 8 presents in live borns with various malformations and retardation levels. Mosaic trisomy 8 in the fetus associated with multiple defects and developmental delay. CPM associated with normal infants. Chromosome 8 carries two oncogenes, which may account for the development of cancer among some patients with trisomy 8 mosaicism https://rarediseases.info.nih.gov/diseases/5359/mosaic-trisomy-8
9	Trisomy 9 syndrome (full trisomy), mental retardation, multiple anomalies,facial features include bulbous nose, small, deep set eyes, ear anomalies, small jaw and large fontanels. Mosaic trisomy 9 can be CPM or in the fetus(Sanchez Zahonero *et al*., 2008)
10	Trisomy 10 is detected in approximately 1.8% of SABs. Full trisomy is lethal. Mosaic trisomy 10 common features: growth retardation, feeding problems, failure to thrive, distinct facial features, high arched palate, a long slender trunk, cardiac defects, renal, skeletal and central nervous system abnormalities, and early death.
11	Mosaic trisomy 11 rare but has been seen. Renal anomalies (Balasubramanian *et al*., 2011)
12	Mosaic trisomy 12 presents with facial abnormalities, behavior disturbances, developmental delay etc.(Bischoff *et al*., 1995)
13	Trisomy 13 (Patau syndrome) occurs in approximately 1 in 10,000 live births and mosaic trisomy 13 is thought to account for about 5% of these cases (Eubanks *et al*, 1998). Full trisomy exhibits mental retardation, multiple anomalies mosaicism has variable traits
14	Trisomy 14 lethal. Mosaic trisomy and monosomy 14 features in offspring, multiple facial anomalies, and short neck.(Eventov-Friedman *et al*., 2015; Johnson *et al*., 1979)
15	Mosaic trisomy 15 has been reported in an IVF fetus, multiple anomalies.(Bennett *et al*., 1992)
16	Full trisomy 16 occurs in 1% of all clinical pregnancy losses. Usually caused by UPD. Mosaic trisomy 16 tissue specific (Garber *et al*., 1994)
17	Mosaic trisomy 17, rare but reported in live born (Baltensperger *et al*., 2016)
18	Mosaic chromosome 18q partial deletion syndrome with bilateral full-thickness corneal disease(Cammarata-Scalisi *et al*., 2017; Galvin *et al*., 2015)
19	Mosaic Trisomy 19 with ring chromosome, spectrum of anomalies (Novelli *et al*., 2005)
20	Mosaic trisomy 20, Rare in live borns, common detection prenatally, case report of live born: (Powis and Erickson, 2009) Trisomy 20 in amniocytes, normal fetus (Maeda *et al*., 2015)
21	Mosaic trisomy 21 associated with Alzheimer's (Potter *et al*., 2016) 1-2% of live born offspring with Down Syndrome are mosaic (Leon *et al*., 2010)
22	Mosaic trisomy 22, rare but some live born offspring (Abdelgadir *et al*., 2013)
Sex chrom	XO- 7% of losses, less than 1% of XO conceptions survive to term. Mosaic XO exists with varying phenotypes. Mosaic XXY, XYY, XXX, all present in live borns with varying issues.

Table 5: examples of mosaic live born offspring for all chromosomes.
SAB= spontaneous abortion, CPM=confined placental mosaicism

1.7.7 Reanalyzing DNA samples from transferred embryos diagnosed as euploid with aCGH

When using aCGH for aneuploidy determination, over 40% mosaicism was classified as aneuploid and less than 40% the embryo was classed as normal. Next Generation Sequencing (NGS) is able to categorize more definitively a mosaic result and has been validated for mosaicism detection by analyzing different ratios of normal to abnormal cell lines even stating percentages of mosaicism in a sample.

All embryos tested in our own clinic prior to November 2014 were tested with aCGH. Amplified DNA from 136 transferred embryos originally diagnosed as euploid with aCGH, was reanalyzed with NGS on the Ion Torrent platform (Life Technologies). Only embryos resulting in a live birth or miscarriage, were re-analyzed for this retrospective study. Mosaicism was detected in 30% of embryos that miscarried and 9% of embryos resulting in live births (Table 6).

Retesting of DNA from aCGH tested embryos with NGS has been carried out by 2 other groups. Fragouli *et al* re-analyzed the DNA from 94 embryos that had been transferred and had a clinical outcome (Fragouli *et al*., 2015a). Forty three of these embryos were found to be mosaic after reanalysis with NGS. Of these mosaic embryos, 26 (60%) did not implant, 6 (14%) miscarried and 11 (26%) gave rise to ongoing pregnancies. Grifo *et al* reanalyzed only patients who had a miscarriage post transfer of supposed euploid embryos tested by aCGH (Grifo *et al*., 2015b). Some miscarried pregnancies had testing on products of conception (POCs) and some did not. Of those

who did not the amplified DNA from each original biopsy was reanalyzed with NGS. Of the 39 embryos that miscarried and on retesting gave a valid result, 17 embryos were euploid (43%) and 22 were found to be mosaic (56%). These studies give us a unique insight into how embryos designated as mosaic may behave post embryo transfer as the embryos since found to be mosaic would not have been transferred if they had been originally tested using NGS.

	Embryos transferred resulting in a Biochemical or miscarriage	Embryos transferred resulting in a Live birth
# FET cycles		
# embryos transferred ("euploid" with aCGH)	69	67
# mosaic post retest with NGS (% of embryos transferred)	21 (30%)	6 (9%)
Types of mosaicism Exhibited and outcomes	Mosaic Partial Trisomy 20pter-q13.2 Mosaic Monosomy 10q26.13-qter Mosaic Monosomy 5 Mosaic Monosomy 21 Mosaic Monosomy 14 Mosaic Partial Trisomy 5q32-qter, Mosaic monosomy 1 Mosaic Partial Trisomy 10pter-p13 Mosaic Monosomy 13 Mosaic Partial Monosomy 4q34.1-qter Mosaic Monosomy 16 Mosaic Monosomy 15 Triploid Mosaic Partial Monosomy 18q21.32-qter Mosaic Trisomy 9 Mosaic Partial Trisomy 1 Mosaic Monosomy 15 Mosaic Monosomy 3pter-q12.2 Mosaic Monosomy 6 Mosaic Partial Monosomy 5 Complex Mosaic Mosaic Nullisomy Y (Y copy number 0.63)	• Mosaic Partial Trisomy 7pter-p21.1, Mosaic Monosomy 15= 7lb 8oz boy • Mosaic Monosomy 14 = boy VSD • Mosaic Partial Monosomy 5pter-p15.1,mosaic partial Trisomy 8q11.21-qter= 6lb 14oz boy • Mosaic Partial Trisomy 8q11.21-qter = 6lb boy • Mosaic Monosomy 6, Mosaic Monosomy 21 = 7lb boy • Mosaic Trisomy 4= 5lb baby, has either 2 vessel cord or umbilical cyst

Table 6: Profiles of embryos designated as euploid prior to transfer and diagnosed as mosaic post retrospective analysis of the same DNA products with NGS (own unpublished data)

1.8 Supplementing PGS with non-invasive testing

All embryos defined as euploid do not have the ability to create a live born offspring. The search for an adjunct to PGS that would further define embryo viability is ongoing. In this section I describe the studies known so far that have combined PGS with other embryo screening methods.

1.8.1 Combining Morphokinetics and PGS

As far as is known, there only been one study utilizing both PGS and morphokinetics to choose embryos for transfer. Yang *et al* (Yang *et al*., 2014) recruited 138 patients undergoing IVF with PGS and cultured half the sibling zygotes in a regular big box incubator (Group B) and half in an Embryoscope time lapse incubator (Group A). All embryos reaching the blastocyst stage were biopsied and screened using aCGH. The study showed a significant difference in ongoing pregnancy rate and implantation rate in favor of euploid embryos cultured in the Embryoscope before biopsy however the numbers in the study were small. Of the 127 transfers, 45 transferred a mixture of one embryo from each group.

While there was an increase in implantation and ongoing pregnancy rates in the time lapse group, this may have been attributable to the very different culture conditions between the two groups. Group A was grown in 5% Oxygen and Group B was grown in atmospheric oxygen (20%) and embryos grown inside a time lapse incubator remain relatively undisturbed for the culture period compared to a conventional incubator.

A better design of the study would have been to reduce the number of variables by also culturing the control embryos (Group B) in a time lapse incubator and blind the embryology team to the time lapse data for that group. Although benefits were essentially seen from this study using both technologies in conjunction, more research is needed to find one morphokinetic event that may add to the selection process between known euploid embryos.

1.8.2 Combining static morphology and PGS

A study by Irani *et al* (Irani *et al*., 2017) showed that there was a association between blastocyst morphology in euploid embryos and pregnancy outcome. Embryos graded as "excellent" before biopsy (38 transfers) resulted in an 84.2% ongoing pregnancy rate vs 61.8% for "good" (76 transfers) 55.8% for "average" (197 transfers) and 35.8% using embryos graded as "poor" (106 transfers). These differences all reached significance. Capalbo however (Capalbo et al., 2014a), in a study with 213 patients showed that the only predictive value for embryo quality was aneuploidy rate which also concurred with our finding in section 2.1.1 of this thesis. In Capalbos' study, once an embryo was deemed to be euploid, the quality prior to biopsy seemed to have no effect on its' ability to progress to an ongoing pregnancy.

1.9 Limitations of the embryo biopsy and testing process

Removing 3-10 cells from the trophectoderm layer on day 5 or 6 of development is a small representation of the embryo as a whole and only the cells present in the biopsy tube can be analyzed for any particular embryo that is destined for clinical use. Patients need to be aware that there is a small error rate when sampling such small numbers of cells.

1.9.1 Risks of misdiagnosis

Establishing error rates in embryos that have been diagnosed as euploid is not simple. In order for an embryo to be tested post transfer, products of conception post miscarriage have to be analyzed or live born offspring would have to be karyotyped. Even if a live born baby is apparently phenotypically normal, there may be underlying chromosomal aberrations that may be in mosaic form and may go undetected until later in life. Many embryos transferred will never implant but whether the cause of that is chromosomal or other factors are involved is near impossible to establish.

A 2014 study by Werner *et al* (Werner *et al*., 2014) re-examined the transfers of 4,794 CCS euploid embryos diagnosed by qPCR. Of the embryos that implanted 10 were identified as aneuploid conceptions. Only embryos that were able to be analyzed post miscarriage were able to be considered and the dataset was certainly not complete. To have an accurate picture of possible misdiagnosis for whatever reason, analysis would have to be done on all the embryos transferred rather than just the few which were able to be sampled at miscarriage. In this study, while 2979

embryos developed a fetal sac, there were 1818 embryos that did not result in a clinically recognized pregnancy. One could assume that at least some of these 1818 embryos were undiagnosed mosaics or misdiagnosed in some way. The group did not state how many products of conception or live born offspring they were able to analyze which would have given a percentage of the samples analyzed rather than the percentage of embryos transferred (10/4794 a rate of 0.2%).

Wilton *et al* (Wilton *et al*., 2009) reviewed a large ESHRE PGD consortium dataset of day 3 biopsy. 15,158 cycles, had 24 misdiagnoses and adverse outcomes reported: 12/2538 cycles after polymerase chain reaction (PCR) and 12/12,620 cycles after fluorescence *in-situ* hybridization (FISH). The causes of misdiagnosis include confusion of embryo and cell number, transfer of the wrong embryo, maternal or paternal contamination, allele dropout, use of incorrect and inappropriate probes or primers, probe or primer failure and chromosomal mosaicism. Unprotected sex during IVF treatment was also mentioned as a cause of adverse outcome not related to technical and human errors.

A retrospective cohort study of 520 embryo transfers using embryos designated as euploid after trophectoderm biopsy and aCGH by Tiegs *et al* (Tiegs *et al*., 2016) showed a less than 1% error rate in live born offspring and 17% error rate in miscarried embryos. Cycles were included in the analysis if the outcome could confirm the PGS diagnosis, such as live birth after single embryo transfer, or in the case of spontaneous abortion where products of conception (POCs) were

obtained and sent for cytogenetic analysis. Cycles without available outcome data were excluded. Of 282 live births, 280 were apparently normal and the gender concurred with the original PGS diagnosis. Of POCs available for testing, 14 of 17 karyotypes agreed with the PGS result. The 3 miscarriages with discrepant results all showed autosomal aneuploidy post karyotype and the 2 live born infants had sex chromosome errors; one was 47XYY (PGS 46XY) and the other was 46XY (PGS 46XX).

1.9.2 Risks of using Whole Genome Amplification (WGA) for PGS

The majority of techniques used for whole chromosome copy number determination require Whole Genome Amplification (WGA) to create more DNA for testing (section 1.6.2). WGA is accomplished using Polymerase Chain Reaction (PCR) techniques such as Sureplex (Illumina) and multiple displacement amplification (MDA) (Spits *et al.*, 2006) which are both routinely used for PGS. These methods create copies from copies which has the potential to perpetuate mistakes made during original copying stages. This risk is greater when using WGA for single gene mutation detection as a smaller part of the chromosome is under detection (Kung *et al.*, 2015).

1.9.3 Risks of biopsy to the embryo

The removal of cells from the trophectoderm is an invasive procedure which appears to cause minimal damage to the developing embryo. Embryos that have been biopsied continue to develop once transferred to the uterus at a high rate and 60% of euploid embryos will give rise to a live born offspring (own data). During the biopsy, a laser pulse is applied to the cell junctions

of the trophectoderm in order to remove the tissue for analysis. Cells in contact with the laser can be damaged possibly disrupting the chromosomes. In theory this could lead to an erroneous mosaic result. This has been postulated by Ilkevitch *et al* (Illkevitch Y1, 2017) who developed a laser free method of biopsy. However in their small dataset they did not detect any mosaic results in the laser or alternate biopsy method groups.

The biopsy procedure will rarely result in loss of an embryo at the time of biopsy. In our own laboratory there have only been 2 instances out of over 12,000 (0.01%) embryos biopsied where the embryos have degenerated at the time of the biopsy.

Scott *et al* (Scott *et al*., 2013b) studied the effects of day 3 and trophectoderm biopsy on subsequent implantation rates. Day 3 biopsied embryos had significantly lower implantation rates than non-biopsied day 3 embryos (30% vs 50%) whereas trophectoderm biopsied blastocysts implanted at a similar rate to non-biopsied blastocysts (51% vs 54%) concluding that removing cells from the embryo at the blastocyst stage at least does not seem to interfere with sustained implantation.

There has not been a study to date of the long term outcome of blastocyst biopsy on resulting development of offspring.

1.10 Referral categories for PGS

The reasons for patients undergoing testing as part of their infertility treatment are diverse. There are two overall categories of patient, those that have higher risk factors for embryonic aneuploidy and receive medical advice to have their embryos tested and those patients who elect to have testing and have a lower risk of embryonic aneuploidy.

1.10.1 Higher risk patients

The first group includes repeated pregnancy loss patients, patients of advanced maternal age, patients who have had failed IVF cycles and patients who have had a previous aneuploid conception and wish to avoid that experience again.

1.10.1.1 Advanced maternal age

As the incidence of aneuploidy is lower in embryos of younger women, the pregnancy rate with embryos of known or unknown ploidy status is almost equal. Without pre-screening embryos, as maternal age increases the implantation and ongoing pregnancy rates decrease (Franasiak *et al*., 2014a, b; Harton *et al*., 2013; Munne *et al*., 2007a).

The graph below shows that replacing only euploid embryos in an embryo transfer cycle removes the maternal age effect on live birth rates. A euploid embryo from a 40 year old woman will implant with the same frequency as a euploid embryo from a 25 year old woman. PGS has been shown to reduce miscarriage rates in older patients (Forman *et al*., 2012b). Aneuploidy is the

major cause of miscarriage (van den Berg *et al*., 2012) therefore only transferring euploid embryos in theory should result in fewer miscarriages.

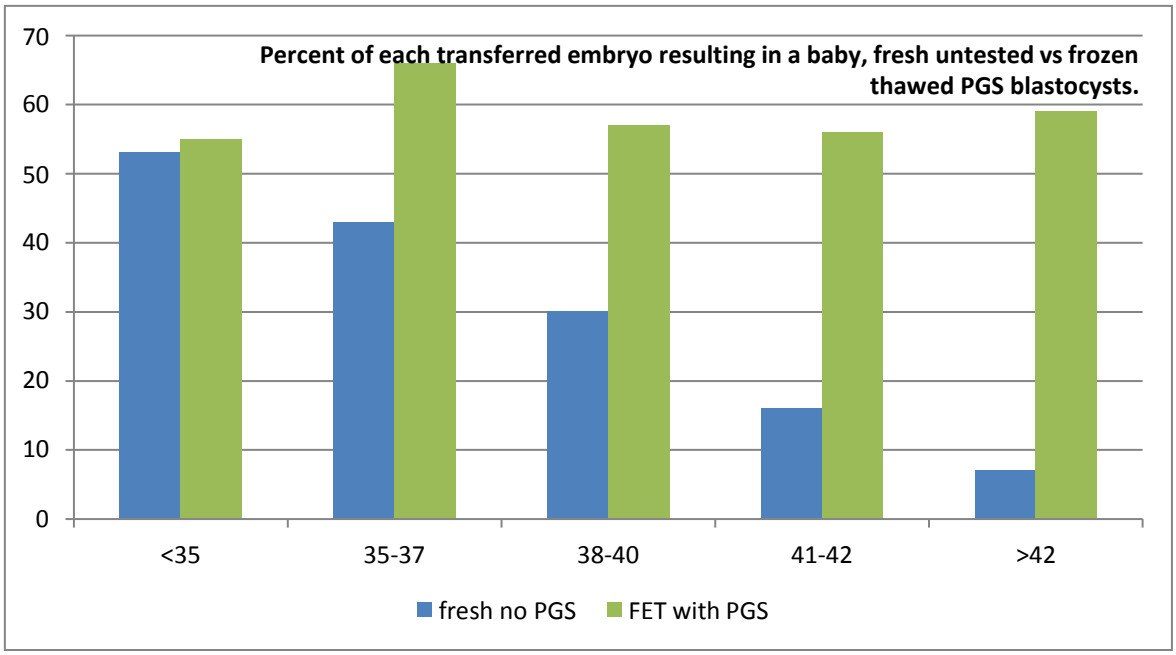


Figure 20: Live birth rates of fresh untested embryos vs frozen thawed PGS embryos. Own data showing rate of live born offspring per transferred embryo comparing fresh untested blastocysts (n=2356) vs frozen thawed PGS blastocysts (n=597). Illustrating beautifully how the maternal age effect can be removed if a euploid embryo can be found for transfer.

Patients of advanced maternal age have fewer euploid embryos than younger women and therefore testing these embryos allows the laboratory to find a chromosomally normal embryo for transfer or avoids replacement of an abnormal embryo to the uterus. These patients are less likely to have a normal embryo in their cohort and therefore more patients in this category will not have anything to consider for transfer (see chapter 2.6).

1.10.1.2 Previous history of aneuploid conception

Patients who have had a termination of an aneuploid pregnancy in the past may psychologically benefit from the early detection of another aneuploid conception before the embryo is replaced in the uterus, thus lowering the chances of a second possible termination or miscarriage due to aneuploidy.

1.10.1.3 Failed IVF cycles

Patients who have had previous failed IVF cycles may elect to have subsequent embryos tested to see if they are aneuploid and if this is the reason they are not achieving a pregnancy. If all embryos are abnormal this can be useful information that may encourage the patient to move on with other options for family building.

1.10.1.4 Recurrent Pregnancy Loss

Recurrent pregnancy loss (RPL) is categorized as 2 consecutive losses before 20 weeks gestation. In theory, the ideal patient to be offered aneuploidy testing during the IVF cycle is the repeated pregnancy loss patient who produces multiple embryos. Cytogenetic analysis of products of conception from spontaneous abortions in late first trimester demonstrate around 60% of conceptuses are aneuploid (Zhang *et al*., 2009). Aneuploidy screening during the IVF cycle is used as a tool to choose the correct embryo for transfer and reduce the chances of miscarriage happening again if aneuploidy were the cause (Farahmand *et al*., 2016; Munne *et al*., 2005; Shahine and Lathi, 2014).

A recent paper by Murugappan *et al* (Murugappan *et al*., 2016) compared IVF with PGS versus expectant management (EM) for the treatment of RPL. They compared one cycle of IVF with PGS to 6 months of EM. They reported that per attempt, PGS is not effective in improving LB rate or decreasing clinical miscarriage rate compared with expectant management except in those PGS cycles that complete transfer of a euploid embryo. Once a euploid embryo was transferred however the live birth rates were higher than spontaneous attempts at conception. They concluded that in the intention to treat analysis, the time to viable conception was 6 months for PGS and 3 months for EM however the parameters of study participants were not equally matched in each group. Partners of females with a history of RPL have been found to have increased rates of sperm aneuploidy for certain chromosomes such as chromosome 16 which is associated with miscarriage (Neusser *et al*., 2015).

1.10.2 Lower risk patients

Patients in this group have a lower risk of embryonic aneuploidy than the previous group of patients and they opt for testing by personal choice. These patients include maternal age of less than 35, use of young donor oocytes (fresh or freeze all cycles) and fertile patients wishing to balance the gender of their family.

1.10.2.1 Young maternal age (less than 35 using own oocytes and donor oocyte)

The incidence of aneuploidy in blastocyst embryos in young women less than 35 is around 40%. (own data) Therefore the risk of replacing an aneuploid embryo without screening with PGS is still 40%.

1.10.2.2 PGS before cryopreservation

Frozen embryo transfer without determining ploidy status has historically been lower than fresh transfer in all age groups. If biopsy is carried out before embryos are vitrified the implantation rate and ongoing pregnancy rates are significantly higher in some patient categories than cryopreserving without testing. In the dataset below all culture and vitrification protocols were the same. The method used for chromosome analysis changed from aCGH to NGS in January 2014.

	<35	35-37	38-40	41-42	>42	Donor egg IP* uterus	Donor egg GC** uterus
FET with PGS	55% 91/167	66% 114/174	57% 100/176	56% 35/63	59% 10/17	60% 87/145	69% 202/291
P value	0.2	<0.0001	0.04	-	-	0.0003	0.2
FET no PGS	48% 82/172	38% 38/99	36% 10/28	2/6	0/2	36% 34/95	62% 43/69

Table 7: Data showing percentages of live births of FET with and without PGS (ORM own data 2013-2015) IP = Intended parent. **GC = gestational carrier*

1.11 Perspectives on the potential to further improve IVF

IVF is a treatment that has improved over the last 30 years to a point where in the best programs we can achieve live births at a rate of 60% per transferred euploid embryo. However this still leaves around 40% of embryos diagnosed as euploid that will either fail to implant or will result in a miscarriage. There is clearly room for further improvement.

2 Specific aims of this thesis

IVF is a major part of reproductive medicine and has become far more widespread since its inception in 1978. Clinics are constantly seeking to improve the efficiency of the IVF procedure and basic research is essential (much of which can be gleaned from existing data in clinics) to advance the field further. Much of this can be achieved by original studies derived from retrospective analysis of clinic data, or by prospectively designed studies. Indeed a number of fundamental biological questions pertaining to chromosome abnormalities and their relationship to IVF embryo development remain unanswered. With this in mind the overall aim of this thesis is to provide further insight into the cytogenetic basis of early human development (with a view to improving IVF care for practitioners and patients) by pursuit of the following specific aims:

1. To test that hypothesis that we can predict aneuploidy levels in human IVF embryos without direct PGS by the following approaches:
    a. Analysis of basic morphokinetic criteria
    b. Analysis of spent media from cell-free embryonic DNA
2. To test the hypothesis that patients undergoing ICSI (many of whom have previously been demonstrated to have elevated sperm aneuploidy levels) display significantly elevated embryo aneuploidy levels.
3. To establish novel patient populations that may benefit from the use of PGS:
    a. Population number 1: Male factor infertility patients
    b. Population number 2: Young oocyte donors

4. To establish the optimal transfer strategy (fresh vs frozen) for euploid embryos in patients using their own oocytes
5. To test the hypothesis that using NGS for PGS increases live birth rates and lowers miscarriage rates by detecting mosaicism compared to using aCGH which does not.
6. To provide a framework for creating realistic expectations for patients planning for their fertility future

2.1 General Materials and Methods

Patients undergoing IVF treatment at Oregon Reproductive Medicine (ORM), Portland, Oregon, USA, were counseled to have their embryos tested as part of our routine protocol to assess the chromosomal status of pre-implantation embryos before transfer to the uterus. Indications for PGS included advanced reproductive age of the female patient, history of repeated pregnancy losses, history of failed IVF cycles, history of previous aneuploid pregnancy, diminished ovarian reserve, or patient request. All embryos were created from super-ovulated IVF cycles to create multiple embryos.

2.1.1 Timeline of data collection

The data collection for the research featured in this manuscript was an ongoing process. Each chapter includes data from different time periods during November 2010 to December 2016 and the specific dates are stated in each chapter summary. Data has been included in multiple studies between different dates and these dates are stated in each chapter of this thesis.

2.1.2 Changes to protocol during the data collection timeline

Changes to protocols over that time frame were as follows:

DNA analysis platform:

- November 2010-January 2014 array CGH used exclusively
- February 2014- November 2015 a mixture of aCGH and NGS (Ion Torrent platform) used. This was the start of the RCT described in specific aim 4 where non study

patient's embryos underwent aCGH and the study patients embryos underwent NGS to determine aneuploidy.

- December 2015-December 2016 all patients underwent NGS to determine aneuploidy

How results were affected: Euploidy rates are lower with NGS than with aCGH due to the increased detection of mosaicism with NGS.

DNA sample transportation:

- aCGH was carried out offsite at a reference lab in Los Angeles. Embryo biopsies were sent from Portland, Oregon via courier on gel cold packs to LA where they were amplified and analyzed.
- NGS was carried out onsite on the ORM premises in Portland, Oregon. All embryo biopsies were amplified and analyzed here onsite.

Vitrification method:

- Vitrification using a non-DMSO method was used from November 2010-December 2012
- Vitrification using DMSO as a cryoprotectant was used from January 2013 onwards

How results were affected: Vitrification using DMSO results in higher survival rates post thaw (97% DMSO vs 85% non-DMSO)

All other culture methods and stimulation protocols remained the same for the entire data collection period.

2.1.3 Stimulation and embryo culture protocols

Oral contraceptive (OC) administration was initiated 2–3 weeks before stimulation. A GnRH antagonist protocol was preferentially used unless the patient had previously had a suboptimal response to this protocol. The antagonist was started on day 6 of stimulation. Ovarian stimulation was achieved with both FSH and hMG preparations. When the lead follicle was ≥18 mm, 10,000 IU of hCG (Novarel) was used for final oocyte maturation. Serum progesterone levels were obtained on the day of trigger. Oocyte retrieval was performed 36 hours after trigger shot. On completion of the retrieval procedure, oocytes were placed in Quinn's Advantage Fertilization Medium (Origio, USA) supplemented with 5% Human Serum Albumin (HSA) (Irvine Scientific, USA) under oil (Ovoil, Vitrolife, USA).

Fertilization was achieved by either Intra Cytoplasmic Sperm Injection (ICSI) (Palermo *et al*., 1992) or standard insemination performed approximately 4 hours after retrieval . The criteria for each method of insemination were as follows:

- Normal sperm (>20 million per ml with normal morphology and % motility) would undergo standard insemination
- Suboptimal sperm/ frozen sperm/ patients with long history of unexplained infertility would undergo ICSI

Once all oocytes had been either inseminated or injected, they were returned to the incubator for overnight culture. All embryos were moved to Quinn's Advantage Cleavage Medium (Sage, Origio, USA) supplemented with 10% HSA (Irvine Scientific) from days 1-3 and subsequently

moved to Quinn's Advantage Blastocyst Medium (Sage, Origio, USA) supplemented with 10% HSA from days 3-6.

Assisted hatching was performed on all embryos on day 3 post-retrieval using a Hamilton Thorne Zilos^{tm} laser with 1-2 800um pulses to breach the inner and outer zona layers. The embryos were transferred back to culture media until day 5 or 6 of development.

2.1.4 Embryo grading and morphological analysis

Blastocysts were graded using the Gardner method (Gardner DK and WB, 1999). This method relies on a 3 part grading system. A number indicates the degree of expansion of the blastocoel cavity and 2 following letters indicate the quality of the inner cell mass and the trophectoderm respectively. So a good quality blastocyst would be denoted as a 4AA, (fully expanded cavity =4; good quality ICM =A; good quality TE=A). Embryos were not biopsied if an ICM was not seen. "Early blastocyst" describes an embryo showing the first signs of blastulation and is not graded at this point. Compacting embryos with no visible sign of blastulation were classified as morulae, which is the stage before the embryo begins to cavitate and cell outlines begin to merge. Embryos classified as "poor quality" with a C grade of either ICM and/or TE were not typically biopsied and tested as we routinely only freeze embryos of average or good quality that have a good chance of implanting.

2.1.5 Embryo biopsy

Embryos were only considered suitable for biopsy when at least 10% of the trophectoderm (TE) was protruding from the breach in the *zona pellucida* made on day 3. All embryos that were not hatching by day 5 were cultured until day 6 and then biopsied. Embryos were only biopsied if there was a visible Inner cell mass (ICM) and multi-celled TE protruding from the *zona pellucida*. Embryos that grew to an expanded blastocyst stage had an estimated 3-8 TE cells excised using a Hamilton Thorne Zilos^{tm} laser with 1-2 800um pulses to break apart cell junctions in the trophectoderm layer for tissue removal . Tissue samples were placed in 2ul of non-stick buffer solution in individual microfuge tubes labelled with the appropriate embryo number. If embryos were to undergo fresh embryo transfer, biopsied day 5 embryos were kept in culture overnight to await results of the PGS results before 12 midday of day 6. If embryos were to be cryopreserved post biopsy, they were individually vitrified using Irvine Vitrification media with DMSO (Irvine Scientific, CA) on Cryotops (Kitazawa, Japan) and stored in liquid nitrogen for future use.

2.1.6 Reducing contamination of samples

Face masks, hats and sterile gloves were worn at all times during biopsy and tubing of the cells to eliminate operator DNA contamination of the embryo tissue sample. Cumulus cells were removed at the time of ICSI on egg collection day, or for standard insemination, at the time of fertilization check on day 1 of development. Standard insemination results in multiple sperm attaching to the *zona pellucida.* These residual sperm remain attached throughout the growth

period and can be a possible source of sperm DNA contamination during embryo biopsy. However, as the blastocyst expands and extrudes from the *zona pellucida***,** the *zona* is left behind and the trophectoderm does not come into contact with the outer layer of the *zona.* If a rogue sperm becomes detached in the biopsy media it can be observed easily and avoided when tubing the trophectoderm sample.

2.1.7 DNA analysis

Biopsied cells were whole genome amplified (WGA) using the Sureplex DNA Amplification System (Bluegnome) according to the manufacturer's protocol. The WGA products were then processed for aneuploidy analysis by NGS or aCGH.

Testing by aCGH was processed using Bluegnome 24sure V3 protocol (Illumina, Inc.) according to the manufacturer's protocol. WGA products were fluorescently labeled with Cy3 and Cy5 dyes and random primers and subsequently were prepared to be hybridized to 24sure V3 array slides. Aneuploidy data analysis was performed using BlueFuse Multi Software.

Testing by NGS was processed using Ion Torrent PGM (Ion Torrent) technology. Libraries were prepared by fragmenting WGA products with DNA concentrations of 100 ng using Ion Xpress Plus Fragment Library Kit (Life Technologies). Library fragments were selected at 200 bp using E-Gel SizeSelect Gels (Life Technologies) and then were normalized to 100 pM using Ion Library Equalizer kit (Life Technologies). Libraries were subsequently pooled together into a mastermix,

and clonal amplified on the Ion One Touch 2 system. The template was then loaded into a 316 V2 chip (Life Technologies) and sequenced at 200 bp. Aneuploidy data analysis was performed using Ion Reporter software, using Low-coverage Whole-Gnome workflow.

2.2 Specific aim 1

To test the hypothesis that we can predict aneuploidy levels in human IVF embryos without direct PGS by the following approaches: analysis of basic morphokinetic criteria and analyzing spent media from cell free embryonic DNA

2.2.1 Specific aim 1a

To establish if static morphology correlates with ploidy status

Static morphology as a predictor of ploidy status is considered in a paper, entitled ”Morphological and Kinetic Embryological Criteria and Association with Aneuploidy Rates: How Might they Be Used to Choose the Best IVF Embryo for Transfer?” Authors: Alison Coates, Breanne Coate, Elizabeth Holmes and Darren K Griffin. Published in Human Genetics & Embryology. (Coates A, 2015)

2.2.1.1 My Personal Contribution to the Work

For this study I cultured and biopsied embryos, I conceived the idea for the study, I retrospectively analyzed the data and I wrote and edited the paper.

2.2.1.2 Chapter Summary

The purpose of this retrospective analysis was to establish if static morphology of blastocysts at the time of biopsy could be used to predict aneuploidy in our own patient population. The original

study submitted for publication in 2014, included 1686 blastocysts from 295 IVF cycles undergoing PGS for aneuploidy during July 2010 to August 2013. A subsequent analysis of data post manuscript publication,from September 2013 to December 2015 was added to the original data, to assess day 5 vs day 6 aneuploidy rates resulting in a total of 8,015 embryos. Embryos were graded prior to trophectoderm biopsy using the Gardner grading system. Once results PGS results were received the data was analyzed.

Speed of growth and its' effect on ploidy status was studied by analyzing aneuploidy rates of day 5 vs day 6 embryos without consideration of static morphology. Day 5 ready embryos had a significantly higher chance of being euploid than day 6 embryos. While the differences between day 5 and day 6 embryos was small (Donor egg: 5%, <35: 5%, 35-37: 6%, 38-40:13%, 41-42: 11% and >42:4%) all the differences noted reached a level of significance apart from the <35 group which almost reached significance and the >42 age group which was not significantly different possibly due to the small data sample.

A subset of embryos from the original study that had not reached expanded blastocyst stage by day 5 were analyzed separately. Embryos at the morula stage on day 5 but which continued on to make a blastocyst by day 6, were more likely to be euploid than an embryo that had reached the early blastocyst stage by day 5. This finding was statistically significant for the younger (donor oocyte) group only (although in the 35-39 and the 40-42 age groups the difference was borderline significant).

Static morphology of the inner cell mass (ICM) and trophectoderm (TE) was considered and correlated with rates of aneuploidy. AA grade (excellent quality) embryos had the lowest rate of aneuploidy in all maternal age groups. In poorer quality embryos (AB,BA,BB) the rates of aneuploidy were higher. The conclusion of the study was that static morphology alone is unable to predict aneuploidy in blastocysts.

2.2.1.3 Introduction

The ability to culture human embryos *in-vitro,* ultimately to lead to viable live births, has improved over the last 20 years, mostly through the evolution of culture media and methods. The development of physiological culture media similar to human tubal fluid improved culture conditions of human embryos (Quinn, 1995) and further modifications using sequential media allowed for successful extended culture to the blastocyst stage (Gardner and Lane, 1997; Marek *et al*., 1999). Unlike the sophisticated development of culture media the method of choosing which embryos to transfer using basic morphological criteria has remained similar since the inception of IVF. That is, the subjective judgment of the embryologist is still the major driver in embryo selection and, if this aspect could be improved, there is still potential for further increases in IVF pregnancy rates. Other more empirical, approaches to inform embryo choice have, in recent years, gained in popularity: preimplantation genetic screening (PGS) for aneuploidy, and morphokinetic analyses following time-lapse photography.

There are a number of referral categories for PGS in a clinical IVF setting, advanced maternal age recurrent pregnancy loss, recurrent implantation failure and prolonged periods of infertility (e.g. related to elevated sperm aneuploidy). Of these the maternal age effect for aneuploidy is the most well-described in preimplantation embryos as well as oocytes, spontaneous abortions and live born individuals and thus remain the largest referral category for PGS (Harton *et al*., 2013; Qi *et al*., 2014). Moreover, while the appearance of an embryo tells us which, among a cohort, may be the most likely to be viable in early embryonic stages, aspects of visual assessment have yet to be fully established as reasonably accurate predictors of ploidy status. Blastocyst biopsy followed by PGS for aneuploidy has been shown to reduce, but not eliminate the risk of miscarriage (Munne *et al*., 2005; Munne *et al*., 2006b) when transferring chromosomally normal embryos and the chance of choosing a karyotypically normal embryo for transfer based on appearance alone becomes less likely as the patient ages. Embryo biopsy followed by PGS thus remains the only method of accurately detecting aneuploidy in pre-implantation embryos.

Studies involving transfer of chromosomally normal embryos have resulted in higher implantation rates per embryo transferred in older patients than transfer of non-tested embryos chosen by morphology alone (Alfarawati et al., 2011; Capalbo et al., 2014b; Hardarson et al., 2001; Schoolcraft et al., 2011; Scott et al., 2012; Scott et al., 2013b; Yang et al., 2012). A further aspect of PGS is that it tells us which chromosome is involved and this can be important in predicting obstetric outcome. For example, the absence of monsomies (apart from XO) among spontaneous abortion material suggests that monosomy is a leading cause of embryo arrest or

implantation failure. The preponderance of trisomy of most chromosomes (and XO) in first trimester miscarriages makes aneuploidy the leading cause of pregnancy loss. The potential for stillbirth or congenital abnormalities in live borns for certain aneuploidies (e.g. trisomy 21, 22) make chromosome specific screening useful to minimize the likelihood of an aneuploid embryo being transferred to the uterus (reviewed in Griffin 1996).

Recent advances in time-lapse technology with cameras installed inside incubators have allowed constant, undisturbed observation of embryo growth patterns. Evidence suggests that timing of one cell division to the next at the early cleavage stage is somewhat predictive of blastocyst formation but not ploidy status (Chavez *et al*., 2012; Ebner *et al*., 2003; Fenwick *et al*., 2002; Kroener *et al*., 2012; Lundin *et al*., 2001). Campbell and colleagues demonstrated that the most predictive morphokinetic events were the timing of the beginning to the end of blastulation and that was able to give an overall risk (i.e. low vs. high), of an embryo being aneuploid.

Despite these impressive results, although we may be able to provide crude estimates of the risk of embryos being chromosomally normal or abnormal based on morphokinetics, further refinements are needed if non-invasive methods are to replace invasive ones for preimplantation genetic screening. In other words, there is still a need to investigate further morphological and morphokinetic parameters (either by static or time-lapse analysis) to establish which, if any, might be correlated with specific aneuploidy risk. While direct analyses of time-lapse images offers the potential to correlate these morphokinetic events and potentially establish thresholds

and ranges within which a lab can predict ploidy status of an embryo, there is not a definitive event which predicts aneuploidy accurately.

While direct analyses of time-lapse images offer an opportunity to address the above questions, static analysis in the traditional manner nonetheless remains useful in identifying the criteria through which time-lapse studies might be directed. This is particularly true for clinics that have not yet installed a time-lapse facility and/or if pre-existing data, that can be analyzed retrospectively, is used to identify factors that might pre-dispose to aneuploidy. Moreover, it is essential that such analysis is performed in specific maternal age windows, given the association of maternal age and aneuploidy. For this reason we have compared aneuploidy rates following PGS by static analysis of specific morphological and morphokinetic parameters; namely the quality of the trophectoderm and inner cell mass individually and the speed of growth as manifested by the day that the embryo achieves full expansion of the blastocoel cavity (i.e. is it “blastocyst biopsy ready” by day 5). Because of the link to maternal age, each analysis is performed on 5 distinct age groups.

2.2.1.4 Materials and Methods

(See general Materials and Methods for stimulation, culture, vitrification and DNA quantification protocols. Section 2.7)

Chesapeake IRB review (September 6th 2013) determined IRB approval was not required; the University of Kent Local Research Ethics Committee approved this study.

Statistical analysis:

Mann-Whitney (for 2 way comparison) and Kruskal-Wallis tests (for 3 way comparison) (non-parametric ANOVA tests) were used to determine significance. P values of <0.05 were considered statistically significantly different.

2.2.1.5 Results

In the initial study, a total of 1686 Embryos from 295 cycles of IVF carried out from July 2010 to August 2013, were successfully biopsied; successful array CGH traces were seen in 97% of embryos. Mean age, number of cycles and mean embryo biopsied per cycle are noted in Table 8. There was no significant difference in the proportion of embryos that did not give a result in each age group. In the subsequent analysis, data from a further 6,329 embryos generated from September 2013 to December 2015 was added to the original dataset.

Group number	1	2	3	4	5
Maternal Age	Donor oocyte*	<35	35-39	40-42	>42
Mean age	24.8	31.4	35.5	41	43.5
No. of cycles	82	46	107	53	7
No. of embryos biopsied	685	284	509	236	25
Mean no. of embryos	8.4	6.2	4.8	4.5	3.6
# embryos with no result	15	12	16	8	1
	2%	4%	3%	3.8%	4%

Table 8: Patient parameters for study participants broken down into maternal age groups.

2.2.1.5.1 Aneuploidy and developmental stage at a fixed time point

In the first analysis, for 4 of the age groups, we compared the overall aneuploidy rates of "day 5 ready" vs. "day 6 ready" embryos, the latter being the slower growing. Results in the donor oocyte (youngest) and 40+ oldest age groups show that faster growing embryos i.e. those that were structurally ready for biopsy on the morning of day 5 ("day 5 ready") were more likely to be chromosomally normal than ones that were slower growing and ready for biopsy by day 6 ("day 6 ready"). In the <35 and 35-39 groups rates of aneuploidy between the two groups were not signficantly different. There were too few "day 5 ready" embryos (only 2) in the >42 age group and therefore this group was not included for this particular analysis.

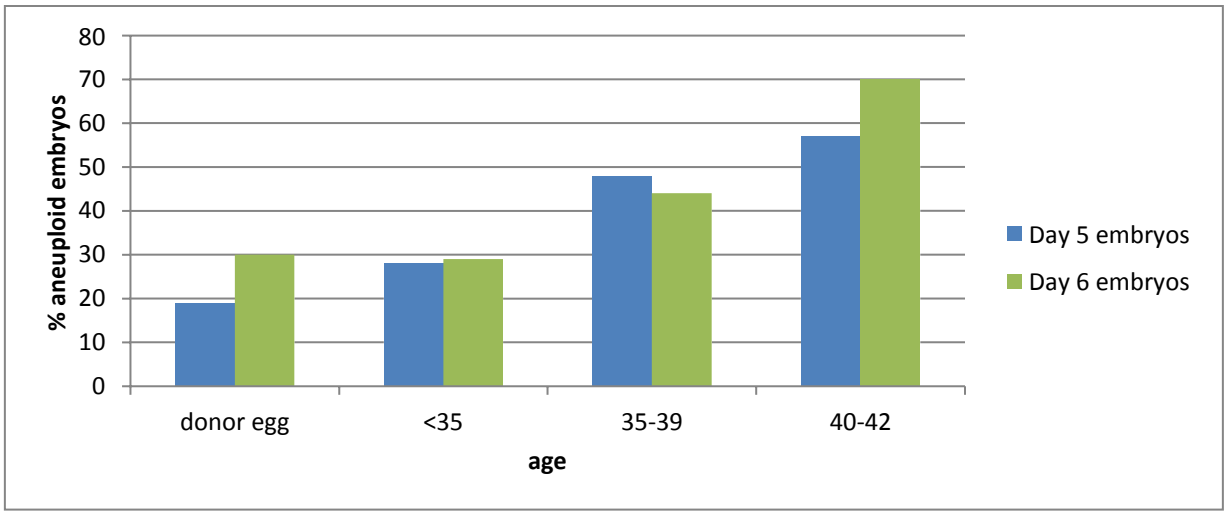


Figure 21: Aneuploidy rates vs developmental stage. % Aneuploidy rates (Y axis) of embryos according to the day they reached the stage of development ready to biopsy (day 5 or 6). Donor oocyte: p= 0.001 (significantly different); <35: p=>0.05 (not significant); 35-39:p=>0.05 (not significant); 40-42: p=0.04 (significantly different - Mann-Whitney test).

In this particular dataset, results thus suggest an association between aneuploidy rate and speed of growth, but only in embryos of younger and older mothers.

A larger dataset was subsequently analyzed from data post publication of this manuscript:

	Donor oocyte (ave 25)	<35	35-37	38-40	41-42	>42
Total # embryos biopsied	3907	1191	1199	1139	455	124
% day 5 biopsied	62%	51%	46%	51%	46%	30%
% day 5 euploid	77%	71%	66%	35%	36%	19%
P value (5 vs day 6) :	0.0004	0.07	0.04	<0.0001	0.01	0.6
% day 6 euploid	72%	66%	60%	22%	25%	15%

Table9: Rate of growth compared to aneuploidy rates according to maternal age

While the differences in aneuploidy rates between day 5 and day 6 embryos was small (Donor egg: 5%, <35: 5%, 35-37: 6%, 38-40 :13%, 41-42: 11% and >42:4%) all the differences noted

reached a level of significance apart from the <35 group which almost reached significance and the >42 age group which was not significantly different possibly due to the small data sample.

In a second analysis in the original study, a subset of the embryos not ready for biopsy until day 6 were taken forward for analysis (figure 22). Aiming to correlate aneuploidy and speed of growth, levels of aneuploidy in each age group were compared. This particular subset of embryos was either morulae or early blastocysts on day 5. They were individually tracked and the ones that subsequently grew to fully expanded blastocysts by day 6 were able to be biopsied. In all age groups the early blastocysts (quicker growing embryos) were more likely to be aneuploid than the morulae (slower growing). Again there were too few embryos biopsied (only 5) in the >42 age group and therefore this group was not included. This finding was statistically significant for the younger (donor oocyte) group only (although in the 35-39 and the 40-42 age groups the difference was borderline significant).

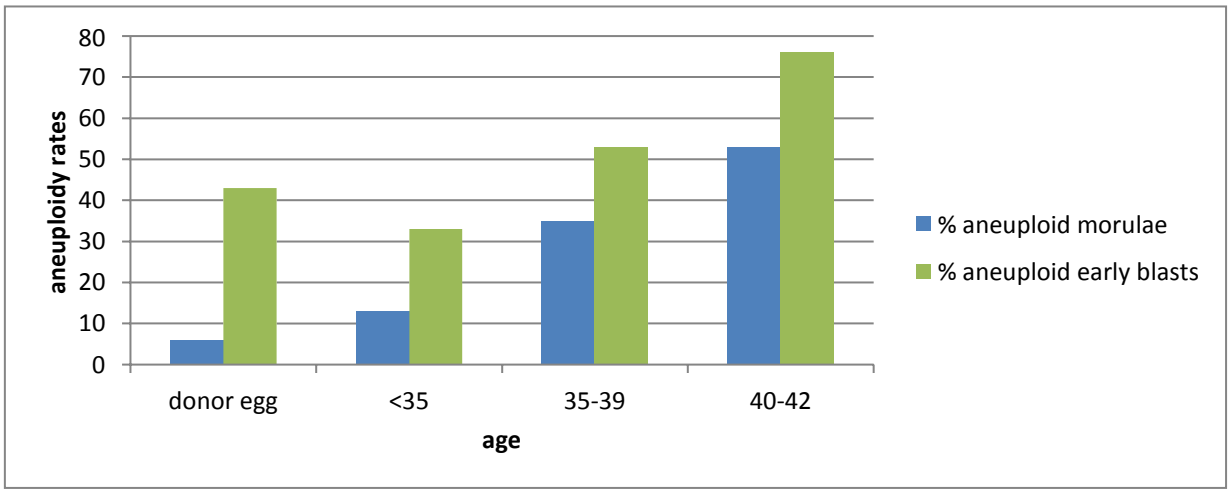


Figure 22: Aneuploidy rates (y axis) of embryos that were early blastocysts or morulae on day 5.

The aneuploidy rates of blastocysts resulting from morulae on day 5 appeared to be lower in each age group than aneuploidy rates from embryos that had just begun to cavitate on day 5 (early blastocysts). Donor oocyte: p=0.009 (significantly different); <35: p=>0.05 (not significant); 35-39: p=0.08 (not significant); 40-42: p=0.08 (not significant -Mann-Whitney).

Taken together therefore, a complex pattern emerges in that, in the whole data set, it is the slower growing embryos that appear to be more likely to be aneuploid. By isolating the slower growing cohort (those not ready to biopsy until day 6) however, it seems that the relatively faster ones that continue to grow to day 6 are more likely to be aneuploid.

2.2.1.5.2 Aneuploidy rates compared to overall embryo grade

For this analysis, the quality of the embryo was classified by the quality of its trophectoderm (TE) and inner cell mass (ICM) separately. With AA grade as the highest quality and BB the lowest for both germ layers AB or BA referred to embryos with one good quality layer. Embryos classified as good quality (AA) had the lowest rate of aneuploidy in all age groups: (19%, 24%, 42%, 52% and 57% respectively) compared to embryos with at least one poorer quality part (AB or BA), which had higher rates (37%, 36%, 45%, 73% and 58% respectively). Embryos with the poorest quality overall (BB) had the highest aneuploidy rates (36%, 42%, 61%, 76% and 92% respectively).

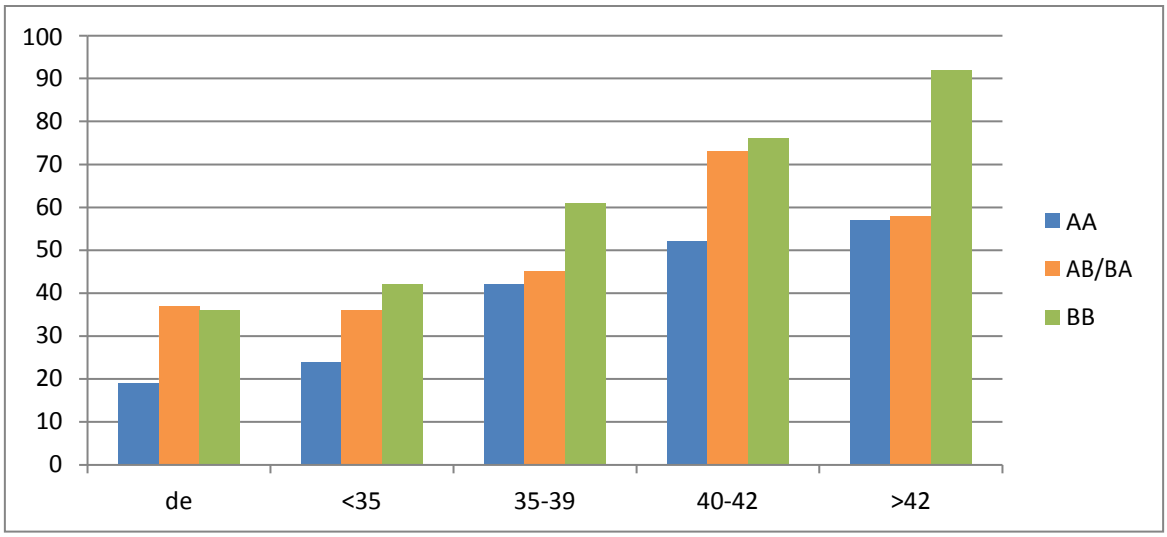


Figure 23: Rates of aneuploidy according to overall grade of embryos. Donor oocyte: p=0.0001 (significantly different), <35: p=0.06 (not significant); 35-39:p=0.02 (significantly different); 40-42: p=0.006 (significantly different); >42: p=0.04 (significantly different - Kruskal-Wallis test).

2.2.1.5.3 Inner cell mass and trophectoderm quality compared to aneuploidy

The quality of the inner cell mass correlated with aneuploidy levels in three of the five age groups (the <35 and 35-39 age groups not being statistically significantly different). The quality of the trophectoderm correlated with aneuploidy status in four of the five age groups (the 40-42 age group not quite reaching statistical significance (p=0.06)). In all cases the aneuploidy levels in the B grade germ layers were higher than the A grade. Taken together therefore the results seem to suggest that TE quality Is a more accurate predictor of aneuploidy.

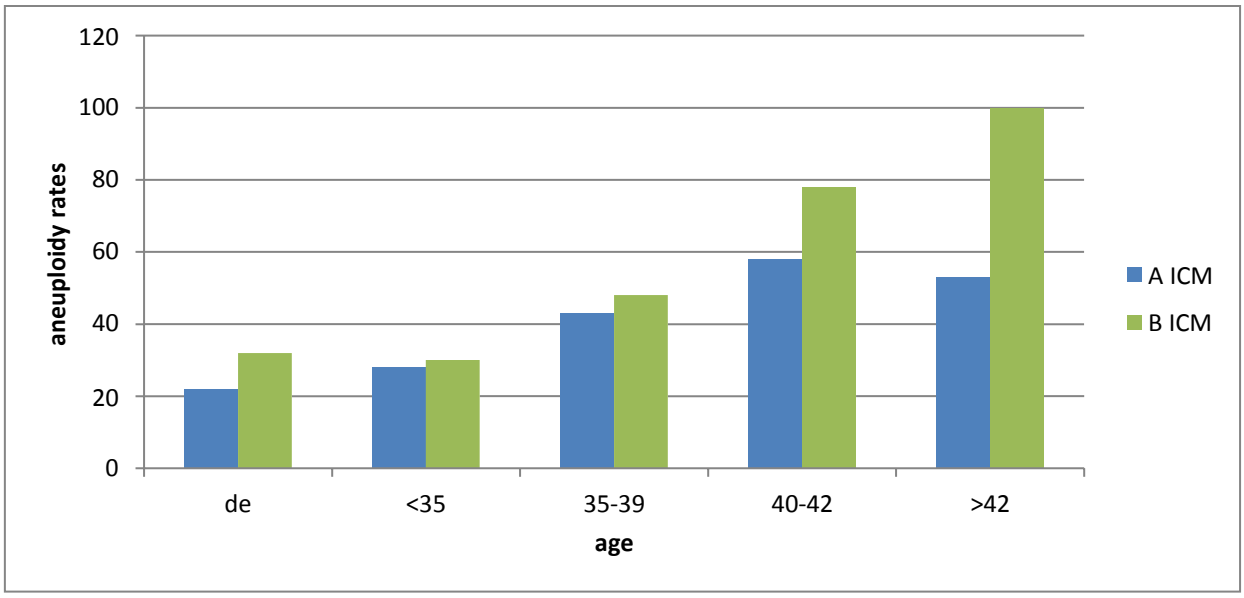


Figure 24: Inner cell mass (ICM) quality and rates of aneuploidy.

Aneuploidy rates of embryos with a B grade ICM were significantly higher than A grade ICM in the donor oocyte group (average age 24) and the over 40 age groups. Donor oocyte: p=0.048 (significantly different); <35: p=>0.05 (not significant), 35-39: p=>0.05 (not significant), 40-42: p=0.005 (significantly different), >42: p=0.03 (significantly different - Mann-Whitney test)

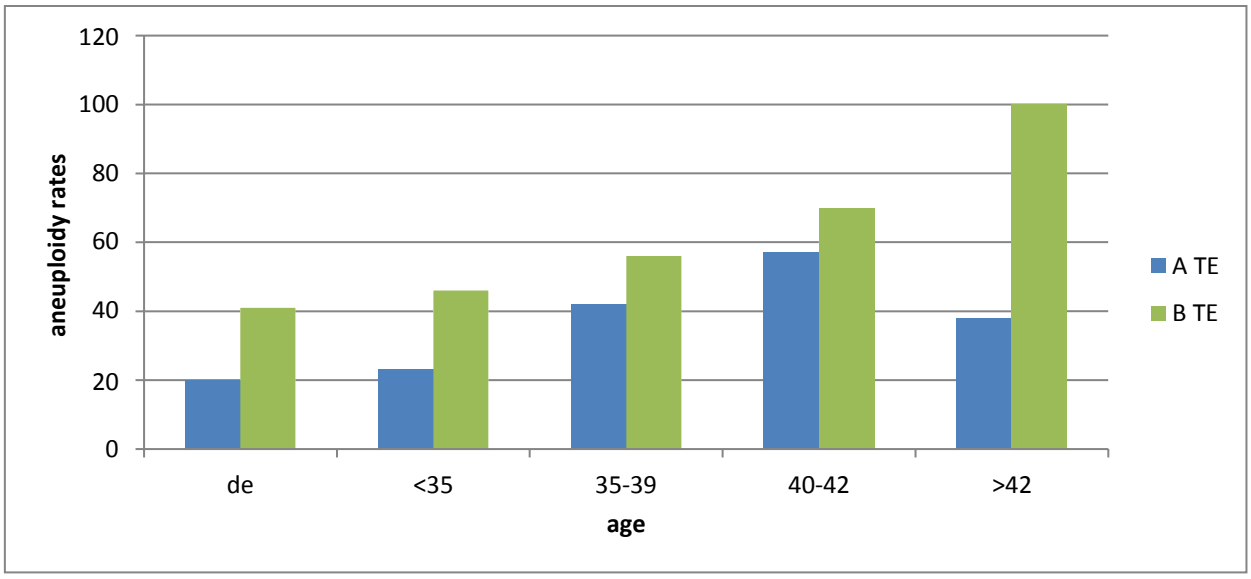


Figure 25: Rate of aneuploidy and quality of trophectoderm (TE).

Aneuploidy rates of embryos with a B grade TE were significantly higher than A grade TE in all but one age groups. Donor oocyte: p=0.0001; <35: p=0.0007; 35-39: p=0.039; 40-42: p=0.06 (not significant); >42: p=0.0014

2.2.1.6 Discussion

Choosing the embryo most likely to lead to a baby is the ultimate goal in any IVF cycle. The development of more physiological culture media (Marek *et al*., 1999; Quinn, 1995) has enabled successful growth of embryos to the blastocyst stage. Better culture conditions eliminate the cohort of embryos that arrest at day 3 and improves implantation rates per embryo. In our own hands (current data 2009-2012) implantation rates of fresh untested blastocyst embryos are typically 56% in patients <35 compared to 35% if day 3 embryos are transferred. However, although extended embryo culture narrows down the choice of embryos it still does not

accurately predict which embryo to choose based on its appearance. Embryo grading by an experienced embryologist also remains a subjective art rather than an exact science. The difference between an A grade and a B grade TE or ICM can be open to interpretation and the exact grading of embryos is acknowledged to be of questionable utility.

A recent study illustrated beautifully how choosing an embryo based on appearance alone is a fairly arbitrary choice if there are many to choose from (Forman *et al*., 2013c). Embryos were chosen using morphological criteria and then tested the embryos before transfer to see if the morphological choice was correct. The embryos were biopsied to assess chromosome complement and transferred on day 6 if the embryo was euploid. If the original choice was aneuploid another embryo would be selected for transfer. Selection of an aneuploid embryo by chance based on appearance alone was higher in patients ≥35 compared to <35 years old (31% vs 14%) purely because there is a higher percentage of chromosomally normal embryos in younger patients.

Morphology was related to aneuploidy in a study of 500 embryos from 93 patients (Alfarawati *et al*., 2011). A association was found between embryo quality and ploidy status but this was not a strong association. That is, 50% of higher grade embryos were euploid compared to 37.5% of poorer quality embryos. Inner cell mass and trophectoderm grades were also affected by aneuploidy in a negative way. However some of the best quality embryos were aneuploid and some of the poorer quality embryos were chromosomally normal. A moderate relationship was shown in a retrospective study between blastocyst morphology and aneuploidy using PGS data

(Capalbo et al., 2014b) but implantation ability was governed by the chromosomal complement of an embryo rather than conventional morphology assessment. This is confirmed in data in this study.

Trophectoderm and ICM quality have been correlated with live birth outcome in two single embryo transfer studies. The conclusions were that TE quality was the most important factor in predicting live birth outcome but neither study specified the age of patients. (Ahlstrom *et al*., 2011; Hill *et al*., 2013) In the current study, TE quality appears to be more indicative of ploidy status than ICM quality whereas in patients 40 and older, ICM quality is more indicative of ploidy status when choosing embryos for transfer. However, when using a more detailed classification of ICM morphology, implantation potential has been predicted more accurately, with the shape and size being clinically important as a predictor of success (Richter *et al*., 2001). Day 5 expanded blastocysts with slightly oval ICMs implanted at a higher rate (58%) compared with those with either rounder ICMs (7%) or more elongated ICMs (33%). Implantation rates were highest (71%) for embryos with both optimal ICM size and shape. In the current study a detailed classification of the ICM was not performed and a gross overall appearance of the ICM as a distinct cohesive clump of cells was classed as a good quality ICM.

The technique for choosing embryos that perhaps has received the most attention in recent years is the study of morphokinetics. Precursors to the latest morphokinetic studies using time-lapse photography, involved taking the embryo out of the incubator at certain developmental junctions

and observing if an event had occurred. One study showed that if the first cleavage division occurred before 25 hours post insemination an embryo was twice as likely to develop to an expanded blastocyst compared to an embryo that failed to cleave within 25 hours post insemination (32% vs 16%) (Fenwick *et al*., 2002). Recent advances in time-lapse technology have allowed constant, undisturbed observation of embryo growth patterns. Timing of early cleavage events predict with some accuracy embryos which will ultimately form a blastocyst but not embryos that are chromosomally normal (Herrero and Meseguer, 2013; Meseguer *et al*., 2012; Paternot *et al*., 2013). The development of a risk classification system using time-lapse photography in conjunction with biopsy and chromosomal analysis showed that the timing from the start to the end of blastulation is predictive of ploidy status and resulted in live birth rates per embryo similar to those seen after replacing known euploid embryos (61% with morphokinetics blastulation assessment vs 69% using chromosome analysis)(Campbell *et al*., 2014a). Interestingly using our own data , morulae on day 5 (pre-blastulation) that continued to grow to fully expanded blastocysts on day 6 had a much lower rate of aneuploidy than embryos that had just begun to blastulate on day 5. This supports previous findings (Kroener *et al*., 2012) which found that delayed blastulation does not in itself result in higher rates of aneuploidy but that, if blastulation does not occur at all the rates of aneuploidy are high. Therefore if a morula carries on growing to an expanded blastocyst then it has a good chance of being chromosomally normal as shown by our own data. The convention has been to choose more advanced embryos using traditional static morphology for transfer from a cohort. If all that were available on day 5 were early blastocysts or morulae then an embryologist would be far more likely to choose the

early blastocyst over a morula. One might conclude that if all that are available on day 5 are early blasts or morulae then maybe the transfer should be carried out on day 6 if the embryos continue to progress and choose for transfer the embryos that were at the morula stage on day 5.

Our own data set suggests that, although there may well be associations between growth rate and aneuploidy, the relationship is by no means a simple one. Finding, as we did, that faster growing embryos ready for biopsy by day 5 were more likely to be chromosomally normal than slower growing embryos not ready for biopsy until day 6, suggests a simple association, albeit in only certain age groups. Finding an apparently paradoxical phenomenon when looking at the day 6 ready group only (i.e. that the ones that reached early blastocyst on day 5 were more likely to be chromosomally abnormal compared to embryos that had not yet begun to cavitate on day 5) however suggests a complex pattern that needs further investigation. In other words, growth rate criteria (whether determined by time lapse or, as in this case, by static criteria) need to be viewed with caution and skepticism when trying to draw conclusions about levels of aneuploidy.

In conclusion, it seems that there is yet to be found a morphokinetically based assessment that will predict with accuracy which embryo to choose without analyzing the chromosome complement of an embryo; this can currently only be achieved by embryo biopsy. Moreover, correlating the end point morphology of an embryo to its ploidy status tell us that embryos that we may have dismissed as poorer quality may often be euploid and embryos that we deem to be good quality may be aneuploid (Alfarawati et al., 2011; Capalbo et al., 2014b). Static Embryo

morphology assessment thus has a place in predicting embryo viability but has limitations in predicting the likelihood of an embryo being euploid. It is possible that we should be looking at different parameters for different patient populations, perhaps for instance choosing embryos with better quality ICMs for the >40 patients (if there is a choice) but focusing on the trophectoderm in other age groups. Time lapse evaluation of morphokinetic events should be viewed with caution when using that alone to attempt to decide ploidy status as further studies need to be performed to establish association between morphokinetic event timing and ploidy status (Natesan S, 2014). A combination of chromosomal analysis and time-lapse observations of morphokinetic criteria or more detailed static morphological observations may help inform future studies aimed at choosing the best embryo for transfer. For the moment however, the use of morphology or growth rate for the prediction of aneuploidy remains ineffective.

2.2.2 Specific aim 1b

To test the hypothesis that we can use cell free DNA in spent media to quantify ploidy status non-invasively: A study of 3 different amplification methods and cell free DNA stability.

An abstract submitted to ESHRE 2017 entitled “Development and application of a novel strategy to explore blastocoel fluid and spent culture media as a source of embryonic DNA” by Babariya, D, Manoharan, A,Welch, C, Spaeth, K, Munne, S, **Coates, A**, Wells, D . It was accepted as an oral abstract.

Abstract submitted to ASRM 2017 entitled “Utilization of a novel DNA amplification method allows non-invasive testing of preimplantation embryos based upon spent culture media”
D. Babariya A. Kung, A. Manoharan, **A. Coates**, C. Welch, S. Munne, D. Wells;

2.2.2.1 My Personal Contribution to the Work

I collaborated with the Oxford laboratory team, thawed and biopsied embryos, tubed spent media, directed clinic study protocol. Contributed to abstracts and wrote this manuscript.

2.2.2.2 Chapter Summary

This is an ongoing project designed to establish if cell free DNA in spent culture medium correlates with ploidy status of the blastocyst. The study was performed on 33 cryopreserved embryos from 7 patients that had been previously diagnosed as euploid or aneuploid by NGS and

were donated to research according to patient wishes and 45 fresh embryos from 4 patients analyzed as part of the IVF cycle. All of these embryos were created using intra-cytoplasmic sperm injection (ICSI). Cryopreserved embryos were thawed and individually cultured in 3ul droplets for 24 hours. Fresh embryos were individually cultured in 3ul droplets from day 3 to day 5 for 48 hours. All spent media samples were collected using single use stripper tips(origio)once the embryo had been removed and were placed in individual microfuge tubes labelled with the corresponding embryo number. Samples were frozen in a -20^{o}C freezer post biopsy for varying lengths of time before amplification with 3 different methods. The novel amplification method based on modified MDA resulted in the highest rate of successful reads (38/40 95%) followed by Sureplex (12/19 63%) and lastly traditional MDA (5/19 26%). All the amplification methods fared best when the time between sampling and amplification was only 12 hours compared to 17-28 days. From this initial experiment, the most concurrent results were from frozen thawed embryos cultured for only 24 hours using the novel amplification method. 32/36 (89%) embryos amplified with the NAM gave a concurrent ploidy status between the spent media and the biopsy sample. When gender was inconcurrent, all of the spent media samples were female and the biopsies were male. Non concurrent gender was only seen in the fresh embryos cultured for 48 hours. As all of these were female in spent media and male in the biopsy proper, this could be attributed to cumulus contamination. This would be more likely to have been present in the spent media on day 3-5 compared to embryos that had been biopsied, frozen and thawed before placing in spent media, thus more thoroughly removing potential cumulus contamination before placing in

the media to be subsequently tested. Further experiments are needed to refine the technique but results are encouraging for this novel strategy for noninvasive PGS.

2.2.2.3 Introduction:

Spent culture media has been considered as a source of embryonic DNA. If concurrence could be achieved between nuclear DNA from a trophectoderm biopsy and cell free DNA in the spent media then the need for invasive biopsy to determine ploidy status may be superfluous. Only 2 studies have been published to date. The first study was a proof of concept study by Shamonki *et al* (Shamonki *et al*., 2016). They placed day 3 embryos in single 15ul culture drops and grew to day 5, at which point the embryo was removed and the spent media was tubed and the embryo was biopsied. The spent media and biopsied cells were amplified using Repli-G (Qiagen). 55 of the 57 spent media samples had detectable levels of DNA but only 6 of these were suitable to be analyzed. One of the 6 gave a reliable result with aCGH which was concurrent with the nuclear DNA samples from the trophectoderm biopsy. The only other study investigating cell free DNA in spent media was by Xu *et al* (Xu *et al*., 2016). They completed the first clinical transfers of embryos to patients based on the spent media DNA analysis alone. They termed the method Non-Invasive Chromosome Screening or NICS. They carried out an initial study establishing concordance between biopsy results and NICS. To validate their results, 43 embryos were cultured from day 3 to day 5 and the spent media drops and biopsy samples were analyzed concurrently. 21 embryos diagnosed as euploid from the biopsy had a concurrent result from the NICS. 16 embryos diagnosed as aneuploid from the biopsy had an aneuploid result from the NICS (although only 6/16 had the exact same losses or gains). There were 2 false negative results

where the NICS was euploid and the biopsy was aneuploid and 4 false positives where the NICS was aneuploid and the biopsy was euploid. 86% of the embryos therefore had a concurrent result between the biopsy and the NICS and 14% of embryos had a non-concurrent result overall. Their clinical application involved 7 patients and 9 embryo transfers replacing embryos solely diagnosed as euploid by NICS. The 9 transfers resulted in 6 live born allegedly chromosomally normal babies (authors stated that a karyotype had been performed). The DNA amplification method used in this study was the MALBAC (Multiple Annealing and Looping Based Amplification Cycles) WGA method, which amplifies the original DNA strands rather than amplifying copies of copies, and the products were analyzed on an Illumina HiSeq NGS platform.

Cell free DNA is fragmented in segments of around 200bp in size(Fan *et al.*, 2010). These DNA fragments are not as stable before amplification as nuclear DNA. The present study considered the length of time from sampling to the time of processing to achieve successful DNA amplification. Traditional amplification methods may not be as accurate in amplifying these smaller fragments and may be the cause of incongruous results between nuclear DNA and cell free DNA when using methods of amplification routinely used for nuclear DNA. An alternative modified MDA based amplification method is currently being investigated (here referred to as "novel amplification method" or "NAM") and the results are presented in this chapter.

2.2.2.4 Materials and Methods

(See general Materials and Methods for stimulation, culture, vitrification and DNA quantification protocols. Section 2.7)

Patients who had consented to the use of leftover DNA as part of their IVF cycle with PGS for aneuploidy were included in the study. All embryos were created using ICSI as a method of fertilization which eliminates sperm contamination.

Cell free DNA in 78 spent media samples from blastocyst culture were amplified utilizing three different whole genome amplification (WGA) strategies. Fresh Embryos were placed in individual 3ul pre-equilibrated drops of Sage Blastocyst culture medium (Origio, US) with 10% HSA (Irvine, CA) under oil (Ovoil, Vitrolife), for culture from day 3 until day 5. On day 5, all embryos were examined for stage of growth.

Embryos reaching the expanded blastocyst stage were allocated a number which was written by the drop under the dish. Biopsy drops were made using 37^{o}C HEPES buffered media (Sage, Origio) with 5% HSA under oil. Each Blastocyst designated for biopsy was removed from its culture media drop in a minimal amount of fluid using a new stripper tip for each embryo and placed in its appropriately labelled biopsy drop. Each spent media drop was placed in a microfuge tube labelled with the embryo number again using a fresh stripper tip for each drop. The embryos were biopsied as usual and vitrified for future use.

Vitrified embryos with known PGS results were warmed as per protocol and cultured for 24 hours before sampling spent media. Control media droplets (no embryo exposure) were collected to confirm an absence of DNA in the media itself.

Tubing of samples was carried out with the tube rack on a cold pack and the tube rack was placed in a -20 freezer as soon as possible after tubing. Samples were amplified as soon as possible post sampling and this ranged from less than 12 hours to 28 days. Amplification in the genetic lab was in house and then amplified DNA was hand carried on cold gel packs as carry-on luggage from the USA to the research lab in the UK for analysis. Samples in the experiment were sent in one shipment.

Samples were split into 3 groups for amplification: 1) Sureplex amplification (Illumina); 2) Multiple Displacement Amplification (MDA, Qiagen); 3) novel amplification method (NAM). Following amplification, spent media samples were tested for aneuploidy using Next Generation Sequencing (NGS).

2.2.2.5 Results

Fourteen of 19 samples of spent media amplified with MDA had no reads (74%), 7 of 19 samples amplified with Sureplex had no reads (37%) independent of the amount of time the samples had been stored at -20^{o}C. The novel amplification method (NAM) resulted in 2/40 (5%) no reads (1/30

(3%) processed after 0.5 days post sampling, 1/10 (10%) processed after 17-28 days post sampling), therefore 95% had a spent media result. With the NAM 36 embryos had a result for both the biopsy and the spent media. Of those, the spent media and biopsy diagnosis of aneuploid or euploid was concurrent in 32/36 embryos (89%). Three of these samples were diagnosed with a concurrent euploid diagnosis but with the opposite gender. 5 of the samples gave inconcurrent results. (3 x aneuploid biopsy vs euploid spent media, 1 x euploid biopsy vs aneuploid media and 1 x euploid biopsy vs aneuploidy media but different genders). Three of 30 media samples processed within a day of sampling had a complete discordance of the euploid/aneuploid diagnosis (10%) vs 2 of 10 (20%) of samples stored for 17-28 days.

Results for the spent media incubated for 24 hours were more likely to have concurrent results with the original biopsy compared to 48 hours of incubation. Of the 25 frozen thawed samples using the NAM, only 2 gave a non-concurrent diagnosis and these 2 embryos had a mosaic only profile in their original biopsies. Of the 28 frozen thawed samples with all 3 amplification methods none had an opposite gender result but all 6 of the embryos with opposite genders in the spent media result to the biopsy were from fresh embryos. 3 embryos had a result for the spent media but a no read result for the biopsy. Amplification and analysis of the control media droplets confirmed absence of human DNA.

NAM

Embryo state	Fresh (48 hour culture)			Frozen (24 hour culture)	Total
Time in -20°C prior to amp	28 days	17 days	0.5 day	0.5 day	
# embryos screened	4	6	5	25	40
# results from spent media	3	6	4	25 (100%)	38 (95%)
# with results for both media and biopsy	3	6	3	25	37
# concurrent ploidy results %/embryo with both results	2	5	2	23/25 (92%)	32/37 (87%)
# non concurrent ploidy status	1	1	1	2	5
# concurrent ploidy status but non concurrent gender	0	3	0	0	3
# non concurrent ploidy status and gender	0	0	1	0	1
# no read bx but results from spent media	0	0	1	0	1

Table10: spent media results of novel amplification method (NAM)

Sureplex

Embryo state	Fresh (48 hour culture)			Frozen (24 hour culture)	Total
Time in -20°C prior to amp	28 days	17 days	0.5 day	0.5 day	
# embryos screened	4	6	5	4	19
# results from spent media	1	5	4	2	12(63%)
# with results for both media and biopsy	1	5	4	2	12
# concurrent ploidy results	1	3	4	2	10/12 (83%)
# non concurrent ploidy status	0	0	0	0	0
# concurrent ploidy status but non concurrent gender	0	2	0	0	2
# non concurrent ploidy status and gender	0	0	0	0	0
# no read bx or spent media	0	0	1	0	1

Table 11: spent media results of Sureplex amplification

MDA

Embryo state	Fresh (48 hour culture)			Frozen (24 hour culture)	Total
Time in -20°C prior to amp	**28 days**	**17 days**	**0.5 day**	**0.5 day**	
# embryos screened	**4**	**6**	**5**	**4**	**19**
# results from spent media	**1**	**1**	**2**	**1**	**5(26%)**
# with results for both media and biopsy	**1**	**1**	**0**	**1**	**3**
# concurrent ploidy results	**0**	**0**	**0**	**1**	**2/5 (40%)**
# non concurrent ploidy status	**1**	**1**	**0**	**0**	**2**
# concurrent ploidy status but non concurrent gender	0	0	0	0	0
# non concurrent ploidy status and gender	0	0	0	0	0
# no read bx but results from spent media	0	0	2	0	2

Table 12: spent media results of MDA

2.2.2.6 Discussion

As aneuploidy is the main cause of implantation failure or miscarriage in human reproduction (Franasiak *et al*., 2014b; Harton *et al*., 2013; Kushnir and Frattarelli, 2009; Lathi and Milki, 2004; Munne *et al*., 2007a; Taylor *et al*., 2014a; Werner *et al*., 2012), establishing which pre-implantation embryos are euploid before transfer in a clinical setting has improved sustained implantation rates for patients undergoing IVF with PGS (Forman *et al*., 2013b; Schoolcraft *et al*., 2010; Scott *et al*., 2012). Biopsy of embryos is currently the only reliable method of establishing ploidy status in pre-implantation embryos before transfer.

Day 3 biopsy has been studied to assess the effects of single cell removal on embryo growth, implantation and effects on offspring. Bar-El *et al* (Bar-El *et al*., 2016) used Embryoscope time

lapse incubators to compare growth patterns of 366 embryos biopsied for PGD with 385 embryos that had not been subjected to blastomere removal. They demonstrated that day 3 biopsied embryos had significantly delayed compaction and blastulation compared to the non- biopsied embryos.

The removal of a portion of the trophectoderm layer (3-10 cells) for analysis seems to have little negative effect on the embryos ability post biopsy to give rise to viable offspring. Implantation rates of biopsied and non-biopsied day 3 and blastocyst stage embryos were compared in an elegant study by Scott *et al* (Scott *et al*., 2013b). This randomized controlled trial transferred 2 embryos to each patient. Some patients were allocated to day 3 transfer and some patients were allocated to day 5 transfer according to the clinics standard protocol. Two embryos of equal morphological appearance were chosen for transfer and labelled as A or B before randomization. A and B were then randomized to either biopsy or control by way of sealed envelopes. One of the embryos was biopsied just before transfer and the other embryo remained intact. Ploidy status of the biopsied embryos was discovered several weeks post transfer. If a singleton pregnancy resulted the infant underwent DNA fingerprinting to establish which embryo it arose from. Day 3 embryo biopsy decreased sustained implantation rates significantly compared to non-biopsied day 3 embryos (30% biopsied vs 50% control). Blastocyst biopsied embryos showed no significant decrease in implantation rates compared to the non- biopsied blastocyst controls.

BhCG levels of pregnancies resulting from biopsied euploid blastocyst stage embryos have been shown to be lower than from non- biopsied embryos. (Olcha *et al*.) This suggests that we are indeed altering the implantation process in some way by removing cells from the trophectoderm.

Follow-up studies of offspring post embryo biopsy have only been carried out post day 3 biopsy. (Desmyttere *et al*., 2009; Liebaers *et al*., 2010; Sunkara *et al*., 2017) and the conclusions have been that there is no apparent evident negative effects seen in offspring. Outcome studies of offspring resulting specifically from embryos biopsied at the blastocyst stage have not been carried out to date.

Each individual transferred euploid embryo biopsied at the blastocyst stage has a 60% chance of giving rise to a live baby independent of maternal age (own data). If the effects of the biopsy procedure were removed and embryos were diagnosed as euploid by analyzing spent media without biopsy then there is a theoretical possibility that live birth rates could be improved further.

The potential for creating mosaic results during the biopsy procedure by the application of laser pulses to separate trophectoderm tissue for sampling has been hypothesized by Illkevitch *et al* (Illkevitch Y1, 2017). They developed a novel biopsy technique where the cells were removed by using the surface tension between the media droplet and the mineral oil overlay to pull cells from the trophectoderm layer. They stated that the cells were less sticky than after laser removal. 55

embryos were biopsied using the novel method and 87 embryos were biopsied with a laser. There were no mosaic results for any of the embryos in either group. As the numbers were small they were unable to conclude that this was due to the novel biopsy technique. This is the first inkling that the biopsy process may have the potential to create errors in the sample itself.

The potential for contamination with extra-embryonic DNA of the media drops to be tested is not an insignificant risk. DNA contamination can be from the culture media as human serum is used as a supplement, it can be from cumulus cells or sperm remaining on the *zona pellucida,* residual sperm stuck to the *zona pellucida,* or it could be from the operator handling the samples and loading the sample tubes. This study included analysis of control drops of media that had not come into contact with embryos. All of the controls came back with no DNA contamination. The embryos all underwent ICSI as part of their IVF cycle therefore reducing possible sperm contamination. All 8 embryos from the 3 groups that had the opposite gender in the spent media to the original biopsy sample, were from fresh embryos cultured for 48 hours. All of these spent media genders were female and the biopsies were male. It is possible that cumulus cell contamination remaining in the culture drop could have been responsible for the gender confusion. Frozen thawed embryos would have less cumulus cell contamination than fresh as they pass through many drops of media, from the biopsy drop to a holding drop, through 5 vitrification solution drops, and then plunged into liquid nitrogen (LN^2). During the warming procedure they are again passed through 4 drops of diluent until they are placed in a culture

media drop. Therefore during fresh culture, embryos would have to be extensively rinsed to remove all cumulus cells from the *zona pellucida*e.

The length of time that embryos remain in the media drops before testing the media may be important. Results for the 24 hour period of culture were more concurrent than the fresh samples cultured for 48 hours. Cell free DNA degrades quickly compared to nuclear DNA, therefore if embryos remain in the same droplet for 48 hours, the cell free DNA from day 1 of micro drop culture may degrade and mix with the newly generated cell free DNA giving spurious results. A longer time in culture may give rise to more cell free DNA for testing but this may be of poorer quality.

The cost of spent media sampling and handling would potentially cost less than the cost of the biopsy procedure. There would be no micromanipulation and therefore no holding or biopsy pipettes needed to purchase. However, the processing of the spent media is still very detailed and time consuming for the lab. When removing the embryo from the media drop, extreme care has to be taken to aspirate a minimal volume along with the embryo so as to leave behind enough for testing. Each 3ul drop of spent media still has to be carefully sampled and placed into individual tubes therefore the cost of sampling would not fall to zero. However the cost of the analysis of the spent media would remain the same as for an actual biopsy as each sample would be processed in the same way as a cellular biopsy. The embryos would still have to be vitrified to allow for results of the spent media analysis to be concluded. Therefore the only time saving over biopsy would be on average 15 minutes per case depending on the number of available embryos.

Three of the results in this study had a no read result for the biopsy sample but did have a result for the spent media. When biopsying an embryo, there is a chance of obtaining a no read or chaotic result in 2-3% of biopsied blastocysts. This means that, either the cells were degenerating, the sample was too small or the sample was not placed in the testing tube. If needed, we thaw, re-biopsy and refreeze the embryo in order to get a definitive result. Taylor *et al* (Taylor *et al*., 2014b) showed that thawing non screened embryos for PGS and then refreezing, had a negative effect on subsequent survival of the embryos (87% for twice frozen/thawed vs 98% for once frozen thawed) but once they had survived the twice frozen group resulted in pregnancies with the same rate as their once frozen thawed counterparts. There is little data on the effect of biopsying blastocysts twice in addition to multiple freeze/ thaw events.

It is possible if we can reach a stage of confidence with analyzing spent media that we could use the spent media as a secondary result and reduce the need for re-biopsy of no read result embryos. The other practical use for this methodology may be that we could use spent media analysis for a previous no read biopsy result and just culture the embryo overnight rather than re-biopsying and then re-freezing. This would reduce the amount of micromanipulation to the embryo.

Further studies are needed to assess the potential for non-invasive PGS in fresh cycles as a replacement for biopsy. The protocol needs to be optimized to decide the following: length of

time in culture before sampling, temperature to store the samples before amplification, time from sampling to amplification and fine tuning of processes to minimize cumulus contamination.

Noninvasive PGS would be revolutionary by reducing potential risks to the embryo. As the basic tenet of any good embryology lab is to keep the process as simple and as near to physiologically normal as possible then to reduce micromanipulation to the embryo can only be a good thing. It may in fact give rise to more accurate representation of the embryo proper if cells are being damaged during the biopsy process. A further trial is planned to optimize the process and to further correlate biopsy and spent media samples in the initial embryo creation cycle.

2.3 Specific aim 2

To determine if the technique of ICSI creates embryonic aneuploidy

This is addressed in the paper entitled ” The use of suboptimal sperm increases the risk of aneuploidy of the sex chromosomes in preimplantation blastocyst embryos ” Written by Alison Coates, John Hesla, Amanda Hurliman, Breanne Coate, Elizabeth Holmes, Rebecca Matthews, Emily Mounts, Kara Turner, Alan Thornhill and Darren K Griffin . Published in Fertility and Sterility (Coates *et al*., 2015)

2.3.1 My Personal Contribution to the Work

I biopsied some of the embryos, retrospectively analyzed the data generated during clinical IVF cases and I wrote and edited the paper.

2.3.2 Chapter Summary

This chapter discusses the hypothesis that the ICSI procedure may contribute to embryonic aneuploidy. Patients undergoing IVF with normal semen parameters were advised to have standard insemination or ICSI to achieve optimal fertilization rates based on the following criteria:

- Standard insemination: Couples using fresh sperm where the male partner had had previous naturally occurring pregnancies.

- ICSI: Patients using frozen sperm or fresh sperm from male patients without a history of natural conception.

568 patient cycles were into 2 main groups: Patients less than 40 years of age using their own oocytes and patients using young donor oocytes. Within these groups, aneuploidy rates with and without ICSI were considered. Aneuploidy rates were similar for patients using ICSI or standard insemination proving that the ICSI technique itself does not create aneuploidy in preimplantation embryos.

2.3.3 Introduction

Intra-Cytoplasmic Sperm Injection (ICSI) is used routinely during the IVF cycle to achieve fertilization. Originally used for male factor infertility it is increasingly used for patients with normal semen parameters for example if the sperm is frozen, which allows analysis of data from ICSI created embryos that have not been created with sub-optimal sperm . As there is evidence that sperm from patients with male factor infertility have higher rates of aneuploidy than patients with normal parameters (Bernardini *et al*., 2005; Tempest and Griffin, 2004) the assumption remains that this could translate into a higher rate of aneuploidy in resulting embryos. Removing this population from the data analysis therefore refines the parameters and only considers the ICSI technique alone as a possible mechanism or not for increasing rates of aneuploidy.

As the technique itself is invasive , it has been suggested that it may have the potential to be detrimental to embryo development in several ways (Griffin *et al*., 2003). ICSI has been shown to

compromise sperm nuclear de-condensation, possibly leading to aneuploidy in the embryo (Terada Y1, 2000). The ICSI process has the potential to disrupt the oocyte spindle apparatus when the ICSI needle passes through the oolemma into the center of the oocyte, possibly leading to abnormal patterns of chromosome segregation (Van Der Westerlaken *et al*., 1999). Finally, the oocyte may be handled outside of the incubator for a longer period of time during ICSI as compared to standard insemination, during cumulus cell removal with hyaluronidase, and injection of sperm. Slight temperature and pH changes during micromanipulation may increase the possibility of stress-induced aneuploidy (Dumoulin *et al*., 1999; Munne *et al*., 1997; Swain *et al*., 2016). All of these interventions associated with the ICSI technique have the potential to impair chromosome segregation in the oocyte and subsequently the cleaving embryo.

If the ICSI process itself induces aneuploidy, one would expect that all ICSI treatments would show an increase in embryo aneuploidy as compared to standard insemination cycles, regardless of sperm quality. To date there has not been a published study definitively establishing whether the ICSI process itself creates aneuploidy in pre-implantation embryos. ICSI conceived products of conception (POC) have been analyzed and compared to naturally conceived and IVF conceived POCs. (Lathi and Milki, 2004) (Wu *et al*., 2016) (Bingol *et al*., 2012) and showed no increase in overall aneuploidy rates between the two groups.

Testing the ploidy status of pre-implantation embryos via trophectoderm biopsy and preimplantation genetic screening (PGS) is now a routine practice in many IVF centers throughout

the world. The objective of this study was to test the hypothesis that aneuploidy frequency (sex chromosome or autosome) in human pre-implantation embryos increases as a result of the ICSI procedure itself.

2.3.4 Materials and Methods

(See general Materials and Methods for stimulation, culture, vitrification and DNA quantification protocols. Section 2.7)

Between August 2010 and March 2015, 586 couples underwent IVF using either ICSI or standard insemination with normal semen parameters. A total of 3683 embryos resulting from these cycles were tested for aneuploidy by trophectoderm (TE) biopsy followed by preimplantation genetic screening (PGS) using array comparative genomic hybridization (aCGH) in a single tertiary fertility center.

All male patients underwent semen analysis before the IVF cycle commenced and again on the day of the oocyte retrieval to determine whether ICSI or standard insemination would be employed to achieve fertilization. Parameters measured included sperm density/mL, forward progression, speed of progression, percent normal forms (WHO 4th edition), and anti-sperm antibody binding. (WHO, 2010) Patient history was also taken into account when deciding which method of insemination was to be used on the retrieval day. Only patients with normal semen parameters were included in this analysis and the reason for performing ICSI with normal semen

parameters were: the use of frozen sperm, many years of unexplained infertility without a pregnancy, or previous poor fertilization using standard insemination in an earlier IVF cycle.
For standard Insemination, 15,000 sperm were placed with each cumulus-oocyte complex on the day of oocyte retrieval. The ICSI procedure was performed on all mature oocytes as described elsewhere (Van Der Westerlaken *et al*., 1999).

Any possible effect of the ICSI procedure itself was controlled for by comparing rates of aneuploidy in embryos derived from the control group involving standard insemination with normal sperm and ICSI with normal sperm. Finally, in order to control for maternal age, the groups were further sub-divided into "own oocytes" and "donor oocytes" with the mean maternal age near identical in both cases (35.3-35.5 for "own oocytes" and 24.9-25.0 for "donor oocytes"). Thus, four groups were examined in total and differences in aneuploidy rates for each chromosome pair evaluated by Fishers exact test (significance set at $p=<0.05$).

Institutional review board approval was obtained for review of patient charts and laboratory data for this study. The study was also approved by the University of Kent Local Research and Ethics Committee.

2.3.5 Results

Our results are summarized in table 13. Each chromosome pair is considered individually and the data set broken down into couples in which their own oocytes were used and in which donor oocytes were used. In both groups, standard IVF (first column) is compared to ICSI with normal semen parameters (second column). For both “own oocytes” and “donor oocytes” the mean maternal age was near identical within the three groups i.e. 35.5 and 35.3 respectively for the “own oocytes” group, and 24.9, 25.0 for the “donor oocyte” group. The greatest number of cycles involved ICSI with normal sperm (262 and 222 respectively). When standard insemination was compared to ICSI with normal sperm, no significant difference was seen, either when considering overall aneuploidy, overall autosomal aneuploidy, sex chromosome aneuploidy or aneuploidy of any individual chromosome.

	Group 1 own oocytes <40		P value	Group 2 donor oocytes		P value
	std insem	ICSI normal sperm		Std insem	ICSI normal sperm	
Ave age	35.5	35.3		24.9	25	
# cycles	77	262		25	222	
# embryos	385	1300		208	1743	
Ave # embryos for biopsy	5	5	>0.05	8	7.9	>0.05
# total aneuploid %/embryo	158 41%	477 37%	>0.05	44 21%	394 23%	>0.05
# with autosomal aneuploidy %/embryo	155 40%	466 39%	>0.05	42 20%	384 22%	>0.05
# with sex chromosome aneuploidy	8 2.1% **XXX,XO** x3 **XXY** x3,**XYY**	22 1.6% **XO** x 12,**OY** **XXYY,XXX** x4 **XXY** x2,**XYY** x2	>0.05	3 1.4% **XO** x2,**XXY**	35 2.0% **XO** x20,**OY** x2 **XYY** x2,**XXY** x9 **XXX** x2	>0.05
1	2.3	1.5		1.4	1.4	
2	2	1.7		1.4	1.5	
3	1.6	1.5		1.4	1	
4	1	1.9		1.4	1.4	
5	2	1.6		1.4	1.4	
6	2.6	2.8		2.4	1.8	
7	2.6	2.8		2.4	1.8	
8	2.9	1.5		1.4	1.8	
9	1.8	1.7		1	1.2	
10	2.6	1.4		1	1.3	
11	0.8	1.8		1.4	1.1	
12	0.8	1.1		1.4	0.9	
13	3.1	2.4		0.5	1.5	
14	1.3	2.6		0	1.4	
15	4.9	3.9		1.9	1.4	
16	7.5	7		2.4	3.4	
17	1.6	1.2		1.4	0.5	
18	3.6	2.3		1	1.3	
19	4.7	2.6		1.4	1.3	
20	2.9	1.8		0	1	
21	6.2	5.5		1.4	1.5	
22	3.9	5.8		1.4	1.6	

Table 13: Comparing embryonic aneuploidy rates for ICSI vs sta>0ndard insemination with normal sperm

2.3.6 Discussion

Since the implementation of ICSI over 20 years ago, IVF babies born using both ICSI and standard insemination techniques have been followed closely to assess rates of major birth defects as compared to spontaneously conceived babies. There appears to be an equal, modest increase in major birth defects with IVF-derived offspring as compared to naturally conceived offspring, whether ICSI or standard insemination are used to achieve fertilization *in-vitro* (Wen *et al*., 2012). This suggests that the complex IVF process as a whole, or an intrinsic aberration in the infertile population, is responsible for this increase, rather than the insemination method used. Indeed, follow-up of babies conceived via ICSI and standard insemination thus far have been relatively reassuring in that there appears to be no difference in cognitive or motor development, pubertal development, or major birth defects between standard insemination and ICSI-derived offspring (Basatemur *et al*., 2010; Bonduelle *et al*., 2005; Leslie *et al*., 2003; Ponjaert-Kristoffersen *et al*., 2005).

Aneuploidy frequency in spontaneously aborted conceptuses conceived via ICSI compared to standard insemination has been examined with varying conclusions. Bonduelle *et al* reported increase in sex chromosome abnormalities and *de novo* chromosomal aberrations in pregnancies derived from ICSI with suboptimal sperm parameters as compared to standard insemination-conceived pregnancies (Bonduelle *et al*., 2002). Lathi *et al* also observed a marked increase in overall aneuploidy frequency in products of conception (POC) from ICSI patients compared to those derived from standard insemination (76% ICSI vs 41% IVF, $P<0.01$).

Kushnir *et al*. observed similar overall rates of aneuploidy in POC between standard insemination and ICSI, although a small increase in sex chromosome abnormalities in the ICSI group was found (Kushnir and Frattarelli, 2009; Lathi and Milki, 2004). Again these studies did not consider sperm quality in their analysis. In one of the few studies analyzing aneuploidy rates of ICSI vs. standard insemination-derived embryos, Munné *et al*. found that ICSI embryos did not show an increase in aneuploidy rates compared to embryos created using standard insemination, unless the parents had a balanced chromosomal abnormality (Munne *et al*., 1998c). However, in that report, embryos were biopsied on day 3 and analyzed using FISH for chromosomes X, Y, 13, 16, 18, and 21. Only a subset of the karyotype was therefore analyzed and the use of FISH for determining aneuploidy in human embryos has come under considerable scrutiny due to its limitations for chromosomal screening of pre-implantation embryos.

In the results presented here, we controlled for any effect that the ICSI process might have on aneuploidy frequency by separately analyzing data of embryos created from normal sperm using ICSI and standard insemination techniques. Our study also suggests that, at least in terms of the risk of aneuploidy, the ICSI procedure using sperm with normal parameters is no more risky in creating aneuploid embryos than standard insemination. With the current findings and past studies in mind, patients undergoing ICSI as part of their IVF cycle with normal semen parameters, need not be counselled for increased embryonic aneuploidy rates based on the use of ICSI alone.

2.4 Specific aim 3

To establish novel patient populations that may benefit from the use of pre-implantation screening

PGS is most commonly applied to patients of advanced maternal age and patients who have had repeated miscarriages, as they have a high risk of aneuploidy occurring in each embryo created (Harton *et al*., 2013; Shahine *et al*., 2016). Other categories of patient may also benefit from PGS and two novel patient populations are discussed in this chapter.

2.4.1 Population number 1: Male factor infertility patients

This is addressed in the paper entitled ” The use of suboptimal sperm increases the risk of aneuploidy of the sex chromosomes in preimplantation blastocyst embryos ” Written by Alison Coates, John Hesla, Amanda Hurliman, Breanne Coate, Elizabeth Holmes, Rebecca Matthews, Emily Mounts, Kara Turner, Alan Thornhill and Darren K Griffin. Published in Fertility and Sterility (Coates *et al*., 2015).

This manuscript answers the question: do male factor infertility patients warrant counselling to consider utilizing PGS during the IVF cycle? The study design is described in the previous chapter addressing if the ICSI technique itself creates aneuploidy.

2.4.1.1 My Personal contribution to the work

I biopsied and cultured some of the embryos, retrospectively analyzed data generated as part of regular IVF cycles and I wrote and edited the paper.

2.4.1.2 Chapter summary

Aneuploidy rates of 3,242 ICSI generated blastocysts, created with oligozoospermic and normozoospermic semen samples were compared between July 2010 and December 2013. Overall aneuploidy rates between the 2 groups were similar however there was a significant increase in sex chromosome aneuploidy in embryos generated from males with oligozoospermia (6.6%) compared to embryos from normozoospermic males (3.3%). Data from January 2014 to December 2015 from a further 2127 donor egg generated embryos were subsequently added to the dataset post publication of the manuscript. Patients presenting with male factor infertility should be aware that the rates of sex chromosome aneuploidy when using suboptimal semen samples may be increased therefore offering PGS to this group of patients is warranted.

2.4.1.3 Introduction

Studies have shown evidence of a significantly higher proportion of aneuploid sperm in the ejaculates of men with male factor infertility when compared to normal controls (Bernardini *et al*., 2005; Mehdi M1, 2012; Vegetti W1, 2000). A review of the literature by Tempest *et al*, demonstrated a clear association between suboptimal semen parameters, principally oligozoospermia, and increased sperm aneuploidy rates for most chromosomes examined,

although it was noted that patient parameters were not always clearly defined (Tempest and Griffin, 2004). Sperm of poor quality are frequently unable to penetrate an oocyte, either *in-vivo* or through standard insemination techniques *in-vitro*, possibly leading to failure of fertilization. To overcome this barrier to conception, intra-cytoplasmic sperm injection (ICSI) was developed and has found widespread, worldwide use as the treatment of choice for male factor infertility (Palermo *et al*., 1992; Van Steirteghem *et al*., 1993). Partly because of the increase in sperm aneuploidy associated with some infertile men, the rates of aneuploidy in the products of conception of nonviable pregnancies and the prevalence of birth defects and achievement of developmental milestones in children conceived through assisted reproduction have been studied. This has enabled the comparison of the outcomes of standard IVF, ICSI and natural conception and results have been relatively reassuring (Bonduelle *et al*., 2005; Kushnir and Frattarelli, 2009; Lathi and Milki, 2004; Sazonova *et al*., 2011).

If the genetic component of sperm is the predominant risk factor for aneuploidy in the blastocyst embryo, one may expect that aneuploidy would be the most prevalent in embryos derived from the sperm of more severely oligozoospermic males (as the potential for sperm aneuploidy appears to be directly correlated with the severity of abnormalities in semen parameters).

2.4.1.4 Materials and Methods

(See general Materials and Methods for stimulation, culture, vitrification and DNA quantification protocols. Section 2.7)

For the purposes of testing the hypothesis that it is the effect of compromised semen parameters that increase embryonic aneuploidy rate, patients were divided into 2 treatment groups: The first underwent ICSI with normal semen parameters (>19 x 10^6/ml, >30% gross normal morphology, >30% motility, WHO 4th edition). The second underwent ICSI but male patients were diagnosed with severe oligozoospermia (<6 x 10^6/ml sperm).

2.4.1.5 Results

In both the original published data and the updated dataset the sex chromosome aneuploidy rate was significantly greater in the oligospermic patient group than the group with normal sperm. In the original dataset, it was 6.1% in the oligozoopsermic group compared to 1.6% in the normal semen category (p=0.007) for the “own oocyte” group; and 5.9% in the oligozoopsermic group compared to 2.0% in the normal semen category (p=0.04) for the “donor oocyte” group. In the reanalysis of newer data using donor oocyte only to minimize the maternal age variable, overall aneuploidy was similar in both the ICSI with normal sperm and ICSI with oligozoospermic sperm, but the original findings were reconfirmed in that the oligozoospermic group had a statistically significantly higher rate of sex chromosome aneuploidy at 5.5% of embryos biopsied compared to 3.3% in the embryos created with sperm with normal sperm parameters (p=0.03).

There was no significant increase in overall aneuploidy as a result of ICSI with oligozoospermic males, nor in the overall rate of autosomal aneuploidy. In the original dataset, three individual autosomes however did show significantly increased levels of aneuploidy in the oligozoospermic group, namely chromosomes 1 and,2, (own oocytes) and chromosome 18 (donor oocytes).

For each of these chromosomes in turn, the aneuploidy levels in the oligozoospermic samples were: Chromosome 1 - 7% (compared to 2.3 and 1.5% - p=0.01); Chromosome 2 – 6.0% compared to 2.0 and 1.7% - p=0.006; Chromosome 18 – 4.7% (compared to 1.0 and 1.3% - p=0.02). Other autosomes showed greater aneuploidy levels in oligozoospermic males but not at the level of statistical significance (threshold $p<0.05$).

More data has been analyzed since the publication of the paper. This extended data set shown in table 15, using only donor oocytes to control for maternal age, confirms the previous finding at the time of publication of an increase in sex chromosome aneuploidies in embryos created from oligozoospermic samples vs normal semen parameters. In this larger data set than the original data there showed no increase in aneuploidies for any of the autosomal chromosomes.

		Group 1 own oocytes		P value	Group 2 donor oocytes		P value
		ICSI normal sperm	ICSI oligo sperm	P value	ICSI normal sperm	ICSI oligo sperm	P value
	Ave age	35.3	35.3		25	25	
	# cycles	262	31		222	12	
	# embryos	1300	114		1743	85	
	Ave # embryos for biopsy	5	3.7		7.9	7.1	
	# total aneuploid %/embryo	477 37%	53 46%	P=>0.05	394 23%	23 27%	p=>0.05
	# with autosomal aneuploidy %/embryo	466 39%	51 45%	P=>0.05	384 22%	19 22%	p=>0.05
	# with sex chromosome aneuploidy	22 1.6% **XO** x 12, **OY,XXYY, XXX** x4 **XXY** x2,**XYY** x2	7 **6.1%** **XO** x5 **XXY** **XXX**	P=0.007	35 2.0% **XO** x20 **OY** x2,**XYY** x2 **XXY** x9,**XXX** x2	5 **5.9%** **XO** x2 **XXY** x2 **OY**	p=0.04
	1	1.5	**7**	p=0.001	1.4	2.3	
	2	1.7	**6**	p=0.007	1.5	0	
	3	1.5	0.9		1	1.2	
	4	1.9	4.4		1.4	3.5	
	5	1.6	1.8		1.4	3.5	
	6	2.8	2.6		1.8	1.2	
	7	2.8	2.6		1.8	3.5	
	8	1.5	2.6		1.8	2.3	
	9	1.7	2.6		1.2	1.2	
	10	1.4	1.8		1.3	2.3	
	11	1.8	4.4		1.1	0	
	12	1.1	0		0.9	3.5	
	13	2.4	2.6		1.5	1.2	
	14	2.6	0.9		1.4	2.3	
	15	3.9	2.6		1.4	2.3	
	16	7	5.3		3.4	5.9	
	17	1.2	1.8		0.5	3.5	
	18	2.3	0.9		1.3	**4.7**	p=0.03
	19	2.6	1.8		1.3	0	
	20	1.8	1.8		1	1.2	
	21	5.5	3.5		1.5	1.2	
	22	5.8	7		1.6	2.3	

Table14: Embryonic Aneuploidy rates and chromosomes affected according to sperm quality (original data from the published manuscript)

		ICSI normal sperm	ICSI oligo sperm	P value
	Ave age	25	25	
	# embryos	3590	365	
	# euploid %/embryo	2665 74%	266 73%	p=>0.05 ns
	# with sex chromosome aneuploidy	117 3.3% **OY x2, XO x 55, XYY x 23, XXY x 29, XXX x 8**	20 **5.5%** **XO x 13, XYY x 5, XXY x 2**	p=0.03
	1	1.9	1.6	
	2	2.1	3.6	
	3	1.8	2.2	
	4	2.0	2.5	
	5	1.7	2.8	
	6	2.0	1.6	
	7	1.9	2.7	
	8	2.2	1.6	
	9	2.2	2.2	
	10	1.3	1.0	
	11	1.6	1.4	
	12	1.4	1.9	
	13	2.0	2.5	
	14	2.1	1.4	
	15	1.9	3.0	
	16	3.6	3.0	
	17	0.8	1.6	
	18	1.5	**2.7**	
	19	1.5	0.8	
	20	1.6	2.7	
	21	2.0	2.2	
	22	2.3	3.6	

Table 15: Embryonic Aneuploidy rates and chromosomes affected (donor egg derived embryos only), according to sperm quality subsequent data analysis post publication of the manuscript to further support the differences between sex chromosome defects seen in the original dataset.

2.4.1.6 Discussion:

The frequency of occurrence of sex chromosome abnormalities in live born offspring varies with the type of aneuploidy. Males with Klinefelter syndrome (47,XXY) occur 1 in 500 (0.2%) of live births (Visootsak and Graham, 2006) compared to 5 in 365 (1.4%) blastocyst embryos derived from oligozoospermic sperm in the extended dataset in this study. The prevalence of newborns with Turner syndrome is 32/10,000 (0.03%) and 176/100000 for fetuses at amniocentesis (0.18%) (Gravholt *et al*., 1996). In the extended dataset from the present study the rate of blastocyst embryos with monosomy X from the oligozoospermic group was 13/365 (3.6%).

In humans, the majority of aneuploidies are maternal in origin. In the sex chromosomes however, while 47,XXX is 5% paternal in origin; 47,XXY is 50% paternal in origin and 100% of 47,XYY cases are paternally derived. For 45,XO, 75% of cases indicate that the remaining sex chromosome is maternal in origin, suggesting that the error arose on paternal meiosis (Griffin, 1996; Hassold T, 1996). If increased sperm aneuploidy is indeed the underlying cause of our observations than we would therefore expect the effect to be seen predominantly in the sex chromosomes, rather than the autosomes and to observe proportionally fewer 47,XXX and 45,OY conceptuses. In our updated amended results (table 15), there were zero 47,XXX and zero 45 OY aneuploidies in the male factor group but these anomalies were present in the normal sperm group.

In the original dataset, some autosomes showed a significant increase in the oligozoospermic group compared to the normal sperm group. Chromosomes affected were 1, and 2 in the "own

oocytes" group. Among spontaneous abortions, the larger chromosomes (up to chromosome 12) have greater paternal contributions to trisomy (Griffin, 1996; Hassold T, 1996) and it is noteworthy that all these autosomes fall into these category (including three of the largest four). In the "donor oocyte" group none of the autosomes apart from chromosome 18 showed elevated levels of aneuploidy for the oligozoospermic males. As a smaller chromosome, 18 is predominantly (96%) maternally derived (Griffin, 1996; Hassold T, 1996) so increased sperm disomy is unlikely to be the cause. The result may of course be a statistical anomaly and autosomal aneuploidy was not increased significantly in the latest dataset. One possible explanation if the dataset was representative is that trisomy 18 is the only autosomal aneuploidy of which we are aware that arises predominantly in maternal meiosis II. Human oocytes only complete meiosis II post-fertilization so perhaps a (genetically) sub-optimal sperm may impair subsequent segregation of maternal chromosome 18 more than any other. Some of these questions could be addressed in future studies by a molecular analysis of the phase and parent of origin of the aneuploidy, the ability to do this has recently been reported using SNP microarrays (Gabriel AS, 2011; Handyside *et al*., 2010). Such an approach is not trivial however as it would require taking and sampling parental DNA. This is not currently routine for ethical, logistical and cost reasons however if new approaches such as Karyomapping were implemented (Natesan S, 2014; Thornhill AR, 2015), it might become so in the future.

Since studies have shown that oligozoospermic sperm samples have an elevated proportion of aneuploid sperm, one suggested clinical solution has been to screen the sperm of these patients

using FISH to determine aneuploidy frequency prior to ICSI. One of the limitations of the present study was that only one semen parameter (sperm concentration) was considered for analysis. Further studies might involve asking whether any other specific semen parameters (apart from oligozoospermia) impact on blastocyst aneuploidy. Indeed, for the most part, studies have focused on oligozoospermia and Tempest *et al* (Tempest and Griffin, 2004) suggested that the sperm concentration was most likely to be correlated to the levels of sperm disomy than motility or morphology. A more recent study showed that men with poor sperm morphology that had repeated pregnancy loss had a higher occurrence of sex chromosome and autosome aneuploidy in their sperm than those with normal morphology (Ramasamy *et al*., 2015). These men also showed a higher rate of sperm aneuploidy with chromosome 18, 13 and 21. The clinical value of testing sperm samples with FISH to assess the proportion of aneuploid sperm may only be useful in helping to direct patients towards PGS testing of embryos if they had otherwise not been inclined to do so.

In any event it is very unlikely that patients or clinicians (the groups that this data will affect most) will pay a great deal of attention to whether or not the abnormalities are paternally derived. Moreover, when we presented these preliminary results to the Pacific Coast Reproductive Society recently and 62% of 700 participants said that they would change their practice as a consequence of our findings. A further consideration is that we have only sampled a few cells from the trophectoderm, assuming the ploidy status to be representative of the rest of the embryo. Confined placental mosaicism (where cell derived from the trophectoderm differ

in ploidy status to those derived from the inner cell mass) is nonetheless a well-described phenomenon (Griffin, 1996; Kalousek DK, 1992) with the errors of meiotic origin most likely leading to adverse clinical outcomes. Implementation of an approach that can not only distinguish parent, but also phase, of origin of the aneuploidy (Gabriel AS, 2011; Handyside *et al*., 2010) would ultimately allow us to select against those embryos that arose as a result of fertilization with an aneuploid sperm.

Given that sex chromosome aneuploidy was significantly elevated in both "own oocyte" and "donor oocyte" populations we provide evidence that the observed effect is independent of maternal age. Moreover, as no significant increase was seen for any of the chromosomes in either of the "ICSI with normal sperm" groups, the most plausible explanation for our observations was sub-optimal genetic quality of the sperm (probably increased sperm aneuploidy), rather than any effect of the procedure itself. Our study advances the current knowledge base in providing strong evidence that it is the quality of the sperm that may be the underlying cause that could lead to the slight increase in rates of anomalies associated with ICSI (specifically sex chromosomal aneuploidy) and not the consequence of the ICSI procedure itself.

With the additional data as further confirmation of our original findings, we feel that this provides sufficiently compelling evidence that it is the presence of compromised semen parameters that led to the increase in the rate of sex chromosome aneuploidy in this patient group. Patients should be counselled accordingly and PGS should be offered as an adjunct to IVF treatment for male factor infertility.

2.4.2 Population number 2: Young oocyte donors

Paper entitled: Differences in pregnancy outcomes in donor oocyte frozen embryo transfer (FET) cycles following Preimplantation Genetic Screening (PGS): A single center retrospective study. Published in November 2016 in the Journal of Assisted Reproduction and Genetics.

Authors: Alison Coates, Brandon Bankowski, Allen Kung, Darren Griffin and Santiago Munne (Coates *et al*., 2016a)

2.4.2.1 My Personal Contribution to the Work

I conceived the idea, biopsied some of the embryos, analyzed the data and wrote and edited the paper.

2.4.2.2 Chapter Summary

The data used for this retrospective analysis was from patients undergoing frozen thawed embryo transfers only. Fresh non tested embryo transfers were not included in this study. The goal of this paper was to discuss the merits of PGS on donor oocyte derived embryos prior to cryopreservation. Many patients using donor oocytes in our own program need to use a gestational carrier (GC) to carry the pregnancy. Often the oocyte donor is ready to cycle before the GC is even recruited by the patient therefore embryos have to be frozen to await the GC. Patients using their own uterus also may wish to postpone a transfer for social or economic reasons and also may need to cryopreserve embryos. Both groups of patient (own uterus and GC uterus) were considered separately due to the intrinsically better pregnancy outcomes when

using a known fertile GC uterus compared to the intended parent's own uterus which may have experienced many years of infertility and may have never carried a baby to term.

To maximize live birth rates per embryo post thaw is the goal and this paper analyzed data from frozen thawed embryos with and without PGS to assess live births per embryo to establish if screening prior to freezing increased live birth outcomes. The own uterus group showed a highly significant difference in favor of PGS (59% PGS vs 36% no PGS P= 0.0005) and the GC uterus group showed a 10% difference in favor of PGS which almost reached significance (68% PGS vs 58% no PGS P= 0.07).

In our own data set, PGS before cryopreservation increases live birth rates in patient's using young donor oocytes and should be recommended as part of the treatment cycle.

2.4.2.3 Introduction

Approximately 12% of all IVF cycles in the US are completed using donor oocytes from young anonymous oocyte donors, which equated to over 20,000 cycles in 2013 (SART, 2013). Reasons for using donor oocytes from young women rather than autologous oocytes during cycles of IVF are varied. They include: advanced maternal age, premature ovarian failure, diminished ovarian reserve, multiple failed IVF cycles using own oocytes, oophorectomy, same sex male couples, maternal single gene defects and cancer treatment in the female patient.

As aneuploidy frequency in human pre-implantation embryos increases with maternal age (Franasiak *et al*., 2014b; Munne *et al*., 2007a), implantation and live birth rates decrease (SART, 2013) because most aneuploid embryos either fail to implant (Munne *et al*., 2004) or miscarry and, rarely, are compatible with life (Kim *et al*., 2010; Werner *et al*., 2012). National IVF live birth rates for embryo transfers (ET) using fresh embryos compiled by the Centre for Disease Control in 2013 from patients younger than 35 were 48% per ET compared to patients aged 41-42 with a live birth rate of only 16% per ET (SART, 2013). While fresh embryo transfers using donor oocytes have resulted in high live birth rates without PGS (live birth rate 77%/ET 2012 SART published Oregon Reproductive Medicine (ORM) data (SART, 2012)), frozen ET live birth rates in this group have been lower. Improvements in freezing techniques over the last ten years have led to better survival of embryos and therefore higher pregnancy rates. National US data (SART, 2013) comparing fresh vs frozen ETs in the donor oocyte recipient group showed that in 2003 fresh live birth rate/ET was 51% compared to frozen ET at 30%. In 2013 the national fresh live birth rate/ET rate was 56% compared to 40.5% with frozen ET. The gap between fresh and frozen embryo transfers has thus become less over this time period from a 21% difference in 2003 to a 14.5% difference in 2013, partly attributable to improved freezing methods. In all of the above comparisons it has to be noted that the CDC data does not break down the data into day 3 vs blastocyst stage transfer. However, in data from our own lab comparing slow freezing vs vitrification, using donor egg derived blastocyst stage embryos, 53 transfers of slow frozen balstocysts resulted in 22 live births (40%) compared to 1110 transfers of vitrified blastocysts resulting in 698 live births (63%) ($p= 0.009$, Fishers exact test).

PGS of blastocysts before transfer, it has been suggested, can reduce the maternal age effect on implantation (Harton *et al*., 2013), significantly increase live birth rates, and reduce miscarriage risk in IVF cycles when using autologous oocytes (Dahdouh *et al*., 2015a; Forman *et al*., 2012a; Hodes-Wertz *et al*., 2012; Lee *et al*., 2015; Schoolcraft *et al*., 2010; Schoolcraft and Katz-Jaffe, 2013; Scott *et al*., 2013a; Yang *et al*., 2012). This area is perhaps among the most contentious in reproductive medicine however with significant proponents and opponents on both sides.(Chen *et al*., 2015; Verpoest *et al*., 2009) Meta-analyses and randomized controlled clinical trials have demonstrated the benefits of PGS for high-risk groups (e.g. advanced maternal age, recurrent implantation failure) however analyses and conclusions still attract criticism (Dahdouh *et al*., 2015b; Sahin *et al*., 2014). At the time of writing, the community awaits the results of the Illumina STAR trial for PGS. (Trial registration number: (NCT02268786)

To date, most attention has been on the high-risk referral categories, however many IVF/PGD practitioners have proposed that all IVF embryos should be screened for aneuploidy prior to transfer, including those from younger patients (Munne *et al*., 2006a). Although the percentage of aneuploid blastocysts from young donors is low compared to older patients (25% for donor oocytes vs 60% for 41-43 year old women, ORM unpublished data) it is nonetheless a concern since most aneuploidies arise from maternal meiotic errors in the oocytes of younger women (Ata *et al*., 2012). Sills *et al* determined, using SNP technology, the parental origin of aneuploidy using donor oocytes (Sills *et al*., 2014), finding that 88% of all the aneuploidies were attributable to maternal errors. Despite this, oocyte donor cycles have historically been the least likely group to be offered this screening.

To the best of our knowledge there has only been one study examining the use of PGS in frozen thawed donor oocyte derived embryos (Haddad *et al*., 2015). Their conclusion was that, although their dataset was small, there was an increase (not significant) in implantation rates and pregnancy rates in the PGS group compared to the non-tested group. In the absence of any statistical significance however, results remain unconvincing. A larger study is thus needed, as is stratifying patients into "own" and "gestational carrier" uterus as well as double vs. single embryo transfers. In this study we thus tested the hypothesis that ongoing pregnancy and implantation rates in frozen embryo transfer (FET) cycles are significantly different in FET cycles utilizing PGS compared to FET cycles not utilizing PGS in a single center.

2.4.2.4 Materials and Methods

(See general Materials and Methods for stimulation, culture, vitrification and DNA quantification protocols. Section 2.7)

2.4.2.4.1 Ethical statement

All patients were consented for all procedures as part of routine care. Oocyte donors were all anonymous. Institutional review board approval was obtained for review of patient charts and laboratory data for this study. The University of Kent Research and Ethics Committee also approved this study.

2.4.2.4.2 Overview

Blastocysts resulting from donor oocyte IVF cycles at the Oregon Reproductive Medicine (ORM) clinic between January 2013 and December 2015 either elected to be tested for aneuploidy by trophectoderm (TE) biopsy followed by PGS using aCGH (Gutierrez-Mateo *et al*., 2011) or NGS (Wells *et al*., 2014) before cryopreservation; otherwise, their embryos were cryopreserved without testing depending on patient preference. Post publication of the manuscript a further 89 FET cycles were analyzed and added to the original data. All blastocysts subsequently transferred in this retrospective observational study were at the day 5 stage when biopsied and vitrified. Only embryos vitrified with DMSO as a cryprotectant were included in the data analysis.

2.4.2.4.3 Recipient population and study design

Patients using donor oocytes from Caucasian donors to create embryos for their IVF cycle were included in this retrospective analysis. The patients were divided into 2 main groups: those using their own uterus and those using a gestational carrier (GC) uterus for the embryo transfer. These groups were further divided into those transferring a single embryo (SET) and those transferring 2 embryos (Double embryo transfer, DET). The groups were then finally divided into those transferring apparently euploid embryos screened by PGS (study group) and those transferring non-screened embryos (control group).

2.4.2.4.4 Uterine preparation and blastocyst warming protocol for embryo transfer

Embryo transfers of previously vitrified blastocyst stage embryos were carried out in a medicated uterine preparation cycle as described previously (Roque *et al*., 2015). Embryos were warmed using Irvine warming kits (Irvine Scientific, Irvine, CA) on the morning of the scheduled embryo transfer and allowed to re-expand in equilibrated drops of Embryoglue^{tm} (Vitrolife) under Ovoil^{tm} (Vitrolife) until the time of transfer. Embryo transfer was carried out using a Wallace Sureview Embryo transfer catheter (Cooper surgical) under ultrasound guidance. Patients rested for 45 minutes post transfer.

2.4.2.4.5 Measured outcomes and statistical analysis

Measured outcomes were live birth rate per cycle and live birth implantation rate (number of babies born per embryo transferred). Any statistical differences in each of these parameters for each group were established using Fishers exact test and Mann Whitney test on ranks where appropriate. Statistical significance was determined at $P<0.05$ at the 95% confidence level. The tested and non-tested groups had near identical average oocyte donor age and the numbers of embryos biopsied in each group was not statistically significantly different (table 1). During the study period personnel in the laboratory all remained constant and there were no major changes to protocols during the time period of the study.

2.4.2.5 Results

A total of 398 thaw cycles were included in the original analysis. One thaw cycle resulted in no transfer due to failure to survive of the one available embryo. 397 frozen embryo transfers (FET) were performed using blastocyst stage embryos generated from oocytes from anonymous donors. All embryos were classed as of good morphological appearance (AA grade, Gardner scale) (Gardner DK, 1999) prior to cryopreservation. In the test (PGS) group, 435 known euploid embryos were transferred in 294 frozen ET cycles. In the control (no PGS) group 103 embryos of unknown ploidy status were transferred in 162 frozen ET cycles.

There was no difference in survival of embryos post thaw in both the PGS and control groups (96% in the PGS group and 97% in the control group, p=1.0 NS). No patients using donor oocytes during the time period of the study had a cycle with all aneuploid embryos.

Published results are summarized in table 16.

	Gestational carrier uterus				Own recipient uterus			
	SET		DET		SET		DET	
	PGS (test)	No PGS (control)	PGS (test)	No PGS (control)	PGS (test)	No PGS (control)	PGS (test)	No PGS (control)
# Thaw cycles	95	20	100	26	58	25	41	33
# FET	95	19	100	26	58	25	41	33
# embryos transferred	95	19	200	52	58	25	82	66
# ongoing pregnancy** (%/FET)	66 (70%)	13 (68%)	93 (93%)	22 (84%)	37 (64%)	10 (40%)	34 (83%)	20 (61%)
P value:	1.0 NS		0.2 NS		0.06		0.04	
# live births (%/thaw cycle)	58 (61%)	12 (60%)	87 (87%)	20 (77%)	34 (58%)	9 (36%)	31 (76%)	18 (55%)
P value:	1.0 NS		0.2 NS		0.09		0.08	
Twinning (rate/live birth)	0	0	58 (67%)	9 (45%)	0	0	18 (58%)	6 (33%)
P value:			0.07				0.1 NS	

Table 16: Frozen embryo transfer outcome data comparing PGS vs non PGS donor egg derived embryos

Data from the original dataset show that live birth implantation rates (number of babies born per embryo transferred) were significantly higher in the PGS group in both the GC and own uterus groups, but only after double embryo transfer (DET); (72% vs 56% GC, 60% vs 36% own uterus, P values: 0.03 and 0.005 respectively). The live birth rate per transfer cycle in the DET groups were nominally higher in the PGS group compared to control group but not significantly so (87% vs 77% GC, 76% vs 55% own uterus, P values: 0.2 and 0.08 respectively). Live birth implantation rates and live births per cycle in the GC SET group were the same in the PGS and own uterus group but the control group was small including only 20 cycles. Live birth implantation rates (58% vs 36% p=0.09) and live births per cycle (58% vs 36%, p=0.09) were nominally higher with PGS vs control in the SET own uterus group but not significantly.

Combining SET and DET in a further data analysis post publication of the paper to analyze live birth rates for each embryo transferred is shown in table 17. The own uterus group showed a highly significant difference in favor of PGS (59% vs 36% P= 0.0005) and the GC uterus group showed a 10% difference in favor of PGS which almost reached significance (68% vs 58% p= 0.07).

	Gestational carrier uterus		Own recipient uterus	
	PGS (test)	No PGS (control)	PGS (test)	No PGS (control)
# FET	250	52	117	67
# embryos transferred	375	85	165	107
# babies born (Live birth/embryo)	255 (68%)	49 (58%)	97 (59%)	39 (36%)
P value	0.07		0.0005	

Table 17: Frozen embryo transfer outcome data comparing PGS vs non PGS donor egg derived embryos. Further data analysis post publication of the paper of live birth rates per embryo transferred.

Finally, live birth twinning rates were nominally higher following PGS for DET cases (67% vs. 45% for GC uterus, p= 0.07 and 58% vs 33%, for recipient own uterus p=0.1 in test and control groups respectively) compared to zero monozygotic twinning following SET in either the test or control group.

Table 18 illustrates that 25% of all donor oocyte derived trophectoderm samples tested were aneuploid, 19% with a single aneuploidy and 5.7% with more than one chromosome involved.

	Biopsied Donor oocyte blastocyst embryos	
# embryos biopsied	3393	
Total # aneuploidy (%/biopsied embryo)	835 (25%)	
# with complex aneuploidy - >1 chromosomes affected (%/biopsied embryo)	193 (5.7%)	
# with single aneuploidy (%/biopsied embryo)	642 (19%)	
	Single aneuploidy only:	
Chromosome affected:	# embryos with monosomy	# embryos with trisomy
1	18	8
2	19	10
3	7	9
4	17	8
5	13	14
6	16	11
7	21	6
8	17	12
9	10	17
10	11	9
11	16	8
12	5	11
13	19	**6 (0.2%)***
14	13	6
15	22	**16 (0.5%)***
16	38	**37 (1.1%)***
17	6	3
18	9	**4 (0.1%)***
19	11	10
20	4	9
21	13	**11 (0.3%)***
22	24	**15 (0.4%)***
Sex chromosomes	**35 (XO) (1%)***	**32 (0.9%)***
Total # of embryos with most common aneuploidies * associated with implantation and subsequent miscarriage or live birth (%/embryo biopsied)	156 (4.6%)	

Table 18: Errors per chromosome for donor egg derived embryos. 4.6% of embryos resulting in a chromosomal aneuploidy commonly associated with miscarriage or abnormal offspring (i.e. trisomy's 13, 15, 16, 18, 21, 22, XY and monosomy X). aCGH and NGS methodologies resulted in similar overall aneuploidy rates per embryo (24% for aCGH vs 26% for NGS respectively P>0.05 not significant). Monosomies and trisomy's occurred at similar frequencies (overall and per-chromosome) with chromosome 16 and the sex chromosomes most commonly aneuploid.

2.4.2.6 Discussion

Donor oocyte IVF cycles have many variants: If the female donor oocyte recipient is able to carry the pregnancy herself the embryos are transferred to her uterus. If however she has an abnormal uterus or no uterus at all the recipient may engage a gestational carrier to gestate the pregnancy. Same sex male couples or single men can fertilize donor oocytes with their own sperm and transfer resulting embryos to a gestational carrier. While the live birth rate of fresh donor oocyte embryos is high overall, if the cases which used a gestational carrier are separated out from the recipients who used their own uterus, we find an even higher pregnancy rate in the donor oocyte gestational carrier patients than when using a donor oocyte recipient uterus from an infertile patient. This even higher pregnancy rate when using a gestational carrier who has successfully previously carried a baby to term, could be attributable to the elimination of unknown uterine factors that may contribute to failure to achieve a successful pregnancy in patients who have been infertile for long periods of time.

In this study, patients elected whether or not to test their embryos by PGS before cryopreservation. The people who elected not to test had the same prognosis as the patients who did elect to test i.e. all patients presenting in our anonymous oocyte donor program had a high chance of conceiving and had access to the same pool of young oocyte donors. Patients committed to the testing process before the cycle started and patients did not change their mind depending on how many blastocysts developed. The reasons patients elected not to test were

financial, uncertainty about the biopsy process and a wish to leave the choosing of embryos up to chance.

Oocyte donor cycles have historically culminated in a transfer of fresh, untested embryos to a recipient or gestational carrier uterus or a freeze all cycle for future use. With an average of 7 good quality blastocysts produced each donor oocyte cycle, even if a fresh transfer is undertaken, there are usually surplus embryos remaining to be cryopreserved for future use (Doherty *et al*., 2014). Often an oocyte donor cycle cannot be synchronized for a fresh embryo transfer with a recipient or gestational carrier uterus for social or medical reasons and cryopreservation of the whole embryo cohort ("freeze all") becomes necessary (McLernon *et al*., 2016; Zenke and Chetkowski, 2004). The findings of this study indicate that there are possible benefits in implantation and ongoing pregnancy rates through the use of PGS in FET donor oocyte cycles. The question of whether FET cycles derived from donor oocytes need to include PGS in future should thus now be subject of larger and ultimately randomized studies.

Single center retrospective cohort studies such as this one provide supportive evidence to justify future prospective cohort studies, multi-center meta-analyses and ultimately randomized controlled clinical trials (RCTs). For evidence-based reproductive medicine, each has their value. RCTs remain the gold standard but, in a field where individual operator skills can have such a profound effect on IVF outcome, larger studies can mask effect of good (or bad) practice of individual groups or centers. In this context, smaller single center trials have considerable value

and may point to genuine efficacy of clinical interventions. On the other hand, the absence of randomization leaves an open question of whether any significant differences observed represent selection bias, inadvertent or otherwise. Moreover, different sizes of test and control groups (in this case control groups are much smaller than test groups) mean that statistical analyses need to be viewed with a degree of caution. RCTs are however expensive, time consuming and cannot be justified without sufficient published datasets from retrospective analyses. In our opinion, the current study justifies further work in this area, particularly with the increased interest in IVF and PGS.

With all the above caveats in mind, the current study is nonetheless the first to show significant differences as a result of the use of PGS in patients who elected to have it as part of their FET donor oocyte treatment cycle. Apparent improvements were seen after double embryo transfer (DET), raising the question of whether differences are only likely to be seen in this context. Equally, as the IVF world in most countries moves more in the direction of single embryo transfers (SET)(Forman *et al*., 2014; Styer *et al*., 2016; Ubaldi *et al*., 2015), attention will inevitably turn to means through which the chances of implantation of that single embryo can be maximized. In our SET GC group, there was no difference in live births per cycle nor live birth implantation rates per embryo between the PGS and no PGS embryos, and in the SET group using a recipient uterus there was a nominal increase in those parameters when using PGS but not significantly.

Taking gestational carrier and own uterus cycles together, all measures favored the PGS cycles but, in the absence of statistical significance, the question remains open about whether this represented a genuine difference. The DET results suggest that, had sufficient numbers been analyzed, the numbers might have reached statistical significance for SET *al*so, however this needs to be confirmed by further studies. Elective single embryo transfer (eSET) to reduce multiple pregnancy rates in IVF cycles is a primary goal of the reproductive medicine community (Forman *et al*., 2014). SETs however result in lower pregnancy rates per transfer, and higher rates of complete pregnancy loss (post positive pregnancy test) compared to double embryo transfer (DET). The benefit of transferring a single embryo is the reduction in the number of twin pregnancies because of the obstetric complications associated with multiple births. In future studies therefore we would look closely to ask whether the nominal differences seen for SET in this study reach statistical significance in larger data sets.

Without randomization, the question also arises about whether the statistical differences that were observed reflect patient cohorts that inherently had differing aneuploidy rates. Of course we cannot completely rule this out. In these particular patient groups however, patients had near identical oocyte donor ages, the best known correlate for different aneuploidy rates (Franasiak *et al*., 2014b; Harton *et al*., 2013; Munne *et al*., 2007a). Equally the couples who elected not to test had the same prognosis as the patients who did elect to have PGS i.e. all patients presenting in our anonymous oocyte donor program had a high chance of conceiving and had access to the same pool of young oocyte donors. Patients committed to the testing process before the cycle

started and patients did not change their mind depending on how many blastocysts developed. The reasons patients elected not to test were financial, uncertainty about the biopsy process and a wish to leave the choosing of embryos up to chance. Whether these are confounding factors that might predispose to increased aneuploidy or reduced pregnancy is questionable. In a study of 23 US clinics sending biopsies to a single reference lab (Reprogenetics, a Cooper Surgical Company) Munne *et al* showed that aneuploidy rates in donor oocyte derived embryos screened with aCGH, vary between IVF clinics (Munne S, 2015). Embryos were not reported as a mosaic result from this reference clinic when using aCGH. They were reported as euploid or aneuploid depending on the percentage of cells affected with a loss or gain. The percent of aneuploid donor oocyte embryos per clinic ranged from 20% to 58% with an average rate of 35%. One possible explanation for this is geographical variation in aneuploidy rates however there is scant data supporting this hypothesis. A more likely explanation is subtle differences in ovarian stimulation and lab protocols might lead to marked differences in aneuploidy levels between centers. Such questions are beyond the scope of this study and are not entirely relevant in this case as this is a single center study in which personnel did not change and identical standard operating practices were performed. Indeed, in our own experience, donor oocyte aneuploidy rates are lower than the average shown in the Munne study (Munne S, 2015). Nonetheless we cannot rule out inadvertent, subtle differences between treatments of test vs. control groups, however unlikely we believe it to be. It is perhaps not unreasonable to suggest however that the centers with higher aneuploidy rates in embryos derived from donor oocytes might be the ones that benefit most from PGS, should it ultimately prove effective for this application.

Aneuploidy rates between Oocyte donor cycles within clinics are also variable and can be patient (donor) specific, which is another reason why PGS can be helpful in this patient group.

The reports below illustrate the variability of oocyte donor cycles within our own clinic. The stimulation protocols and lab conditions were the same for each donor cycle.

Donor oocyte pregnancies, although at lower risk for miscarriage or offspring with chromosomal defects than pregnancies from patients of advanced maternal age, are still at risk from adverse pregnancy outcomes. The most common single aneuploidies associated with miscarriage or congenitally affected live births are trisomy's 13, 15, 16, 18, 21, 22, XY and monosomy X (Hook *et al*., 1983; Jenderny, 2014). In this retrospective analysis, 4.6% of blastocysts fall into this category (Table 18). Given that monosomies (other than monosomy X) are rarely, if ever, seen among spontaneous abortions we might expect these and other trisomy's (e.g. trisomy's 1 and 19) not to reach the stage of a clinically recognized pregnancy, perhaps through failed implantation. Others however, such as the more common trisomy's above remain a significant concern for donor oocyte recipients when using unscreened embryos for transfer, especially since prenatal diagnosis is not routinely recommended for this group. Other features also noteworthy from table 18 include: Monosomies and trisomy's occur with equal frequency indicating that embryos are equally likely to survive to the blastocyst stage whether they arise from a nullisomic or disomic gamete. Moreover the most common aneuploidies seen in spontaneous abortions (monosomy X and trisomy 16)(Shen *et al*., 2016) are the most common in this dataset, suggesting

that some patterns of chromosome-specific rates of aneuploidies are laid down before implantation.

PGS costs around $4500 (including biopsy procedure plus testing process) per cycle in addition to the IVF cycle costs. The cost including medication of one FET cycle is also around $4500. As 52% of non PGS frozen embryos fail to implant and result in a pregnancy compared to 35% of PGS embryos, it is more likely that a second or maybe third frozen embryo transfer may be needed to achieve a successful outcome when using untested embryos therefore offsetting the initial cost of embryo screening.

Aneuploidy is a condition that affects all age groups, even women in their twenties (Franasiak *et al*., 2014b). While it has been suggested that PGS is a valuable tool for improving outcome in patients of advanced maternal age (Harton *et al*., 2013), in the current study we provide preliminary evidence that the application of PGS may improve IVF outcomes using younger oocytes from an oocyte donation cycle. This research provides sufficient evidence for increased research in this area, ultimately leading to prospective randomized clinical trials to address this issue further.

This study only examined the outcome of frozen donor oocyte derived embryo transfer cycles with PGS and non PGS embryos. Fresh embryo transfer is also a consideration for this patient group. In the next chapter I attempt to answer the question whether fresh or frozen transfer of

euploid embryos results in higher live birth rates but only in patients using their own eggs. As the live birth rates for patients using fresh donor egg derived embryos without screening is already high with 63% of embryos transferred resulting in a baby, the possibility of biopsying these embryos on day 5 and rush testing overnight for a day 6 transfer into a non-stimulated recipient uterus may result in even higher live birth rates per embryo.

The issue of asynchrony between the uterus and the embryo when transferring autologous oocyte fresh embryos on day 6 in the same cycle as the oocyte retrieval as described in the next chapter is removed when using a recipient uterus. Further studies should be completed to investigate if implantation rates can be improved further using fresh euploid donor oocyte derived embryos.

2.5 Specific aim 4

To establish the optimal transfer strategy (fresh vs frozen) for euploid embryos in patients using their own oocytes

This was a randomized controlled trial (RCT) comparing the two commonly utilized transfer protocols for euploid embryos, fresh vs frozen. The data was presented as an oral abstract at ASRM 2016 in Salt Lake City and won the SART prize paper award. Paper entitled: **"**Optimal euploid embryo transfer strategy, fresh vs frozen, following PGS with NGS. A randomized controlled trial." Authors: Alison Coates, Allen Kung, Emily Mounts, John Hesla, Brandon Bankowski, Elizabeth Barbieri, Baris Ata, Jacques Cohen and Santiago Munne (Coates *et al*., 2017). Comment in Fertility and Sterility (Kort *et al*.)

2.5.1 Personal contribution to the work

I designed the study, developed the protocol, oversaw the recruitment and randomization of patients, analyzed the data, wrote the paper and responded to the reviewers comments before final acceptance for publication.

2.5.2 Chapter Summary

This RCT was developed to determine the optimal transfer strategy using PGS for patients using their own oocytes and uterus. In order to screen embryos for aneuploidy, a biopsy is taken from

the trophectoderm on day 5 or 6 of *in-vitro* development. If ploidy determination is carried out using NGS or aCGH, there is an 18-20 hour turnaround time to obtain results. Because of the length of time needed to get results, the transfer in the embryo creation cycle of a fresh embryo can only occur if a day 5 ready blastocyst is shown to be euploid by day 6. The other option for transferring euploid embryos is to freeze all embryos post biopsy and transfer in a subsequent cycle. These 2 options are routinely used in IVF clinics. The fresh day 6 option is stressful for the patient and the clinic but is perceived to be better for cycle outcome than freezing all embryos. The freeze all option allows for consideration of all embryos in the cohort (day 5 and day 6) for transfer. The results of this RCT showed that live birth rates were superior using frozen compared to fresh euploid embryos (77% frozen vs 59% fresh p= 0.04). The conclusion of this RCT was that patients using their own oocytes with PGS should opt for a freeze all cycle to achieve the best outcome.

2.5.3 Introduction

Embryo aneuploidy is likely the leading cause of implantation failure in *in-vitro* fertilization (IVF) cycles. There is well documented evidence of increasing maternal age directly correlating with an increase in embryonic aneuploidy rates (Ata *et al*., 2012; Franasiak *et al*., 2014a; Hassold *et al*., 1980; Munne *et al*., 2007a). With recent advances in IVF (extended embryo culture, trophectoderm biopsy and vitrification) along with the combination of new and advanced technology in pre-implantation genetic screening (PGS) (the use of array-comparative genomic hybridization (aCGH), quantitative polymerase chain reaction (qPCR) and next generation

sequencing (NGS) to determine all chromosome copy number), ongoing pregnancy rates have improved with the selective transfer of euploid blastocysts (Forman *et al*., 2012a; Forman *et al*., 2012b; Lee *et al*., 2015; Schoolcraft and Katz-Jaffe, 2013). Preimplantation genetic screening (PGS) is routine in some clinical IVF practices in the USA (Schoolcraft and Katz-Jaffe, 2013; Scott *et al*., 2013a). However, despite ongoing advances in reducing error rates (Capalbo *et al*., 2016a) and increasing implantation (Chen *et al*., 2015; Dahdouh *et al*., 2015b), the optimal embryo transfer strategy for euploid embryos still needs to be determined.

The two transfer strategies for euploid embryos currently in clinical practice are to utilize vitrified/warmed ("freeze-all") or fresh embryos for the first embryo transfer. The freeze-all strategy involves cryopreservation of all embryos post-biopsy, and then waiting for the PGS results of the whole cohort (day 5 and day 6 embryos) in preparation for a frozen embryo transfer (FET). The fresh strategy involves biopsy of expanded blastocysts before 10 am on day 5 and culture overnight to await PGS results for a fresh embryo transfer of euploid embryos before 12 midday on day 6. In this scenario, slower-growing embryos may be biopsied on day 6 and frozen for later use.

There are benefits and challenges to each approach. There is evidence that implantation and clinical ongoing pregnancy rates may be higher when transferring vitrified/warmed embryos in a non-stimulated cycle compared to fresh transfer in a stimulated cycle (Bhattacharya, 2016) . The incidence of low birth weight babies and pre-term delivery has also been shown to be lower in

pregnancies resulting from frozen transfers compared to fresh transfers (Maheshwari and Bhattacharya, 2013; Wennerholm *et al*., 2013).

However, success with FET requires that a laboratory's embryo vitrification methods have high survival rates. Even with the latest methods, an embryo still has about a 3% chance of being damaged by either the vitrification or warming processes (Darwish and Magdi, 2016).

Although there are an increasing number of studies supporting improved clinical outcomes after FET (Evans *et al*., 2014; Ozgur *et al*., 2015; Roque *et al*., 2015; Shapiro *et al*., 2014), fresh transfer protocols are typically more affordable, require little to no additional medications, and potentially allow the patients immediate transfer. However, a successful fresh day 6 transfer approach necessitates not only that expanded blastocysts be available on the morning of day 5, but also that at least one of these embryos is euploid, thereby also reducing the chance for a transfer.

The main aim of the present clinical trial was to identify which embryo transfer strategy after PGS by NGS, freeze-all or fresh, would improve implantation and live birth rates or if the strategies were equally successful.

2.5.4 Materials and Methods

(See general Materials and Methods for stimulation, culture, vitrification and DNA quantification protocols. Section 2.7)

2.5.4.1 Patient Population

Participants were recruited at the Oregon Reproductive Medicine Center between December 2013 and August 2015. Patients between the age of 18 and 42, while undergoing IVF and PGS using their own oocytes were eligible to participate in the trial. The exclusion criteria included the following: the need to use surgically retrieved sperm (microsurgical epididymal sperm aspiration (MESA) or testicular sperm aspiration (TESA)), patients utilizing preimplantation genetic diagnosis for a single-gene or chromosomal disorder, oocyte donor cycles, gender selection cycles, decreased ovarian reserve indicated by early follicular phase serum FSH level >10 IU/L or random serum AMH level <1 ng/ml, and any medical reasons occurring prior to recruitment which would not allow a patient to undergo a fresh embryo transfer such as the need for uterine surgery prior to transfer. Patients were excluded post recruitment prior to randomization if they were unable to undergo a fresh transfer for medical reasons such as OHSS or other medical issues.

2.5.4.2 Randomization

At the time of hCG administration, patients were randomized to either a freeze-all cycle or a fresh day 6 embryo transfer during the stimulated cycle. The stratified block randomization sequence

was prepared by a professional third party (sealedenvelope.com). The allocation sequence was stratified for female age (<35, 35 – 37, 38 – 40, and 41 – 42 years) and number of prior ART cycles (≤2 or ≥3). Women were randomized in a 1:1 ratio in blocks of 10, i.e. of every ten women in each stratum five were allocated to fresh and five to frozen transfer in a random order. Principal investigator registered each participant in the designated trial website, and allocation information was disclosed after confirmation of the eligibility criteria.

2.5.4.3 Frozen embryo transfer (FET) cycle uterine preparation

FETs were performed in an artificial cycle. A combined oral contraceptive containing 30 mcg ethinyl estradiol/0.15 desogestrel (Apri, Teva) was administered for 15-21 days starting from the third day of menstrual cycle.

Estradiol valerate 4mg/day (Delestrogen, JHP pharmaceuticals) via IM injection was commenced 5-7 Days after the last contraceptive pill, increasing by 1mg each injection until dosage of 6mg twice weekly was reached and the endometrium measured a minimum of 7.5 mm thickness and had a tri-laminar pattern visualized on ultrasound using a GE S6 device . Then progesterone in oil (Watson) was commenced at a dose of 50 mg/day intra muscularly for the initial two days and increased to 100 mg/daily thereafter. Frozen thawed embryo transfer was performed on the 7th day of progesterone injections. Patients who had a positive pregnancy test were also given 100mg Endometrin®(Ferring) administered vaginally three times a day. A weaning schedule for

estradiol and progesterone in oil was followed until discontinuation of the medication at 11- 12 weeks of pregnancy.

2.5.4.4 Fresh embryo transfer (Fresh ET) cycle uterine preparation

Fresh ETs were carried out during the original oocyte retrieval cycle. On day 2 post–retrieval, supplementary progesterone (Endometrin^{tm}, Ferring), was administered vaginally once/day along with 2mg oral estrogen supplementation (Estrace, Teva) twice a day. Day 3 post-retrieval progesterone increased to twice a day. The transfer occurred on the morning of day 6 of embryo growth. These medications continued up to and beyond the pregnancy test as for the FET luteal support protocol.

2.5.4.5 Statistical and Ethical Considerations

The primary outcome measure was the live birth rate. Live birth was defined as the delivery of a viable fetus. Ongoing pregnancy was defined as viable pregnancy progressing beyond the 8th week of gestation. Embryo implantation rate was calculated as the number of gestational sacs divided by the number of embryos transferred per group.

Continuous variables were defined with mean (standard deviation) or median (25th – 75th percentile) and compared between the groups with t-test for independent variables or Mann-Whitney U test, depending on distribution characteristics. Categorical variables were defined as numbers and percentages and compared between the groups by the chi-square test or its

derivatives as appropriate. Significance was defined as a p value of <0.05. A logistic regression analysis involving live birth as the dependent variable and female age, number of metaphase two oocytes, and embryo transfer cycle (fresh transfer as the reference) was conducted to adjust for possible differences in baseline characteristics in the per protocol analysis including all women who eventually underwent a fresh or frozen embryo transfer regardless of initial allocation.

For reference, the study center had a 63% ongoing FET pregnancy rate with euploid embryos for patients who were younger than 42 years using their own oocytes the year before commencing the study. Seventy-four patients would be required to detect an absolute increase of 20% from 63% with an alpha error level of 0.05 and a beta error level of 0.2. In order to account for possible drop-outs the aim was to recruit up to 186 participants during the study period. Offspring or products of conception were not routinely karyotyped for concurrence with the embryo biopsy results. The protocol was Institutional Review Board approved and registered with clinicaltrials.gov (NCT02000349). All patients that met inclusion criteria and expressed the desire to participate in the study were consented before ovarian stimulation.

2.5.5 Results

183 patients were consented to the study. Four patients were cancelled due to poor response before hCG administration. A total of 179 patients received hCG for oocyte retrieval and were randomized; 88 patients were allocated to the fresh transfer group and 91 to the frozen transfer group. Patients had either 1 or 2 embryos transferred depending on availability of euploid embryos and patient request. Demographics are presented in Table 19:

	Fresh Transfer (n=88)	**Frozen Transfer (n=91)**	**P value**
Average Age (range)	36.6 (25 – 42)	36.7 (27 – 42)	0.7
Average AMH (range)	3.6 (1.5 – 4.5)	3.3 (1.4-4.7)	0.7
Average FSH (range)	7.4 (0.5-21)	7.9 (1.8-15)	0.2
Oocytes collected (range)	14 (0-41)	17 (4-44)	0.1
Average # Metaphase II oocytes	11.5	13.3	0.3
2pn Fertilization rate/mature egg	78%	77%	0.6
% aneuploidy rate/embryo	37%	40%	0.4
Infertility diagnosis:			
Unexplained	23.6%	29.7%	0.4
Female factors	31.4%	30.7%	1.0
Male factor	12.4%	7.7%	0.3
AMA ≥40	11.2%	16.5%	0.4
RPL	3.4%	2.2%	0.6
>1 factor	19.1%	13.2%	0.3

Table 19: Baseline and IVF cycle characteristics of patients randomized to each treatment group

2.5.5.1 Outcome of patients in the intention to treat analysis

The intention to treat analysis considered all randomized patients in their original group of allocation regardless of achieving an embryo transfer, fresh or frozen. 14 of the fresh group allocation failed to achieve a fresh day 6 transfer (9 had only day 6 euploid embryos, 2 had to re-biopsy day 5 embryos and freeze, 1 had OHSS, 1 NGS equipment failure, 1 had pulmonary embolism so could not transfer) but did have euploid embryos available which were transferred in a subsequent FET cycle. These 14 cycles, even though they had their embryos transferred in an FET cycle, were still included in the fresh transfer group according to intention to treat protocol, as this was their original randomization allocation. One patient dropped out of the study post randomization and had a day 3 transfer of untested embryos. 8 patients in the freeze all group did not have embryos available to biopsy on day 5 but had day 6 euploid embryos available and these were transferred in their primary FET cycle. Ongoing pregnancy rates (40.9% vs 62.2% p=<0.01) and live birth rates (39.8 vs 61.5% p= <0.01) per intended treatment was significantly higher for the freeze all group compared to the fresh.

	Fresh (n=88)	Frozen (n=91)	p
Embryo transfer rate/ cycle	61/88 (69.3%)	69/91 (75.8%)	0.40
Number of embryos transferred[a]	1.4 (0.5)	1.5 (0.5)	0.27
Ongoing pregnancy	36/88 (40.9%)	57/91 (62.6%)	<0.01
Live birth	35/88 (39.8%)	56/91 (61.5%)	<0.01
Implantation rate (total number of sacs/total number of embryos transferred)	58/86 (67.4%)	79/104 (76.0%)	0.19

Table 20: Outcome in the Intention to Treat Population (Values are number (percentage) or [a]mean (standard deviation).

2.5.5.2 Outcome of patients receiving the intended transfer protocol

Only patients who had at least 1 expanded blastocyst to biopsy on day 5 were included in this analysis. A total of 46/88 (52.27%) patients underwent a fresh euploid blastocyst transfer and 61/91 (67.03%) patients underwent a frozen thawed euploid blastocyst transfer.

The mean number of embryos transferred was similar in both fresh and frozen transfer groups (1.4 and 1.5 respectively, p = 0.3). The implantation rate (sac formation/embryo transferred) was higher in the frozen group (75%) compared to the fresh (67%) but this difference was not significant (Table 21). The ongoing pregnancy and live birth rates were significantly higher for the frozen group compared to the fresh (ongoing: 80% frozen vs 61% fresh, p value=0.03, live births: 77% frozen vs 59% fresh, p value=0.04).

	Fresh (n=46)	Frozen (n=61)	P value
Embryo transfer rate per cycle	46/88 (52%)	61/91 (67%)	0.03
Average number of embryos transferred	1.4	1.5	0.3
# ongoing clinical pregnancies (%/ET)	28/46 (61%)	49/61 (80%)	0.03
# Live births(%/ET)	27/46 (59%)	47/61 (77%)	0.04
Live birth rate(babies born per embryo transferred)	55% 37/67	66% 62/94	0.1
Implantation rate (% sacs/embryo transferred)	45/67 (67%)	72/96 (75%)	0.2

Table 21: Outcomes for each transfer strategy (only includes patients who had at least 1 day 5 embryo to biopsy)

The analysis was further broken down into single embryo transfers (SET) and double embryo transfers (DET)(table 22). In the SET subset, ongoing pregnancy (52% fresh vs 64% frozen p=0.5), implantation rates (68% fresh vs 73% frozen p=0.7) and live birth rates (52% fresh vs 64% frozen p=0.7) were all trending higher in the frozen group than in the fresh group, but not significantly. In the DET subset, implantation rates (67% fresh vs 73% frozen p=0.5) and live birth rates (67% fresh vs 86% frozen p=0.1) were trending higher but not significantly, however the ongoing pregnancy rate (71% fresh vs 91% frozen p=0.04) was significantly higher in the frozen group compared to the fresh. Despite a strong trend toward improved live birth rates with frozen transfer, embryo transfer strategy did not have a statistically significant effect on the likelihood of achieving a live birth when adjusted for female age and number of metaphase two oocytes in the logistic regression model. Odds of a live birth was 2.1 (95% confidence interval 0.95 – 4.68), (p = 0.68) with a FET as compared to a fresh ET.

	SET		DET	
	Fresh	Frozen	Fresh	Frozen
# ET	25	25	21	36
# ongoing pregnancies (%/ET)	13 (52%)	17 (65%)	15 (71%)	32 (91%)
P value:	0.4		0.04	
# live births (%/ET)	13/25 (52%)	16/25 (64%)	14/21 (67%)	31/36 (86%)
P value:	0.5		0.1	
# twins	0/13	1/15	8/13	15/30
P value:	0.3		0.4	
Implantation (sacs/embryo)	68% (17/25)	73% (19/26)	67% (28/42)	73% (51/70)
P value	0.7		0.5	

Table 22: Further breakdown of analysis into Single (SET) vs double (DET) embryo transfer between the 2 transfer strategies

2.5.5.3 Comparison between fresh ET and Frozen ET of only day 5-biopsied euploid blastocysts

When comparing the use of only day 5 blastocysts, 46 patients underwent a fresh ET, and 37 patients underwent an FET. The average number of embryos transferred in each group was the same at 1.5 (p=0.3). Live birth, ongoing pregnancy and implantation rates were higher in the frozen transfer group but did not reach statistical significance (table 23).

	Fresh ET (n=46)	Frozen ET(n=37)	P value
Average number of embryos transferred	1.5	1.5	0.3
# ongoing clinical pregnancies (%/ET)	28/46 (61%)	29/37 (78%)	0.1
# live births (%/ET)	27/46 (59%)	26/37 (70%)	0.3
Implantation rate (% sacs/embryo transferred)	45/67 67%	45/58 (78%)	0.23

Table 23: Comparison of outcome between the 2 transfer strategies when transferring only day 5 biopsied embryos

2.5.5.4 Comparison between only FET of euploid blastocysts that were biopsied on post retrieval day 5 or day 6

While 37 patients in the freeze-all group exclusively transferred day 5 biopsied euploid blastocysts, 20 patients in the freeze-all group exclusively transferred day 6 biopsied euploid blastocysts. Euploidy rates between day 5 and 6 biopsied blastocysts in the study were similar at 56.5%/embryo for day 5 and 53%/embryo for day 6 (p = 0.54). The average number of embryos

transferred in each group was significantly different at 1.5 for day 5 and 1.2 for day 6 (p=0.009). Despite this, the positive pregnancy, ongoing pregnancy and implantation rates for each group were nearly identical (table 24).

	Frozen day 5 (n=37)	Frozen day 6 (n=20)	P value
Average age of patient: (range)	35.7 (27-42)	37.3 (30-41)	0.1
Average number of embryos transferred	1.5	1.2	0.009
# ongoing clinical pregnancies (%/ET)	29/37 (78%)	15/20 (75%)	0.7
# live births (%/ET)	26/37 (70%)	14/20 (70%)	1.0
Implantation rate (% sacs/embryo transferred)	45/58 (78%)	18/24 (75%)	0.8

Table 24: Comparison of outcome within the frozen transfer strategy group between transferring day 5 vs day 6 biopsied embryos

2.5.5.5 Cycle management of patients undergoing fresh transfer

2.5.5.5.1 Progesterone (P4) monitoring at hCG administration

Progesterone levels in the present study were monitored at hCG administration to capture premature luteinization and therefore possible cancellation of a fresh transfer in the stimulated cycle. P4 levels of >1.5 have been reported to have a deleterious effect on implantation (Bosch *et al*., 2010). Three patients in the fresh arm of the study, exhibited a rise before hCG and had borderline high p4 values of 1.7, 2.2 and 2.4. They were not cancelled because of this premature rise and the patients with levels of 2.2 and 2.4 both had a live birth from the fresh cycle. The patient with a level of 1.7 had a negative pregnancy test. The other patients in the fresh transfer group had a range of P4 of 0.3-1.4 with an average of 1.

2.5.5.5.2 Stimulation protocols in the fresh transfer group

Of the 46 patients who achieved a fresh transfer, 37 had an antagonist stimulation protocol and 9 had an overlap stimulation protocol. 22/37 of the antagonist group and 7/9 of the overlap group had a live birth.

2.5.6 Discussion

This RCT has demonstrated that the ongoing pregnancy and live birth rates were significantly higher in the frozen ET group compared to the fresh. Additionally, a significantly higher proportion of patients are able to attain the desired embryo transfer strategy in the frozen ET group compared to the Fresh ET group. However, although a higher proportion of embryos transferred implanted in the frozen embryo transfer group than in the fresh embryo transfer group, the variations were short of statistical significance.

The two transfer strategies when using known euploid embryos are very different in their execution and each have their own challenges to take into consideration. There is increasing evidence in favor of transferring embryos created during IVF in an unstimulated uterine environment (Gomaa *et al*., 2016; Ozgur *et al*., 2015; Roque *et al*., 2015; Shapiro *et al*., 2014; Shapiro *et al*., 2011; Shi *et al*., 2014). This approach however necessitates embryo cryopreservation during the IVF cycle. With the adoption of vitrification as a cryopreservation method over the past few years the survival rate of blastocyst embryos has improved significantly

making the transfer of frozen thawed embryos a pragmatic option for IVF patients and practitioners alike. While there have been studies comparing fresh vs frozen embryo transfer outcomes and PGS tested vs non PGS tested embryo transfers (Forman *et al*., 2014; Scott *et al*., 2013a), there has not been a study comparing fresh vs frozen/thawed transfer of known euploid embryos.

To confirm that the improvement in implantation and ongoing pregnancy rates in the freeze all group was related to the transfer strategy alone we compared only embryos that had been biopsied on day 5 in the fresh and frozen groups (Table 23). The ongoing pregnancy and implantation rates all showed positive trends in favor of the frozen ET group but these did not reach significance, probably due to the smaller sample size.

As part of the study analysis we assessed if the implantation potential of day 6 blastocysts was different to embryos ready for biopsy on day 5. We found almost identical implantation rates with both groups, 78% with day five embryos compared to 75% for embryos ready for biopsy by day six (p=0.8)(table 24). So to be able to include day 6 embryos in the cohort for transfer is a definite advantage for the freeze all group and leads to more patients reaching the goal of their intended transfer protocol compared to the fresh transfer strategy.

Transferring fresh embryos on day 6 could be regarded as a disadvantage for the fresh transfer protocol, since usual clinical practice is to transfer blastocysts on day 5 of embryo growth.

However, fresh transfer of tested embryos on day 5 is not possible with current NGS technology due to the length of time needed to run the assay (17 hours) before results are obtained. For this reason day 6 transfer is currently the standard procedure for fresh transfer of known euploid embryos when using NGS. Arguably, the ideal comparison for this study may have been to transfer both fresh and frozen embryos on day six, but the point of the present trial was to compare the two currently available options using NGS technology in daily practice. Moreover, the optimal window of implantation where the endometrium is most receptive is not precisely known and may differ from cycle to cycle and from patient to patient (Casper and Yanushpolsky, 2016; Haouzi *et al*., 2009). Thus we elected to conduct fresh transfers before 12 midday on day six, as soon as the PGS results became available. A faster methodology for counting chromosomes is real time quantitative polymerase chain reaction (qPCR) where the turnaround time is 4 hours therefore allowing for same day transfer on day 5. In this study we were comparing the use of NGS for the two transfer strategies as this is the technology of choice for many PGS laboratories and one which is in place for clinical testing in the study center laboratory. Other studies are needed to compare fresh vs frozen transfers of euploid embryos using other technologies and this was outside the scope of this trial.

The consequences of transferring embryos in a high estrogenic environment are higher rates of abnormal placentation (Farhi *et al*., 2010) and possible reduced endometrial receptivity (Arslan *et al*., 2007; Carmona-Ruiz *et al*., 2010) compared to cycles with more physiological levels of estradiol. Abnormal placentation includes incorrect placenta placement in the uterus such as

placenta *previa* and *vasa previa* or abnormal cord insertion such as velamentous or marginal cord insertion. The risk of fetal maternal hemorrhage is high if the placenta or vessels lie over the cervix or if the cord is easily detached from the placenta during the birthing process. Farhi *et al* (Farhi *et al*., 2010) showed in a retrospective single center study that there seems to be a higher incidence of abnormal placentation in fresh ET cycles associated with estradiol levels above 2724 pg/ml at the time of hCG and in a large Australian multicenter study of singleton pregnancies, Healy *et al* (Healy *et al*., 2010) found that there is a higher risk of obstetric hemorrhage after transfers in a stimulated IVF transfer cycle than in frozen transfer cycles or naturally conceived pregnancies.

An insufficient placenta can be associated with an increase in the rate of lower term birth weight infants resulting from fresh ET cycles compared to frozen ET cycles (Farhi *et al*., 2010; Kalra *et al*., 2011; Wennerholm *et al*., 2013). In the present study, maximum E2 levels for the fresh group reached an average of 3489pg/ml compared to an average of 1098pg/ml in the freeze all group. While the FET E2 levels were not as physiologically normal as during natural cycle conception they were three times lower than the fresh transfer group and did not exceed the levels above which an increase in abnormal placentation has been observed (Farhi *et al*., 2010). The present study did not assess placental defects of offspring however and the average weight of singleton offspring in each group was similar.

In addition to biological concerns, infertility is psychologically challenging for patients, and stress related to infertility and undergoing a demanding IVF cycle should be taken into account while making treatment decisions. The uncertainty of possibly having no euploid embryos available for fresh transfer on the morning of day six could significantly increase patient's perceived stress. On the other hand, knowing in advance that a fresh transfer will not be attempted thus allowing time to carefully consider the results of the whole cohort of embryos may relieve some of that stress for the patient.

It should be noted that fresh transfers also have several advantages. A fresh transfer avoids direct costs associated with cryopreservation and the subsequent frozen thawed embryo transfer. A fresh transfer also saves on future indirect costs of treatment, such as loss of earnings for monitoring visits during the frozen transfer cycle and travel costs for patients travelling from another state or even another country. The chance of a positive outcome is more immediate compared to a freeze all cycle where the patients often have to wait weeks or months to even attempt a transfer. Thus, it is also possible that some patients can regard a fresh transfer as less stressful. Either of the treatment protocols investigated in this study can be a reasonable option for patients using their own oocytes. On balance the non-stimulated uterine environment of the FET protocol may result in pregnancies with fewer obstetric complications as shown by previous studies of fresh vs frozen protocols using non tested embryos and IVF vs non IVF pregnancies (Englert *et al*., 1987; Farhi *et al*., 2010; Healy *et al*., 2010).

While this study did reveal a significant improvement in live birth rate per transfer event using frozen thawed euploid embryos compared to fresh, it was unable to demonstrate a statistically significant improvement in live born offspring per transferred embryo between the two transfer strategies. Freezing all embryos allows for inclusion of all blastocysts in the cohort of embryos available for transfer which also results in a higher proportion of patients reaching their primary embryo transfer goal. In conclusion, our findings suggest a trend towards favoring the freeze-all option as a preferred transfer strategy when using known euploid embryos. Further randomized controlled trials using known euploid embryos need to be executed to further substantiate the outcome data described in the present study.

Trial registration number: NCT02000349

2.6 Specific aim 5

To test the hypothesis that using NGS for PGS increases live birth rates and lowers miscarriage rates by reporting mosaicism compared to using aCGH .

This chapter includes a retrospective data analysis of IVF cycles for patients undergoing PGS for aneuploidy.

2.6.1 My personal contribution to the work

I analyzed data generated from IVF cycles carried out in our own clinic. I summarized the data and wrote this chapter.

2.6.2 Chapter summary

This retrospective study of FET cycles using euploid embryos designated as such by aCGH or NGS, analyzed rates of euploidy, average number of euploid embryos per cycle, percentage of cycles with no euploid embryos, miscarriage rates and live birth rates per embryo, between the two platforms. The hypothesis was that as mosaicism is not reported using aCGH, if we are able to classify mosaicism by using NGS and avoid transferring mosaic embryos, live birth rates should be higher, euploidy rates should be lower and therefore less embryos would be available for transfer, the number of cycles with no normal embryos should be higher and miscarriage rates should be lower using NGS compared to aCGH. The only parameter that was significantly different was the overall euploidy rates for each age group which were lower with NGS. All other

parameters were similar between the two platforms. The results of this study therefore may lead us to wonder if mosaicism is a real concern in preimplantation embryos. If it is a true phenomenon then the live birth rate should eventually be slightly higher with NGS with a larger dataset.

2.6.3 Introduction:

As more detailed chromosomal abnormalities are detected in embryo biopsies by more sophisticated testing methods, one would expect a corresponding increase in live birth rates per embryo transferred. In the early days of PGS, FISH was used to detect only a maximum of 12 chromosomes in a single cell. With the implementation of more advanced technologies such as qPCR, aCGH and NGS, the quantification of whole chromosome copy number in preimplantation embryos has now become possible.

Array CGH was used to screen embryos for aneuploidy between 2010 and 2014 until converting to NGS on the Ion Torrent (Thermo Scientific) platform. NGS has higher resolution and a broader dynamic range compared to aCGH and therefore is able to detect mosaicism and smaller segmental anomalies in chromosomes than with aCGH. Historically, it was generally accepted that aCGH lacked the resolution necessary to reliably detect mosaicism. A mosaic result was classed as euploid or aneuploid depending on the percentage of mosaicism seen. Above 50% was aneuploid and below was euploid (verbal confirmation from industry leaders).

As NGS technology detects these anomalies in blastocyst embryos one might assume that once transferred, the percent of babies born from NGS screened embryos would be higher than from those screened with aCGH. In this retrospective study, euploidy rates, percent of cycles with no normal embryos, average number of euploid embryos per cycle, miscarriage rates and live birth rates per transferred embryo were compared between aCGH and NGS screened embryos.

2.6.4 Materials and Methods

(See general Materials and Methods for stimulation, culture, vitrification and DNA quantification protocols. Section 2.7)

2.6.5 Results

2.6.5.1 Euploidy rates between the two screening platforms

Euploidy rates in both the own oocyte and donor oocyte group were significantly lower using NGS compared to aCGH in all but the 38-40 age group which only had a 3% difference in favor of aCGH (Figure 33 and table 25).

2.6.5.2 Percent of cycles with zero euploid embryos per cycle

The two platforms showed similar percentages of cycles that had no euploid embryos at all with only a trend towards NGS resulting a higher percentage in all age groups (table 25)

2.6.5.3 Average number of euploid embryos per cycle

There was no difference between the two platforms of the average numbers of euploid embryos available for each age group. However all of the maternal age groups apart from the 38-40 age group, had more embryos to biopsy in the NGS groups compared to the aCGH groups. This resulted in a lower percentage of euploid NGS embryos compared to those designated as euploid by aCGH but the same average number available for patients using each platform. (table 25)

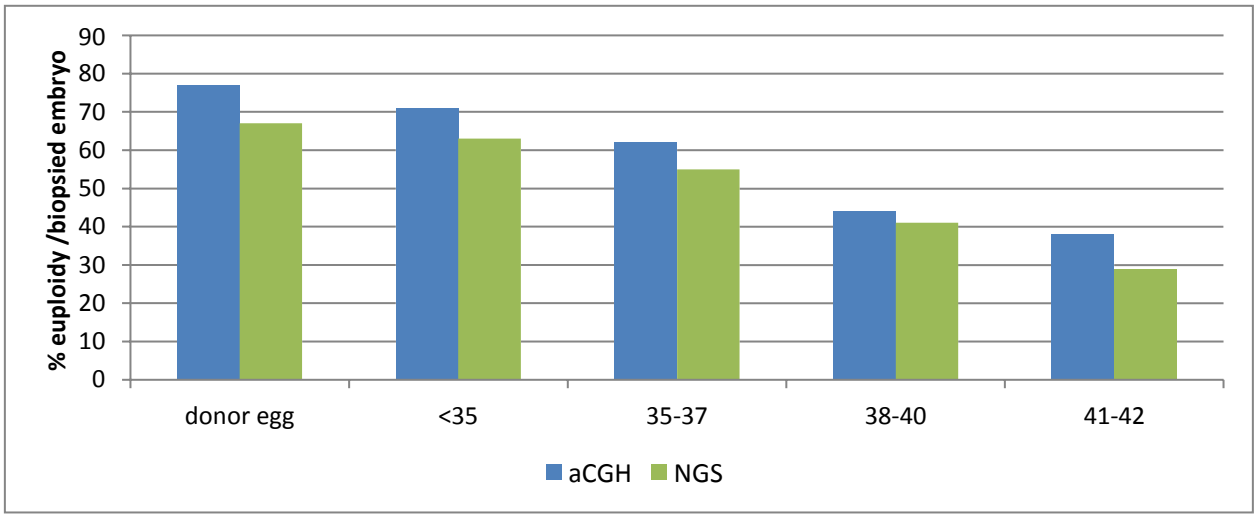


Figure 26: Euploidy rates per age group and platform used for determination- aCGH 2010-2014, NGS May 2015-March 2017

.	<35		35-37		38-40		41-42		Donor egg (ave age 25)		Total	
	aCGH	NGS	aCGH	NGS	aCGH	NGS	aCGH	NGS	aCGH	NGS	aCGH	NGS
# cycles	94	245	106	232	129	211	49	85	171	473	549	1246
# embryos biopsied (ave)	525 (5.6)	1544 (6.3)	484 (4.6)	1247 (5.4)	553 (4.3)	856 (4.1)	177 (3.6)	351 (4.1)	1484 (8.7)	4812 (10.2)	2950	8810
P value	0.3		0.3		0.1		0.5		0.007			
# euploid embryos	374 (71%)	974 (63%)	297 (61%)	703 (56%)	238 (43%)	356 (42%)	67 (38%)	98 (28%)	1142 (77%)	3216 (67%)	2118 (72%)	5347 (61%)
P value	0.0009		0.07		0.6		0.02		<0.0001		<0.0001	
Ave # euploid embryos	4.0	4.0	2.8	3.0	1.8	1.7	1.4	1.2	6.7	6.8		
P values	0.9		0.7		0.1		0.08		0.7			
# cycles with no euploid embryos in the cohort	2 (2%)	11 (4%)	7 (7%)	30 (13%)	32 (25%)	51 (24%)	11 (22%)	32 (38%)	4 (2%)	6 (1%)	56 (10%)	130 (10%)
P value	0.5		0.09		0.8		0.08		0.5		0.9	

Table 25: comparing euploidy rates and number of cycles having no euploid embryos between aCGH (2011-December 2014) and NGS post reporting of mosaicism (May 2015-March 2017)

2.6.5.4 Miscarriage rates:

The percentage of fetal hearts visualized, resulting in a live born baby was compared for each maternal age group between NGS and aCGH. The loss rate per fetal heart was not statistically different between each platform (table 26)

2.6.5.5 Live birth rates:

Comparing the two screening method for Live births per embryo transferred resulted in no significant difference between an NGS screened embryo vs an aCGH screened embryo. There seemed to be a slight trend in favor of NGS screened embryos but to reach significance with a 2%

difference in live births per embryo transferred one would have to have 4900 embryos transferred in each group (figure 27).

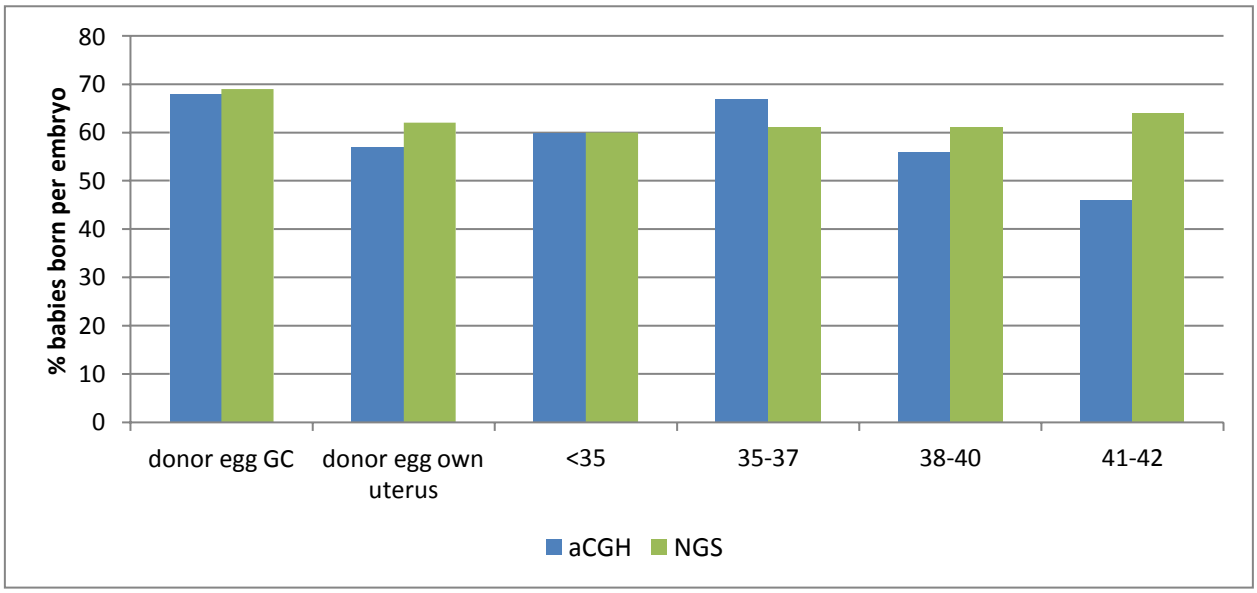


Figure 27: Live birth rates per embryo screened with aCGH or NGS.

	Patient own uterus										GC uterus	
	<35		35-37		38-40		41-42		Donor egg (ave age 25)			
	aCGH	NGS	aCGH	NGS	aCGH	NGS	aCGH	NGS	aCGH	NGS	aCGH	NGS
# FET	52	96	55	82	65	86	26	26	53	67	98	170
# embryos transferred (ave)	73 1.4	146 1.5	81 1.5	127 1.5	80 1.2	125 1.4	35 1.3	33 1.3	70 1.3	99 1.5	149 1.5	258 1.5
# fetal hearts %/embryo	46 63%	104 71%	61 75%	88 69%	48 60%	80 64%	18 51%	24 72%	45 64%	70 71%	118 79%	194 75%
P value	<0.001		0.4		0.6		0.08		0.4		0.4	
# FH lost before live birth %FH lost/FH seen	2 4%	17 16%	6 10%	10 11%	3 6%	6 8%	2 11%	3 13%	5 11%	9 13%	16 14%	17 9%
P value	>0.05 NS											
# babies born – %/embryo transferred	44 60%	87 60%	55 67%	78 61%	45 56%	78 61%	16 46%	21 64%	40 57%	61 62%	102 68%	177 69%
P value	>0.05 NS											

Table 26: Outcomes of transfer cycles using embryos screened with aCGH or NGS (all embryos vitrified with same vitrification protocol)

2.6.6 Discussion

The evolution of molecular technologies brings with it the ability to identify more potential chromosomal abnormalities in preimplantation embryos (Capalbo *et al*., 2016b; Lai *et al*., 2017; Munne and Wells, 2017; Treff and Franasiak, 2017). Unlike aCGH, the NGS platform is able to determine mosaic profiles and distinguish these from full aneuploidies or noisy euploid results. Mosaic profiles can occur along with a full aneuploid profile in the same embryo (for example: monosomy 15, mosaic trisomy 12) or they can occur as a single or double mosaic profile without a full aneuploid result (for example: mosaic monosomy 18, mosaic trisomy 1). These embryos with only a mosaic result fall within a grey area of clinical IVF because they have both euploid and aneuploid cells present. If the euploid cells are present in the inner cell mass and proliferate in preference to the aneuploid cell line then the fetus could be normal. If the aneuploid cell line proliferates then the embryo may not be viable or give rise to an aneuploid fetus.

In our own program, NGS on the Ion Torrent platform identified around 10% of embryos with an isolated mosaic result in patients less than 40. In patients over 40 we found 5% of embryos with an isolated mosaic result (own unpublished data). Older patients have higher aneuploidy rates so even though they seem to have lower mosaic rates, their mosaics exist more often alongside full chromosome aneuploidies.

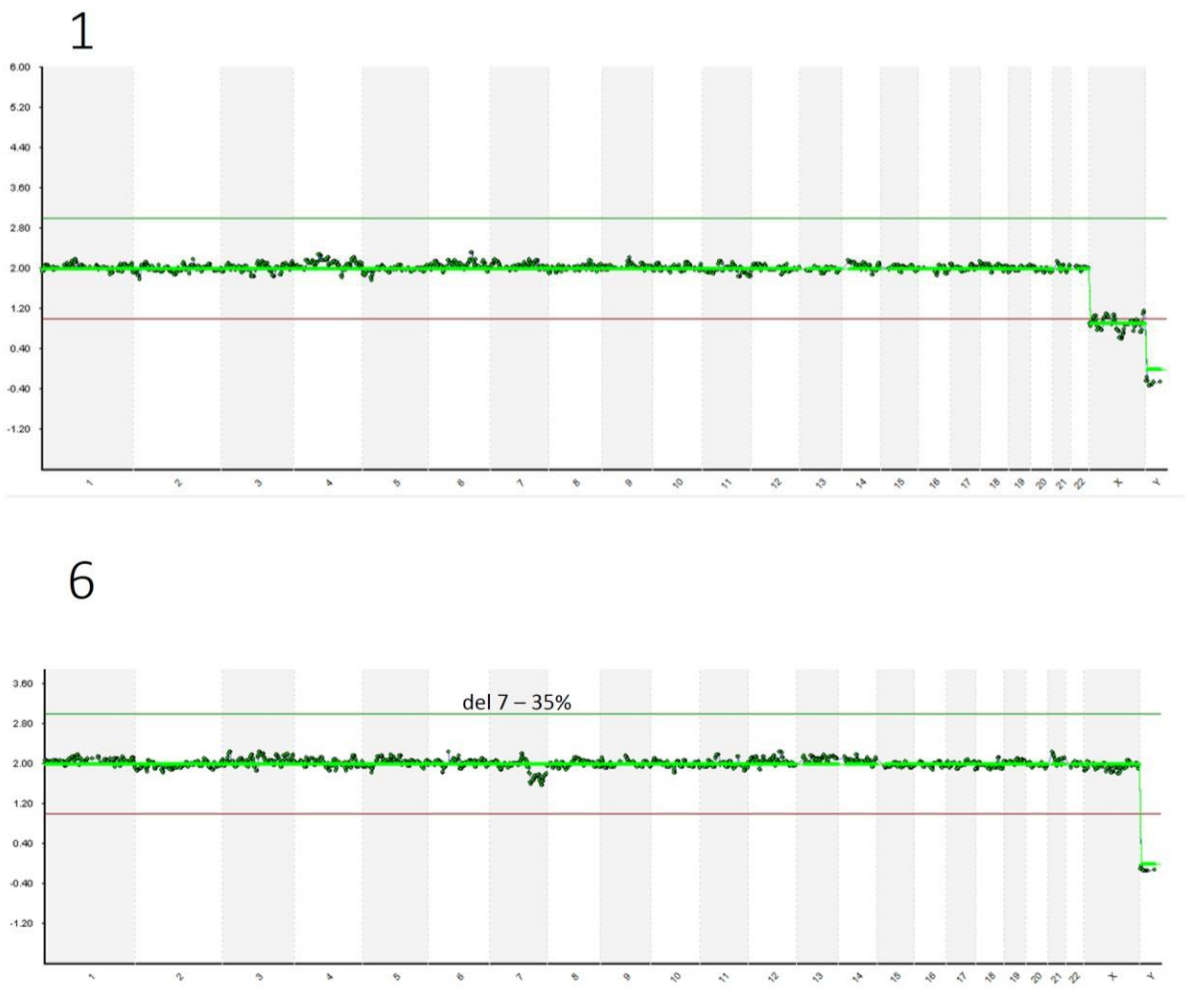


Figure 28: NGS profiles showing a euploid trace (embryo 1) and a mosaic monosomy 7 at 35% (embryo 6)

These 10% of embryos without a full aneuploidy, may have been called euploid by aCGH and may have been chosen for transfer in an FET cycle. These inadvertently transferred mosaics may never have implanted or may have resulted in a miscarriage. Therefore to have the slight at least perceived increase in live born babies per NGS screened embryo may be attributable to the weeding out of mosaic embryos. To expect the percent increase of live borns to match the mosaic embryo rate at 10% with NGS would be unrealistic as not all the undiagnosed mosaic embryos

would be chosen for transfer, so the few that were inadvertently chosen could account for the small decrease in live borns from aCGH screened embryos.

While there have been validation studies comparing the two technologies and their ability to predict ploidy status (Aleksandrova *et al*., 2016; Kung *et al*., 2015) there have been few studies comparing IVF outcomes using NGS and aCGH to determine ploidy. Yang *et al* in 2015 (Yang *et al*., 2015) carried out a study comparing implantation and pregnancy rates between the 2 methodologies showing a similar small increase in implantation and pregnancy rates in the NGS group to the one seen in the present study. The latest study published by Lai *et al*(Lai *et al*., 2017) biopsied embryos from 45 patients and ran the amplified products on aCGH and NGS platforms concurrently to examine the differences in profiles on the same embryo sample.

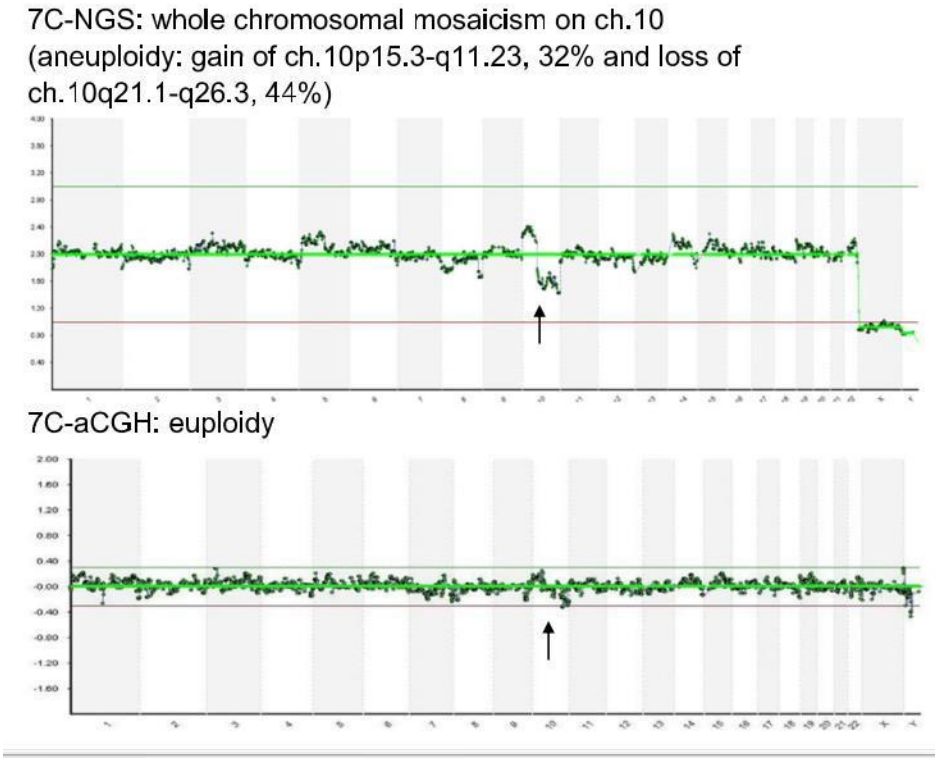


Figure 29: showing different interpretations of the same amplified DNA sample from the same embryo between the two testing platforms. (Lai et al 2017) Open access article distributed under the terms of the Creative Commons Attribution (http://creativecommons.org/publicdomain/zero/1.0/)

As is shown in figure 29, some embryos diagnosed as euploid using aCGH show errors when analyzed by NGS. The second phase of the same study compared outcomes of FET cycles using aCGH and NGS screened embryos. Their findings were similar to the present retrospective analysis, with significantly higher implantation rates with NGS screened embryos compared to aCGH (53% vs 45% respectively, p= 0.04) and a trend towards improved ongoing pregnancy rates with NGS but not significantly (57% NGS vs 46% aCGH, p= 0.1).

While average aneuploidy rates vary according to maternal age, within each age group there will always be outliers who will have no euploid embryos in their cohort. As NGS results in fewer euploid embryos available per cycle in all age groups compared to aCGH, the assumption was made that when using NGS, there would be more cycles that had no euploid embryos at all. As our data shows in table 25, there were no significant increases in the number of cycles with no euploid embryos available.

The Preimplantation Genetic Diagnosis International Society (PGDIS) position statement on chromosomal mosaicism, published in 2016 (PGDIS, 2016), stated that less than 20% mosaic aneuploidy should be considered euploid, greater than 80% mosaic aneuploidy should be considered aneuploid and between 20-80% mosaicism should be classified as a mosaic result. These reporting guidelines, were based on the Illumina NGS platforms (MiSeq, NexSeq)and VeriSeq assay and these thresholds had little clinical data to support their determination.

Reported rates of mosaicism in pre-implantation blastocysts vary depending on which platform and assay is used. When Array CGH was used for PGS, mosaicism was observed but not reported and results were classed as aneuploid (over 50% mosaic aneuploidy) or euploid (less than 50% mosaic aneuploidy). If the NGS result profile is clean and artefacts have not been introduced during sequencing or amplification (see figure 28) then a true mosaic result is easy to categorize but if the results are noisy then a mosaic result is more difficult to call and could be attributed to DNA degradation or sample mishandling. Critics of mosaic results argue that there is not enough evidence that these results firstly are fact or artifact and if they are fact what effect these results have on pregnancy outcome and the phenotype and genotype of live born offspring. Mosaic

thresholds above and below which an embryo will be classified as mosaic rather than aneuploid or euploid have been established within the PGS community. Other NGS platforms such as the Ion Torrent PGM platform will also detect mosaicism but the threshold for classifying a mosaic result is higher than with the Illumina platform. In our own lab, the Ion Torrent platform resulted in isolated mosaic rates of around 10% of all blastocysts screened for aneuploidy compared to 20% with the MiSeq Illumina platform

However, the proportion of aneuploid cells in any mosaic sample may or may not reflect the extent of mosaicism in the whole embryo. If 10 cells are biopsied and one/ten is aneuploid, the percent of mosaicism would be stated as 10%. If 5/10 of the cells are aneuploid then the percent of mosaicism observed would be 50%. These results could have been from the same embryo and the biopsy could have been taken at different places on the trophectoderm.

There are both biological and analytical challenges when detecting mosaicism.

Biological challenges are two- fold: 1) sampling errors; as the mosaic determination can only be made based on the cells sampled from one site on the trophectoderm and 2) reciprocal errors; where deletion in one cell and duplication in another cell for the same chromosome, could be diagnosed as euploid when the total amount of DNA for the sample is analyzed.

Analytical challenges are that the data points of euploid and aneuploid samples often overlap extensively. Smoothing algorithms are used to give a weighted average of the data points to discriminate between aneuploid, euploid or mosaic (Scott and Galliano, 2016).

Because of the challenges involved in the technology, some practitioners question the validity of mosaic results (Gleicher *et al*., 2016; Greco *et al*., 2015) and infer that the results may be artefact or noise. If there is a rise in live birth rates when mosaicism is reported then the issue may indeed be real rather than a statistical average and should be taken into account when looking at embryos suitable to transfer. Further studies on the interpretation of mosaic results need to be performed.

2.7 Specific aim 6

To provide a framework for creating realistic expectations for patients planning for their fertility future

Data presented in an abstract at ASRM 2016, entitled: Planning for the future: how many oocytes do patients need to harvest to achieve their fertility goals**? Alison Coates** Emily Mounts, Brandon Bankowski, Santiago Munne. *Abstract poster ASRM annual meeting 2016* ((Coates et al., 2016b))

2.7.1 Personal contribution to the manuscript:

I retrospectively analyzed PGS data on blastocyst embryos from patients undergoing IVF with PGS in a private IVF clinic in Portland, Oregon, USA and used the data to calculate the number of mature oocytes required to make one euploid blastocyst according to maternal age.

2.7.2 Chapter summary:

The IVF process is an increasingly complex one which can halt at any stage: cancelled cycle before oocyte retrieval, no eggs collected, no mature eggs, failure of fertilization, failure to cleave, failure of all embryos to progress to the blastocyst stage, no euploid embryos post biopsy, failure to thaw and then of course post transfer, failure to implant and failure to reach a live born offspring. As maternal age increases the incidence of an adverse event simultaneously increases as does the aneuploidy rate. Patients need to be aware of all the pitfalls that can occur as part of the IVF cycle and I attempted to provide new tools to show the frequency of these negative

outcomes and by analyzing how many oocytes are needed to create one euploid blastocyst I created a tool for clinicians to use to better counsel patients on how many cycles they should expect to complete to achieve their family goal.

2.7.3 Introduction

One cycle of IVF may or may not result in the desired outcome of a live born infant or the creation of enough embryos to complete an individual patients' desired family size. More than one cycle may be required to achieve these goals. As maternal age advances, the number of euploid oocytes decreases (Harton *et al*., 2013) which results in fewer euploid embryos available for transfer. Patients therefore need to be counselled on how many cycles they should plan to complete to maintain a realistic expectation during the treatment cycle. With the development of successful oocyte vitrification protocols and the subsequent increase in popularity of elective oocyte freezing for fertility preservation, the optimal number of oocytes needed to produce a single euploid blastocyst at any given age, plays a part in fertility planning. Given the increasing rate of aneuploidy with maternal age, it is important to understand how many oocytes are ideally needed to later result in an adequate number of euploid embryos for transfer.

This study includes a retrospective, single center, data analysis of fresh IVF cycles undergoing PGS for aneuploidy using high resolution Next Generation Sequencing (hrNGS) on the Ion Torrent PGM platform, and illustrates how many mature eggs are needed in the fresh cycle to create one euploid blastocyst based on maternal age. In order to help expectations to be managed

appropriately I also present data showing the percentage of oocyte retrievals that result in adverse events during the IVF cycle according to maternal age.

2.7.4 Materials and Methods

(See general Materials and Methods for stimulation, culture, vitrification and DNA quantification protocols. Section 2.7)

2.7.4.1 Calculation of number of mature oocytes required to get one euploid blastocyst

The determination of how many oocytes to target was derived by dividing the total number of mature eggs retrieved for each maternal age group by the final number of euploid blastocysts available post-biopsy, resulting in the number of mature oocytes retrieved per euploid blastocyst. *(Total # mature eggs ÷ Total # euploid embryos = # of mature eggs per euploid blastocyst).* Blastocysts with an inconclusive result were omitted from the analysis which accounted for 2.0% of the total number of blastocysts available. Assuming an approximate 55% live birth rate per transferred euploid embryo, the patient should aim to at least double this number of mature eggs for a goal of one child and quadruple it for a goal of 2 children.

For oocyte cryopreservation cycles the calculation has to compensate for potential losses post thaw. Survival rates of vitrified oocytes are typically between 85-90%. To calculate the number of mature oocytes needed to freeze for future use, the number of fresh eggs should be increased by 10-15% to account for post thaw losses.

2.7.4.2 Calculation of other events occurring throughout the IVF cycle

Data was also analyzed to establish percentages of oocyte retrievals that result in no blastocyst formation and no euploid embryos during each cycle according to maternal age.

2.7.5 Results

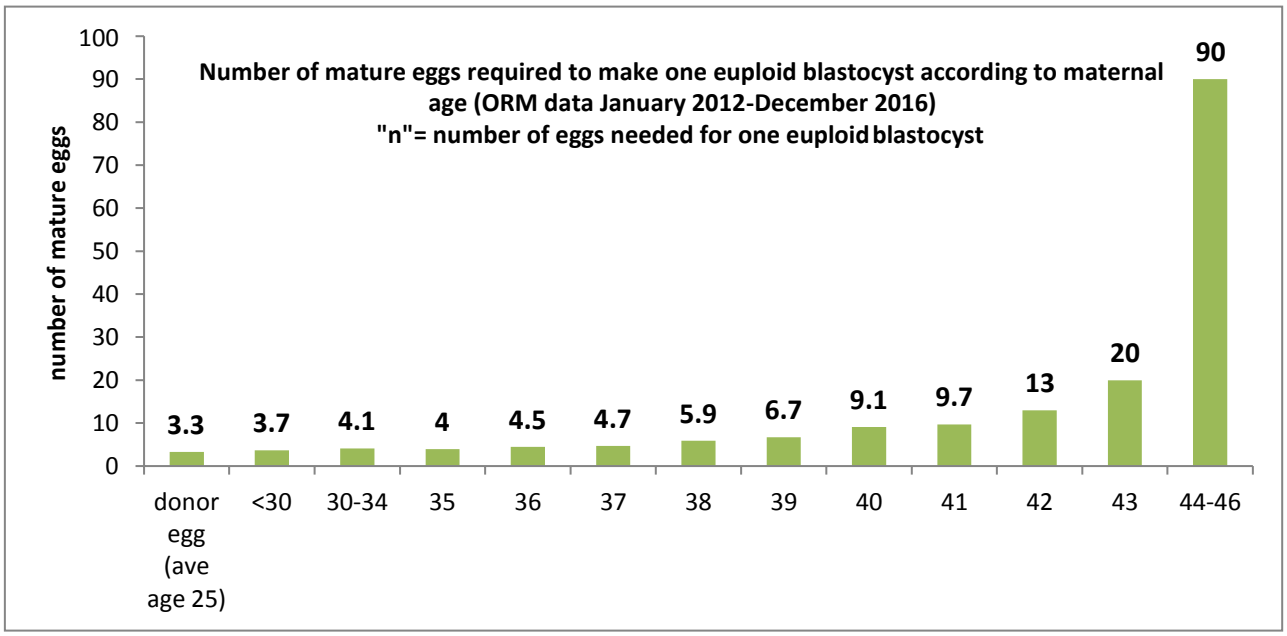


Figure 30: Graph showing number of mature eggs required to create one euploid blastocyst

	Donor egg (25)	<30	30-34	35	36	37	38	39	40	41	42	43	44-46
# cycles	**641**	**68**	**277**	**113**	**123**	**111**	**121**	**135**	**116**	**90**	**50**	**34**	**28**
Ave # M2/cycle	**22.2**	**17.1**	**15**	**13.7**	**13**	**12.8**	**11.6**	**11.2**	**12.3**	**13**	**12.4**	**10**	**10**
Ave # embryos biopsied/ cycle	**9.7**	**6.6**	**5.8**	**5.2**	**4.9**	**5.1**	**4.2**	**3.8**	**3.8**	**4**	**3.8**	**2.6**	**2.3**
Ave # euploid/cycle	**6.8**	**4.6**	**3.6**	**3.4**	**2.8**	**2.7**	**2**	**1.7**	**1.4**	**1.3**	**1**	**0.5**	**0.1**
# mature eggs to make one euploid blastocyst	**3.3**	**3.7**	**4.1**	**4**	**4.5**	**4.7**	**6**	**6.7**	**9.1**	**9.7**	**13**	**20**	**90**

Table 27: Data for figure 36 showing number of cycles and average numbers of oocytes and embryos for each maternal age group

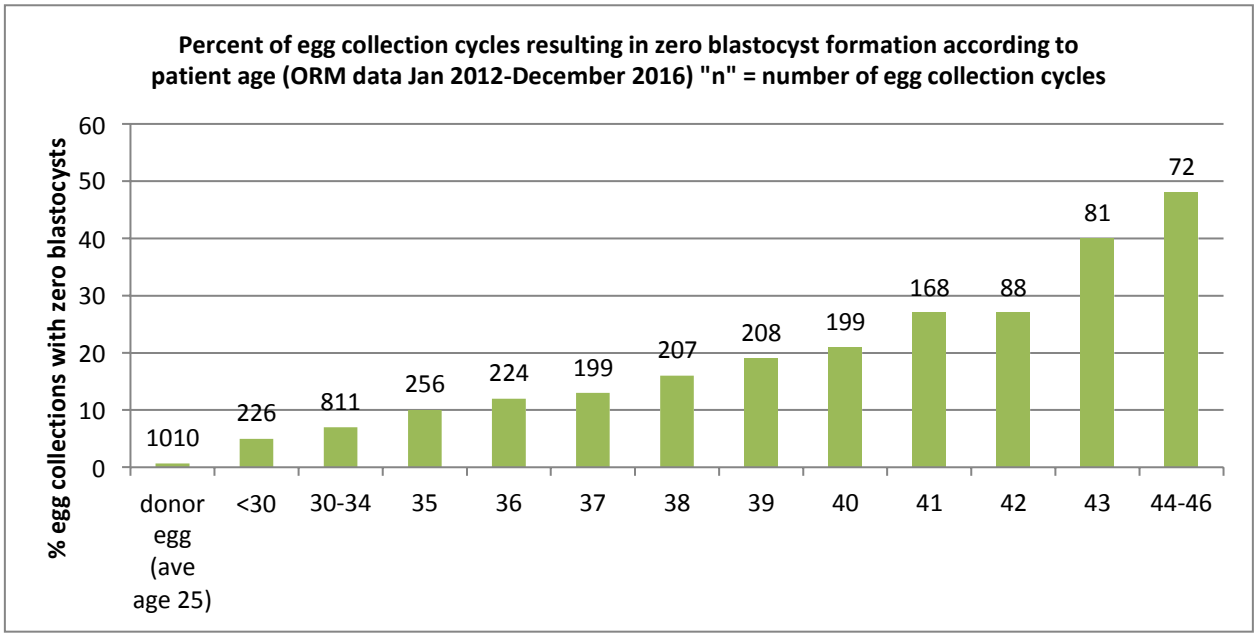


Figure 31: Graph showing percentage of oocyte retrievals resulting in zero blastocyst formation according to maternal age

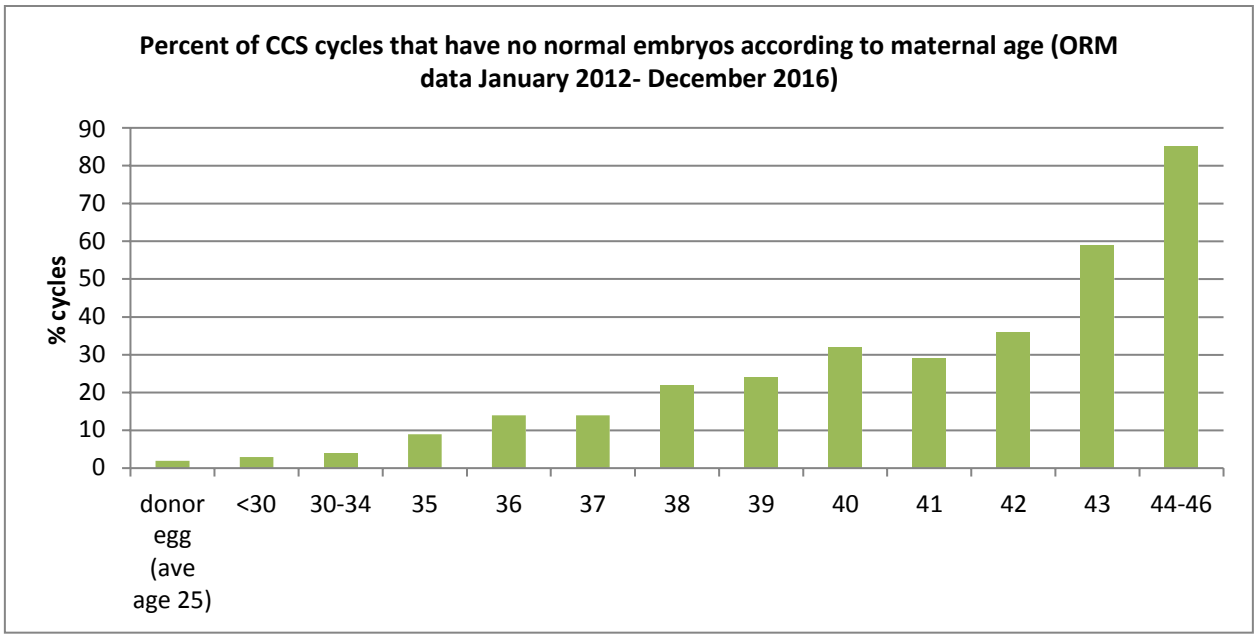


Figure 32: Graph showing percent of PGS cycles that have no euploid embryos post biopsy according to maternal age

	Donor egg (25)	<30	30-34	35	36	37	38	39	40	41	42	43	44-46
% cycles with no eggs	0	0	0	0.4%	0.4%	0.5%	0	0	0	0	2%	0	1.4%
% cycles with no mature eggs	0	0	0.6%	0	0.4%	0	0	0	0.5%	1.3%	0	1.4%	0
% ICSI cycles with zero fertilization	0	0.5%	1.4%	1.4%	2%	0	2%	2%	3%	3%	3%	6%	6%

Table 28: Incidence of adverse events during the IVF cycle.

2.7.6 Discussion

Managing patient expectations during the IVF cycle is challenging. A cycle of IVF is physically, mentally and financially stressful for the patient. The majority of cycles will complete without an adverse event and will fall within the average expectations. However, when a patient falls outside of those expectations it helps to be able to understand what can go wrong before the cycle starts. Patients going through their first cycle of IVF are the least aware of upcoming events in the process. Patients find it difficult to comprehend exactly what is involved in an IVF cycle unless they have physically experienced it themselves even though clinical staff spend many educational hours explaining each part of the process in detail before it occurs.

The IVF process is an increasingly complex one which can halt at any stage: cancellation before retrieval due to poor response to the medication, no eggs collected, no mature eggs, failure of fertilization, failure to cleave, failure of all embryos to progress to the blastocyst stage, no euploid embryos post biopsy, failure to thaw and then of course post transfer, failure to implant and failure to reach a live born offspring. Patients need to be aware of all the pitfalls that can occur as part of the IVF cycle and I attempted to provide educational tools to show the frequency of these negative outcomes.

As maternal age increases so do the number of oocytes required to result in one euploid blastocyst (figure 36). The model presented here is useful when counselling patients vitrifying oocytes for fertility preservation, to facilitate an informed decision regarding the number of egg

retrieval cycles they may require to achieve their future fertility goals. It is also useful to use in patient/ provider discussions for patients undergoing IVF for immediate family creation to plan for the desired family size.

The minimum number of oocytes calculated for each age group is only a guideline as cycles and patients all vary a great deal but patients appreciate any information that may guide them in their estimation of financially and emotionally planning their desired family goal.

3 General Discussion

This thesis was largely successful in the fulfilment of its stated specific aims, namely:

1. Some association between aneuploidy levels and morphokinetic criteria was established in human IVF embryos however this is by no means diagnostic. Similarly, analysis of cell free embryonic DNA found in spent culture media shows great promise as a non-invasive preimplantation genetic screen and further studies are planned and warranted.
2. Patients undergoing ICSI with normal semen parameters showed no increase in embryonic aneuploidy rates compared to standard insemination patients with normal sperm, showing that the ICSI process itself does not create aneuploidy in embryos.
3. Novel insights were provided in terms of the patient populations that may benefit from the use of PGS, specifically, male factor infertility patients and young oocyte donors. Namely that patients with suboptimal semen parameters were shown to have increased sex chromosome aneuploidy rates in embryos, and patients of young maternal age may still benefit from PGS when cryopreserving embryos for future use shown by the increase in live birth rates when PGS embryos are used for transfer.
4. Results demonstrated that the optimal transfer strategy for euploid embryos in patients using their own oocytes was to freeze all embryos and transfer thawed embryos in a subsequent cycle.

5. The use of NGS to screen embryos for aneuploidy should have resulted in higher live birth rates per transferred embryo compared to array CGH screened embryos, as mosaic embryos are reported by NGS but not array CGH (see section 2.6.2). As we are presumably avoiding mosaic embryos by using NGS the final outcome should be improved compared to other platforms. I found this to not be the case and live birth rates remained similar between the two screening platforms.
6. A framework for creating realistic expectations for patients planning for their fertility future was provided which is currently being used clinically in my own IVF clinic. Patients undergoing oocyte cryopreservation find the metric used to calculate the number of oocytes needed to create one euploid embryo very useful and have been able to plan for multiple cycles depending on their response to the stimulation medication based on that equation. Patients also like to be made aware of any pitfalls that may occur during embryo creation cycles and the table of adverse events I created places some perspective on rates of occurrence.

The embryologist's role of choosing embryos for transfer solely on morphology alone is diminishing in many clinics. If we are able to determine ploidy status in an embryo the morphology becomes almost irrelevant. There is great variation in the rate of use of PGS between IVF clinics across the world. In the USA there are still clinics that never test embryos but just transfer untested embryos whatever the patient age. The number of clinics who utilize PGS in the majority of their patient cycles are increasing each year with improvements in testing

technology. As I stated at the beginning of this thesis, in order to optimize embryo choice we have to be able to grow embryos in a stress free environment as closely aligned to the *in-vivo* environment as possible. If a lab is unable to successfully grow embryos to their fullest potential *in-vitro* then all of the innovative adjunct technology currently in use or under research to help choose the best embryo will be wasted.

As aneuploidy accounts for the majority of IVF implantation failures and miscarriages (Spandorfer *et al*., 2004), to reduce the number of aneuploid embryos transferred is of benefit to many patients going through IVF treatment. With fewer failed cycles the length of time from cycle start to successful outcome is reduced and this effect is much greater in patients of advanced maternal age, but only if a euploid embryo can be found. Murugappan *et al* (Murugappan *et al*., 2016) recently stated that embarking on an IVF cycle with PGS for patients experiencing repeated pregnancy loss did not appear to reduce the time to viable conception compared to expectant management. However I would argue that patients who conceive with an aneuploid embryo and subsequently miscarry by definition will prolong the time taken to live birth due to wasted time recovering from miscarriages that could have been prevented by pre-screening embryos before transfer.

In 2007 Mastenbroek published a randomized double blind study comparing the transfer of PGS vs non screened embryos. The biopsies of single cells were carried out on day 3 and the DNA analyzed with 8 probe FISH (Sebastiaan Mastenbroek *et al*., 2007). 408 women were randomized

to the treatment or control group before the follicular aspiration. The results were a significant reduction in ongoing pregnancy rates in the PGS group compared to the control group (25% vs 37% respectively p=0.01). This study was greatly criticized at the time for lack of reporting an error rate, poor biopsy technique, substandard fixation and FISH methods and inappropriate patient selection (Cohen and Grifo, 2007; Munne *et al*., 2007b). In 2011, Mastenbroek published a meta-analysis of PGS studies, again only considering day 3 biopsy with FISH analysis which concurred with the results of his own RCT (Mastenbroek *et al*., 2011). Hardarson *et al* in 2008 (Hardarson *et al*., 2008) also carried out an RCT comparing pregnancy outcomes of PGS tested and non-tested embryos. Again day 3 biopsies were analyzed with 7 probe FISH. Ongoing pregnancies in the PGS group were 9% vs 25% for the non PGS group.

As it has since been shown that the biopsy process itself on day 3 is detrimental to embryo growth (Scott *et al*., 2013b) and as there are limitations to FISH analysis of single cells, the Mastenbroek study conclusions may have had some validity.

As the field has progressed over the last ten years, the development of trophectoderm biopsy followed by 24 chromosome analysis has repaired this damaged reputation to some extent and PGS for aneuploidy is now back in widespread use in many clinics especially in the USA with plentiful data to illustrate the effectiveness of the process. The arguments for the use of PGS in a clinical setting are that that implantation, ongoing pregnancy and live birth rates per embryo transferred is higher for a known euploid embryo compared to an unscreened embryo for

patients of advanced maternal age (Chen *et al*., 2015; Dahdouh *et al*., 2015b; Forman *et al*., 2013a; Forman *et al*., 2013b; Forman *et al*., 2012b; Schoolcraft *et al*., 2010; Schoolcraft and Katz-Jaffe, 2013; Scott *et al*., 2012).

The arguments against the use of PGS are that there has not yet been a large randomized controlled Intention to treat trial, which takes into account all cycles started to get to the desired outcome of a live birth and that the presence of mosaicism translates to an inaccurate picture of the whole embryo therefore why would we test at all (Gleicher *et al*., 2016; Orvieto, 2016; Orvieto and Gleicher, 2016).

In the most experienced labs that are biopsying trophectoderm, vitrifying embryos with a high survival rate and using the latest DNA analysis techniques, one can still only expect around a 60-70% chance that any one blastocyst stage embryo, diagnosed as “euploid”, will give rise to a live born baby.

So why do so many embryos still fail? With the limitations of relying on the small representation of the biopsied cells presented for DNA analysis we are certainly missing some mosaic cell lines. Some of the embryos we transfer may be undetected mosaics and may be doomed to fail before implantation. Other lethal mutations at the gene level that we do not screen for, may be present in embryos.

From the perspective of the embryo, metabolomic/proteomic profiles may eventually be used in conjunction with PGS. It is possible that if particular metabolites or amino acids can be found, that are utilized or excreted by the embryo that strongly correlate with high implantation potential, and if a method of detection could be developed that is translatable to clinical practice, the results could be used to rank euploid embryos amongst a cohort for preferential transfer. An embryo with undetected mosaicism from a trophectoderm biopsy, may have a metabolomic/proteomic profile that differs from an embryo with a pure euploid cell line. To use euploidy determination alongside a metabolomic /proteomic profile may accurately predict which embryo is likely to make a healthy baby and may give a holistic overview of the whole embryo rather than relying on a few cells from the trophectoderm which may or may not represent the chromosomal complement of the embryo as a whole. We need to continue research to find biologic markers, their presence or deficit that predicts a high chance of a live birth in an embryo.

The long term effects (if any) of trophectoderm biopsy of a pre-implantation embryo have yet to be determined. It is possible that removing part of the trophectoderm has an effect on placentation which may affect fetal growth and development by restricting nutrition to the fetus. Offspring with compromised fetal nutrition can develop metabolic issues into adulthood. The future study that needs to be done is to assess placentation in offspring resulting from trophectoderm biopsied frozen thawed embryos. The 2 control groups in the study would be non-biopsied frozen thawed and non-biopsied fresh embryos. The ideal patient population to include in the study would be parous gestational carriers who typically have had normal vaginal

deliveries with no complications to remove the uterine effect. These patients are not large in number in any one center and this data would be difficult to obtain.

By definition, we are disrupting the trophectoderm layer by biopsying. As we do not know if there are any adverse long term effects, to be able to reliably ascertain the ploidy status of a blastocyst by a non-invasive method would be of benefit to our field. The non-invasive spent media PGS study carried out as part of this thesis showed some promise in amplifying cell free DNA with a novel amplification method. Cell free DNA quickly degrades and this novel MDA based method was able to capture these elusive short strands of DNA. The results obtained were only concordant with the nuclear DNA from the biopsied samples of frozen thawed embryos and less concordant when fresh embryos were used. Theoretically any cells damaged by the freezing process would release their DNA into the media which may have explained the difference between the two groups. Non-invasive PGS is still in it's very early stages of development and we may never be able to rely on results from fresh embryo culture. However there may be a place for this technology where the patient wishes to thaw, test and refreeze previously untested embryos, to obtain ploidy status before transfer. For these cases we currently thaw, biopsy and then refreeze the embryos to obtain PGS results. Although there would still be 2 rounds of freezing, the stress to the embryo would be minimized by removing the invasive biopsy from the procedure. There are plans to carry out further studies to validate the method.

Of course, choosing between embryos which is primarily what these screening tools could be used for, is only possible if there are multiple embryos available for a patient. If patients have

very few embryos or only one, there are arguments that the patient should transfer an unscreened embryo. Part of infertility treatment is to psychologically allow each patient to fulfill their own desire to feel that they have given each embryo a chance and this reasoning is mostly seen in older patients with fewer embryos. The younger patient with many embryos in her cohort we could argue would benefit from adjunct screening to best choose between euploid embryos for transfer.

If we do find a proteomic/metabolomic profile to add to PGS that predicts implantation success or failure at a high rate, there would always be patients who would still require a transfer if an embryo was determined to be euploid post PGS but non-viable post non-invasive screen, therefore the goal of 100% sustained implantation may never be achieved for all patients.

There will always be patients who wish to transfer unscreened embryos and some of those will result in a baby and some will not as has always been the case since IVF first began. Hopefully this thesis has provided the basis through which patients can make more informed choices.

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