Optimisation of Embryology Procedures and Clinical Outcomes in Preimplantation Genetic Testing of Human Embryos

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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

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Abbreviations

aCGH	array comparative genomic hybridisation
ADO	allele drop out
AH	assisted hatching
AI	artificial intelligence
AMA	advanced maternal age
ART	assisted reproductive technologies
BAC	bacterial artificial chromosome
BP	base pairs
CCS	comprehensive chromosome screening
CGH	comparative genomic hybridisation
CVS	chorionic villus sampling
DCDA	dichorionic diamniotic
DNA	deoxyribonucleaic acid
DOP-PCR	degenerate oligonucleotide primed-polymerase chain reaction
DZ	dizygotic
ET	embryo transfer
FET	frozen embryo transfer
FISH	fluorescent in situ hybridisation
HLA	human leukocyte antigen
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IVF	in vitro fertilisation
MBP	mega base pairs
MDA	multiple displacement amplification

MZ	monozygotic
NGS	next generation sequencing
niPGT-A	non invasive preimplantation genetic testing for aneuploidy
NORs	nucleolar organising regions
NR	No result
PCR	polymerase chain reaction
PEP	primer extension preamplification
PGT	preimplantation genetic testing
PGT-A	preimplantation genetic testing for aneuploidy
PGT-M	preimplantation genetic testing for monogenic disease
PGT-P	preimplantation genetic testing for polygenic disease
PGT-SR	preimplantation genetic testing for structural rearrangements
PND	prenatal diagnosis
qPCR	quantitative polymerase chain reaction
RCT	randomised controlled trial
RIF	recurrent implantation failure
RNA	ribonucleic acid
RPL	recurrent pregnancy loss
SNP	single nucleotide polymorphism
STR	short tandem repeat
TE	trophectoderm
UPD	uniparental disomy
WGA	whole genome amplification
YAC	yeast artificial chromosome
ZP	zona pellucida

Publications, book chapters and abstracts linked to this thesis

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Lynch, C., Cater, E., Charitou, M., Forbes, H., Griffin, D. & Gordon, T. 2019. Intracytoplasmic sperm injection is not necessary as a preventive measure against paternal contamination in preimplantation genetic testing. Reproductive BioMedicine Online. 39. e24-e25. 10.1016/j.rbmo.2019.04.051.

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Abstract

Since the very first Preimplantation Genetic Testing (PGT) case in 1989, the field has seen many changes; in indications, in diagnosis platforms and in embryology procedures. While PGT-M (monogenic) is widely accepted as an option for couples at risk of having a child with a serious inherited condition, PGT-A (aneuploidy) and PGT-SR (structural rearrangements) remain more controversial, with some still questioning the validity and utility of the data produced.

Some of the disagreement surrounding PGT-A and PGT-SR has stemmed from variation in approaches and results from different genetic laboratories and different assisted reproduction technology (ART) centres. As with all ART procedures, there are many variables that contribute to the success or failure of a treatment cycle, beyond the chromosome data produced from a trophectoderm sample. As well as technical aspects in the genetics and embryology laboratories, patient decision making contributes. Maximising clinical outcomes also depends on providing patients with reliable data and helping them make informed choices and treatment decisions, and minimising financial and emotional cost by not subjecting them to unnecessary procedures with little chance of success.

In order to investigate and, in part, redress variability in the field of PGT, this thesis had 7 specific aims:

- To liaise with diagnostic labs, embryology labs and medical affairs to create a unique set of guidelines for embryology labs wishing to use diagnostic services (chapter 2). Such a set of guidelines was created.

- To test the hypothesis that intracytoplasmic sperm injection is necessary as a preventive measure against paternal cell contamination in preimplantation genetic testing. Hence to ask if it is necessary for all patients undertaking PGT– even those with proven fertility – to have to undergo and pay additional fees for ICSI (chapter 3). Here, it was established that ICSI was not necessary
- To provide proof of principle that re-biopsy is, technically, a viable strategy when no result is obtained (chapter 4). This was established.
- To examine the "no result rate" in a leading group of UK PGT diagnostic laboratories since the introduction of next generation sequencing (NGS) to examine how much it varies between referring ART clinics, and assess whether re-biopsy is a viable alternative for most patients (chapter 5). It was established that re-biopsy was indeed a viable strategy.
- To perform a retrospective analysis of over 1,800 PGT-SR samples (479 cycles of 5 years) in order to: a) Provide the biggest dataset to date on PGT-SR outcomes using CCS to date; b) Test the hypothesis that the level and nature of structural chromosomal rearrangements is correlated to age, sex and time of biopsy; c) To test the hypothesis that an interchromosomal effect exists in this dataset; d) To provide a set of practical guidelines for genetic counsellors to advise patients on their likelihood of having euploid embryos for transfer based on the type of chromosome rearrangement, sex of the carrier of the rearrangement, maternal age, and any other factors that may be implicated (chapter 6). A retrospective analysis of over 1,800 PGT-SR samples (479 cycles of 5 years) was performed, to date on PGT-SR outcomes using CCS, finding no demonstrable ICE. A set of practical guidelines was put forward for genetic counsellors to advise patients on their likelihood of having euploid embryos for

transfer based on the type of chromosome rearrangement, sex of the carrier of the rearrangement, maternal age, and any other factors that may be implicated

- To provide novel insight into the mechanism of twinning as a result of a PGT case (chapter 7). Here evidence is presented of twinning occurring later in development – post day 5 development, but prior to implantation - thereby shedding light into a possible novel mechanism. That is, I hypothesise that this mechanism may involve splitting of the embryo as the inner cell mass hatches, with potential involvement of altered zonal lysis and apoptosis.

The data presented in this thesis points to continued differences in practice in the field, that will continue to lead to variable PGT outcomes – as with standard IVF treatment cycles – unless further research is performed, with large data sets presented which can point toward best practice.

1. Thesis Introduction

Preimplantation genetic testing (PGT) of embryos is seen by many, especially the general public, as a new and experimental technology. However, the first babies were born 30 years ago following this treatment and 12 years following the birth of the first IVF baby (Handyside *et al.* 1990). In fact, Robert Edwards, a pioneer of both IVF and preimplantation testing, performed the first PGT, by sexing of rabbit blastocysts (Edwards & Gardner 1967) ten years prior to the words first IVF birth that would eventually lead to his Nobel prize.

1.1 Development of preimplantation genetic testing (PGT)

PGT of embryos is enabled by IVF technologies, given that couples have to undergo IVF treatment in order to produce embryos for testing. Recent advances in both IVF technologies, molecular genetics and cytogenetics have opened the door to offering such treatments to a much wider range of patients.

There are four main categories of preimplantation genetic testing:

- PGT-A, preimplantation genetic testing for aneuploidy, for the identification of chromosomally normal, or euploid, embryos in IVF treatment cycles. Sex may be revealed by inclusion of the sex chromosomes in testing.
- PGT-M, preimplantation genetic testing for monogenic disease, involving testing for monogenic disease and tissue type. Sex may be revealed when testing for X-linked conditions.
- PGT-SR, preimplantation genetic testing for structural rearrangements, for the identification of chromosomally normal, or euploid, embryos for couples with structural chromosome rearrangements

- PGT-P, preimplantation genetic testing for polygenic disorders

In recent years, PGT-M and PGT-SR have garnered wider acceptance as a feasible reproductive option for couples at risk of passing a heritable condition to their children. The selection and use of unaffected embryos is, for most, an acceptable alternative to prenatal diagnosis (PND) and selective termination. For some, however, there is still the issue of what happens to affected embryos and the message it sends to affected individuals. PGT-A remains more controversial however, even within the scientific and medical communities, given different practices and technologies have variously shown the treatment to decrease pregnancy rates, provide no benefit, and increase pregnancy rates (Mastenbroek *et al.* 2011). The theory behind the intervention – the high percentage of aneuploidy in human embryos – remains sound though, and recent studies involving blastocyst biopsy and comprehensive chromosome screening are proving promising (Vinals Gonzalez *et al.* 2019, Gorodeckaja *et al.* 2020). Only one multi-centre double blinded randomised control trial (RCT) has been published, showing a significant increase in ongoing pregnancy rates per embryo transfer in the advanced maternal age group (35-40yrs) with utilisation of PGT-A (Munne *et al.* 2019).

1.1.1 Preimplantation genetic testing for monogenic disease

PGT-M was developed in response to couples at risk of transmitting heritable conditions to their children. The diagnosis of affected embryos prior to implantation and conception would circumvent the need for prenatal testing and selective termination. It is used in the following situations:

- Monogenic disorders inherited from a parent (dominant condition e.g. Huntington's disease, Achondroplasia, Myotonic Dystrophy)

- Monogenic disorders where neither parent is affected, but there may be a background of a wider family history or the birth of an affected child (recessive condition e.g. Cystic Fibrosis, Sickle Cell Anaemia, or X linked conditions e.g. Duchenne Muscular Dystrophy, Haemophilia)
- Medically indicated gender selection, where a genetic condition only affects one gender, or affects one gender more severely
- Human Leukocyte Antigen (HLA) tissue matching, to enable the conception of a child who is HLA compatible with an existing child requiring a bone marrow or stem cell transplant (often performed in conjunction with testing for a recessive monogenic disorder). So called "saviour siblings."

The first PGT-M cases were performed in 1989 for X linked monogenic disorders (Handyside *et al.* 1990). At this time, the specific genes and mutations involved in many conditions were unknown. Thus, in the situation of X-linked monogenic disorders, couples were offered prenatal diagnosis via chorionic villus sampling (CVS) or amniocentesis with cytogenetic analysis to determine the gender of the foetus. Despite the fact only half of all male foetuses would be affected, in some cases the inability to perform specific disease testing would mean the only option of avoiding the condition was selective termination of male pregnancies. PGT-M was developed as an alternative for couples who had already undergone previous terminations or had trouble conceiving naturally.

PGT-M was performed as a clinical treatment for the first time at the Hammersmith Hospital in the UK. Couples at risk of having children with the X linked conditions Lesch-Nyhan Syndrome, adrenoleukodystrophy, Duchenne muscular dystrophy and X linked mental retardation underwent IVF cycles with embryo biopsy at the day 3

stage. Analysis was performed by amplification of a Y chromosome specific repeat sequence detected via gel electrophoresis – in the absence of amplification the embryo was inferred to be female (Handyside *et al.* 1990). This was made possible by the development of the polymerase chain reaction (PCR), allowing exponential amplification of specific DNA targets. The short amount of time required for the analysis protocol meant that embryos could be transferred on the same day as biopsy, as blastocyst culture would not be robust or routine for a number of years. Later, the technique would be improved by amplification of both X and Y linked sequences (Kristjansson *et al.* 1994, Hussey *et al.* 1999) and then the use of fluorescent in situ hybridisation (FISH) allowing for the visualisation of both sex chromosomes, XX or XY (Griffin *et al.* 1991, 1992, 1993; Munne *et al.* 1995), and reducing the possibility of misdiagnosis (Staessen *et al.* 1999).

In 1992, the molecular techniques that first allowed the sex determination of embryos were extended to look at specific disease-causing genetic mutations. While the majority of groups continued to focus on blastomere biopsy, some undertook a combination of polar body and blastomere analysis – a position that was as much to do with legal implications as science. Cystic fibrosis was the first monogenic condition for which PGT-M was undertaken on human embryos to detect a specific disease-causing mutation (Handyside *et al.* 1992) – much research had taken place on mouse embryos prior to this. The deltaF508 mutation is the highest frequency CFTR mutation and is a 3 base pair deletion. A nested PCR of the region and gel electrophoresis allowed the detection of DNA homo- and heteroduplexes and the identification of affected, unaffected and carrier embryos. However, this technique was vulnerable to failed amplification of a specific allele (ADO, allele drop out), as the first gender

selection cases had been, given drop out of the affected allele would appear the same as a homozygous unaffected result on gel electrophoresis.

The introduction of multiplexing protocols with fluorescently labelled primers allowed multiple linked markers to be used to follow disease inheritance, whilst reducing the impact of ADO that had been an issue in earlier simplex approaches (Dreesen *et al.* 2000). ADO results from the preferential amplification of one allele in a heterozygous sample, resulting in the failure of detection of the other allele. Whilst this can be minimised by good practice in cell lysis and amplification, the use of closely linked short tandem repeat (STR) markers had the biggest mitigating impact. Each additional informative marker confirms the diagnosis or points to issues with contamination or ADO. Additional factors still applicable in multiplex strategies include amplicon size, good primer design, the choice of reagents for DNA lysis, the choice of DNA polymerase (Harton *et al.* 2010).

Multiplex fluorescent PCR both increased the accuracy of PGT-M by detecting ADO and contamination (Sermon *et al.* 1998) and allowed more than one genetic condition to be tested for, or more commonly, to test for both disease and HLA type in embryos (Van de Velde *et al.* 2004). Rather than determining PCR fragment size using ethidium bromide gels, as early simplex protocols had done, analysis of fluorescent PCR fragments on an automated sequencer provided greater sensitivity of detection and accuracy of size determination. However, it was labour intensive, requiring identification of informative markers and multiplex PCR optimisation for each family prior to the clinical PGT cycle. Informativity testing involved genotyping family members for STR markers flanking the gene of interest. Ideally, fully informative

markers would be identified, allowing the visualisation of all four parental haplotypes, but some cases also required the inclusion of partially informative markers, where a parental allele was shared. In some cases of multi-generational consanguinity or genetically homogenous ethnic groups, it would not be possible to identify the requisite markers. Each marker for each individual was a separate reaction, and only when enough informative markers had been identified – usually a minimum of two flanking markers on each side of the mutation - could phase be set, with the haplotype shared between individuals carrying the familial mutation assigned as the “at risk” or “affected” haplotype. PGT-M for *de novo* mutations could be designed in the same way, but with phase being assigned with the data generated by the biopsy samples in testing. This requires the incorporation of direct mutation analysis, commonly either via Sanger or mini sequencing (Bermudez *et al.* 2003) or using marker analysis to detect the different fragment lengths produced in cases of small deletions or duplications. Other methodologies including amplification refractory mutation system (ARMS) (Moutou *et al.* 2007), endonuclease restriction (Moutou *et al.* 2007), and quantitative real time PCR (Traeger-Synodinos *et al.* 2007)) have also been employed. However, in cases of complex or large rearrangements – for example, large deletions in the dystrophin gene – this may not be feasible, and the precise details of the breakpoints may be unknown.

The optimisation of the multiplex PCR was equally labour intensive, with single cell models such as lymphocytes and buccal cells used to validate the reaction and ensure amplification of all required loci in tandem. Selection of the cell type was important, as it had the potential to influence amplification efficiency and ADO rate (Glentis *et al.* 2009). Optimisation required the determination of optimal PCR conditions for

combining all primer sets in a single PCR reaction, adjusting primer concentrations to produce distinguishable signals in the same detection range and ensuring no overlap between marker signals and background peaks (Dreesen *et al.* 2000). Validation required assessing the amplification efficiency and ensuring it was at least 90% for each marker and assessing the ADO rates at preferably <10% (Harton *et al.* 2010).

This approach remained unchanged for many years until robust and accurate methods of whole genome amplification (WGA) negated the need for multiplexing and greatly increased the number of linked markers that could be run and the reliability of testing (Ao *et al.* 1998, Spits *et al.* 2006, Renwick *et al.* 2006). WGA enables the production of several micrograms of DNA from single cells, which can then be used as a template for a number of downstream applications. A large number of WGA protocols have been published all with their own advantages and disadvantages (Zheng *et al.* 2011). Early issues included incomplete genome coverage and introduction of sequence errors. No WGA method provides a true representation of the original DNA template and current approaches still vary in terms of ADO and preferential amplification rates, genome coverage, and the incidence of nucleotide errors. Given this, the optimal WGA method of choice is essentially dictated by the intended downstream application, discussed in more detail in sections 1.2.1 and 1.2.2.

Haplotyping continued to remain the gold standard in PGT-M following the introduction of WGA, using a greater number of markers than previous multiplexed approaches. However, the advent of SNP (single nucleotide polymorphism) arrays has been the biggest paradigm shift in the field since its introduction (Natesan *et al.* 2014) and

potentially allows the tandem identification of meiotic aneuploidy, and uniparental disomy.

SNPs are biallelic and the two possible alleles are identified as A (representing the nucleotide A or T) or B (representing the nucleotide G or C) (Illumina 2015). Thus, three genotypes are possible: homozygous AA or BB, and heterozygous AB. The method of SNP genotyping is dependent on the platform used, but the general principle is that following scanning of the array, the SNP genotypes are called based on total fluorescence and the ratio of hybridisation intensities for each allele (A and B). Typically, 20-40% of SNPs genome wide are expected to be informative (Illumina 2015), which can still equate to tens or hundreds of informative markers in the region of interest. For a SNP to be informative one parent must be heterozygous and the other homozygous. The informative allele from the heterozygous parent is used to set phase in the biopsy sample against the reference i.e. if they both inherited or both did not inherit the informative allele, they inherited the same chromosome from that parent.

SNP arrays, therefore, work on the same linkage-based principle as multiplex PCR and haplotyping. However, using a high-density array allows genotyping of hundreds of thousands of SNPs across the genome in a single reaction, cutting the workload and time involved in test optimisation. The use of SNP arrays also enables a more standardised workflow and the introduction of elements of automation, which when coupled with the reduction of workload, reduces the chances of error or variability in the laboratory. The current application of SNP arrays in PGT is discussed in greater detail in section 1.2.1.

1.1.2 Preimplantation genetic testing for structural rearrangements (PGT-SR)

The introduction of fluorescent *in situ* hybridisation (FISH) to single cell work opened up the possibility of PGT-SR to couples where one carried a balanced structural chromosome rearrangement – for example a reciprocal or Robertsonian translocation or an inversion. This was first applied in the mid-1990s, using FISH probes specific to the chromosomal breakpoints and enabling the detection of different unbalanced segregation patterns (Munne *et al.* 1998). A probe set was required to detect all unbalanced forms of the chromosome rearrangement and so, like PGT-M via multiplex fluorescent PCR, pre-examination validation and optimisation was also necessary. The most commonly applied approach in PGT-SR involved investigation of chromosome copy number on interphase nuclei, removing the requirement for cell culture or the preparation of metaphase spreads, important factors in reducing analysis time and allowing the return of results prior to embryo transfer. It involved the hybridisation of fluorescently labelled single stranded DNA probes to a complementary target, which were then visualised using fluorescence microscopy (Munne *et al.* 2002).

The first step of the pre-examination stage was to confirm the breakpoints of the rearrangement and perform a segregation analysis to characterise all potential unbalanced forms of the rearrangement. It would then be necessary to generate metaphase spreads from peripheral blood samples of the couple seeking treatment to ensure the selected probes were specific for the intended chromosomes, assess any polymorphisms of cross hybridisation which would interfere with test efficiency or analysis of results, and to ensure that the selected probes were informative with respect to the rearrangement. The additional scoring of interphase nuclei served to assess signal specificity, brightness, and discreteness (Harton *et al.* 2010b).

Probe selection was therefore of vital importance. Some early strategies involved the work up of patient specific yeast artificial chromosome (YAC) DNA probes (Cassell 1997). While very labour intensive, the use of breakpoint spanning probes allowed the differentiation of normal, balanced and unbalanced samples. Using a breakpoint spanning probe on each chromosome involved in the rearrangement, labelled with, for instance, green and red fluorochromes, would see two green and two red signals in a normal sample. However, in the balanced sample, a green and a red signal would appear, denoting the normal chromosomes, and the derivative chromosomes appear as associations of smaller red or green chromosomes. Any other combination of signals visualised would denote imbalance (Munne *et al.* 2002).

PGT-SR became more feasible with the availability of commercially available sub telomeric probes (Ning *et al.* 1996), and the use of these and probes distal to the breakpoint represent the most commonly adopted, and simple approach (Figure 1.1), despite an inability to distinguish normal and balanced embryos. This required the use of a minimum of two probes distal to the breakpoint and one proximal (or vice versa) otherwise 1:3 imbalances would not be detected (Pierce *et al.* 1998). The use of additional probes increases the reliability of the test in the case of any FISH errors or failures. These could include false monosomies resulting from signal overlap or the loss of micronuclei during fixation, the fixation of multinucleated blastomeres, false positives resulting from split signals, or false positives resulting from initiation of S-phase with non-synchronous chromatid replication (Mucherjee *et al.* 1992, Munne *et al.* 2002). The use of centromeric probes were also still required in cases where a high risk of adjacent 2 segregation had been identified (Handyside *et al.* 1998).

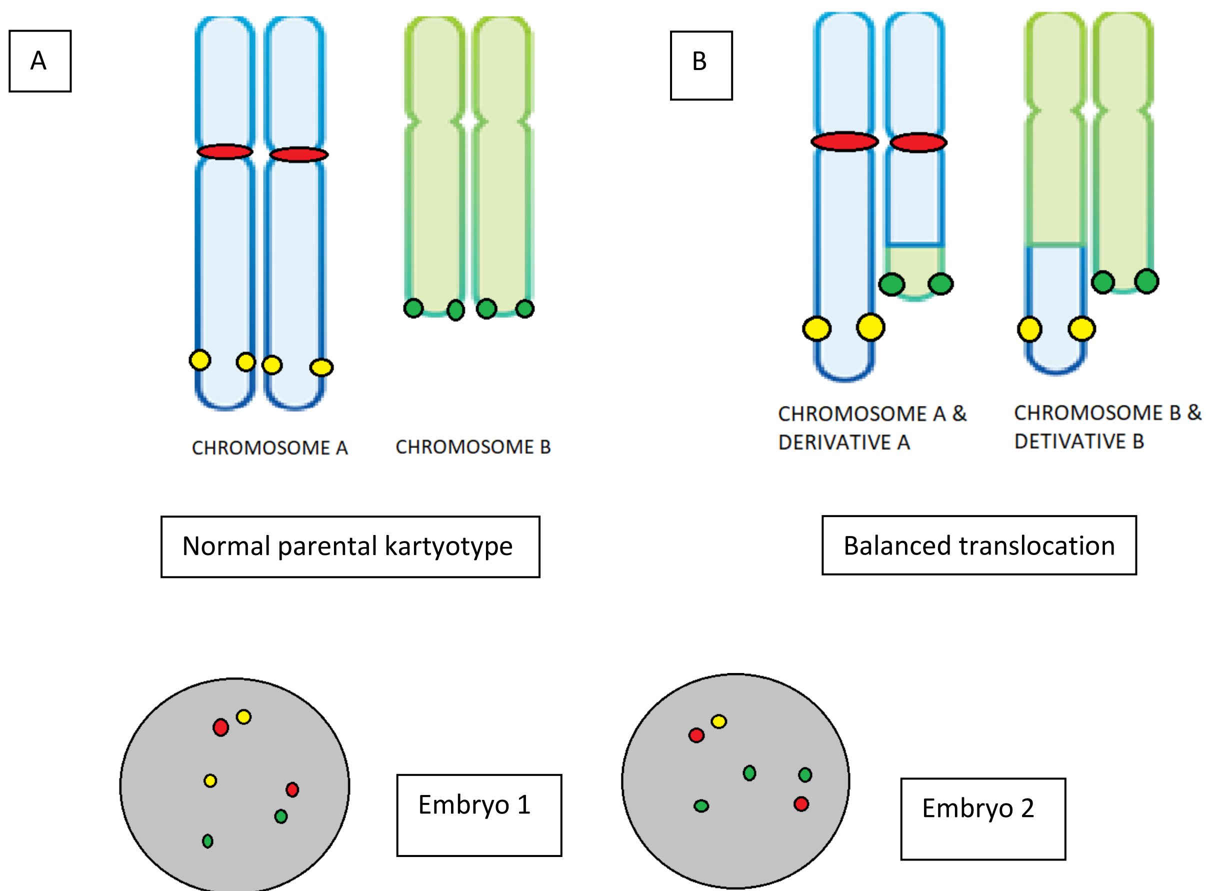

Figure 1.1. Three colour FISH strategy used for PGT-SR (Ogur and Griffin, 2020).

A. A normal karyotype– paired homologous chromosomes – and an abnormal karyotype with a reciprocal translocation - chromosome A is shown paired with a derivative A, and chromosome B with a derivative B. To detect all aberrant segregation patterns that may be present in the embryo, three fluorescently labelled probes are used: sub-telomeric (green), locus specific (yellow) and centromeric (red)

B. The biopsy from embryo 1 displays two signals for each probe, consistent with a normal or balanced complement of the translocated chromosomes. The biopsy from embryo 2 displays two red signals, 3 green signals, and 1 yellow signal, indicating three copies of the translocated segment of chromosome B, 1 copy of the translocated

segment of chromosome A, and 2 copies of the centromere of chromosome A. This is consistent with Adjacent-1 segregation of the translocation, resulting in an embryo with a partial monosomy of chromosome A and partial trisomy of chromosome B.

The use of PGT-SR for chromosome rearrangements was, however, controversial, as some groups showed that couples would be successful in the same time frame naturally when compared with multiple PGT-SR cycles (Scriven *et al.* 2013, Murugappan *et al.* 2015), failing to take into account the emotional impact of recurrent miscarriage for couples. An important limitation of PGT-SR until this point was that only the chromosomes involved in the rearrangement were examined. Fluorescent multiplex PCR was applied to PGT-SR, requiring extensive workup to identify STR markers located on both sides of the breakpoint, but allowing the identification of contamination by exogenous DNA, the detection of uniparental disomy (UPD), and additional aneuploidy testing of chromosomes not involved in the rearrangement (Fiorentino *et al.* 2010). However, it was the advent of comprehensive chromosome screening (CCS) that demonstrated that many embryos scored as balanced/euploid and selected for transfer following PGT-SR via FISH were, in fact, aneuploid for chromosomes that had not been tested (Treff *et al.* 2010).

Metaphase comparative genomic hybridisation (CGH), array based comparative genomic hybridisation (aCGH), and SNP arrays were all applicable to PGT-SR, dependent on the size of the imbalances to be detected (Fiorentino *et al.* 2011, Treff *et al.* 2011) and allowed the detection of aneuploidy in addition to unbalanced segregants. Despite the fact that SNP arrays allowed the differentiation of normal and balanced samples, and identified UPD, aCGH became the more widely adopted

technique in practice. Validation of SNP arrays for PGT-SR demonstrated identification of segmental changes of 13.8 Mb (Treff NR *et al.* 2011), while 2.5Mb segmental imbalances were successfully identified via aCGH using BAC microarrays (Fiorentino *et al.* 2011) and oligo microarrays proved even more sensitive (Ramos et al. 2014).

aCGH involves competitive hybridisation of labelled WGA sample DNA and reference DNA to BAC or oligo microarrays and analysis of the relative fluorescence via software. It provided a simple workflow with a short protocol length, coupled with the possibility of automation. The application of aCGH to PGT-SR identified abnormalities FISH could not detect, with one study demonstrating aneuploidy in 26.4% of samples classed as normal/balanced by FISH (Colls *et al.* 2012). Initial studies showed clinical pregnancy rates increased to 70% (Fiorentino *et al.* 2011), reductions in miscarriage rates (Alfarawati *et al.* 2011), and reinforced the importance of CCS with high percentages of samples displaying incidental aneuploidy unrelated to the chromosome rearrangement (Ghevaria *et al.* 2016, Christodoulou *et al.* 2017).

More recently, next generation sequencing (NGS) has become more widely used ford PGT-SR. Discussed in more detail in section 1.2.2, it allows multiple samples to be pooled and processed on a single sequencing cell. Sequenced fragments are aligned to a reference genome and read depth compared across regions, with trisomy or monosomy resulting in a greater or lower read depth respectively (Handyside 2013, Yin *et al.* 2013, Illumina 2020). Currently, NGS platforms and data pipelines are available allowing identification of segmental changes as small as 5 Mb (Bono *et al.*

2015, Blanca *et al.* 2018), and it has demonstrated greater resolution for the detection of imbalances than SNP arrays (Yin *et al.* 2013, Tan *et al.* 2014).

The major limitation of most CCS molecular cytogenetic techniques is the inability to reliably detect unbalanced derivatives from rearrangements that have breakpoints in the telomere or sub-telomere (Morin *et al.* 2016). While FISH does provide coverage of these regions, the limitations of this approach remain as previously described and misdiagnoses have been reported (Van Echten-Arends *et al.* 2013). It should be noted that no misdiagnoses have been reported or published relating to NGS or SNP arrays. Before any case is accepted for PGT-SR it is important that the testing laboratory is able to examine breakpoints and the size of all potential imbalances to ensure this will be detectable by the platform in use.

While clinical outcomes have been well characterised across different technology platforms, less well studied are the indications for testing, the types of structural chromosome rearrangements that result in referrals for PGT-SR, and what this can tell us about the relationship between these rearrangements and subsequent fertility status, and also what this can tells us about the molecular mechanisms and behaviour of chromosome rearrangements in general. Chapter 7 of this thesis aims to address these issues to some degree.

1.1.3 Preimplantation genetic testing for aneuploidy (PGT-A)

The introduction of FISH to single cell PGT-SR was only subsequent to chromosome screening, PGT-A (Munne *et al.* 1993) and medically indicated sex selection (Griffin et al. 1993). The age-related decline in natural and IVF birth rates is linked to increased

aneuploidy rates, which contribute to failed implantation, miscarriage and increased rates of Down, Edwards and Patau syndromes (trisomies 21, 18 and 13 respectively). Theoretically, therefore, the identification and negative selection of chromosomally abnormal embryos should mitigate the age effect in IVF and increase implantation and reduce miscarriage rates in patients of advanced maternal age. Thus, PGT-A was generally indicated in patients with advanced maternal age, recurrent miscarriage and recurrent implantation failure and was reported to improve success rates (Munne, 2002).

As discussed in section 1.1.2, FISH for PGT-A also involved the hybridisation of fluorescently labelled single stranded DNA probes to a biopsied cell fixed to a glass slide, which were then visualised using fluorescence microscopy (Munne *et al.* 2002). The presence or absence of each targeted chromosome is then inferred by the presence or absence of the associated fluorescent signal. A limited number of fluorochromes limited the number of chromosomes that could be tested. Thus, initially, chromosomes with a high risk of resulting in the birth of child with a chromosomal syndrome were selected for PGT-A probe panels – chromosomes 13, 18, 21, X and Y (Munne *et al.* 2002). However, it was soon demonstrated that a second round of hybridisation could be performed with efficiency of >95% (Martini *et al.* 1997, Bahce *et al.* 2000), and a third round, although with impact of efficiency dropping below 80% (Liu *et al.* 1998). This allowed the incorporation of chromosomes associated with recurrent miscarriage, including chromosomes 15, 16, and 22 (Munne *et al.* 2002). Most panels applied to PGT-A used between 5 and 12 chromosomes, and although 24 chromosome FISH was reported, it was not applicable to clinical use (Ioannou *et al.* 2011).

The limitations of FISH as applied to single cell analysis are well documented and were discussed in section 1.1.2, including signal overlap, the loss of micronuclei during fixation, the fixation of multinucleated blastomeres, split signals, and initiation of S-phase with non-synchronous chromatid replication (Mucherjee *et al.* 1992, Munne *et al.* 2002). In terms of PGT-A, there was the additional issue of mosaicism. While an inherited abnormality as tested for via PGT-M or PGT-SR can be expected to be present and detected in each cell of an embryo, it soon became clear that in many cleavage stage embryos there was a mixture of cell populations in terms if chromosome constitution (Delhanty *et al.* 1993, Munne *et al.* 1994). This was not an issue with FISH as a technology, but a fundamental issue of the biology of early embryo development. Aneuploidy resulting from meiotic errors would be present from fertilisation and detectable in all embryonic cells in subsequent divisions. However, it appeared that there was also a high incidence of post zygotic errors in mitosis, the mechanisms of which are discussed in section 1.5, resulting in mosaicism in embryos.

Cleavage stage mosaic embryos could be classified into three categories (Munne *et al.* 2002):

- Chaotic mosaics with on average 84% chromosomally abnormal cells, with most cells chromosomally different from each other, and accounting for half of all mosaic embryos.
- Diploid/polyploid mosaics with on average 43% chromosomally abnormal cells and accounting for a quarter of mosaic embryos.

- Mosaic embryos produced by mitotic errors such as non-disjunction or anaphase lag with on average 63% chromosomally abnormal cells, and accounting for a quarter of mosaic embryos.

Studies reported in excess of 70% of cleavage stage embryos to be impacted by mosaicism (van Echten Arends *et al.* 2011, Mertzanidou *et al.* 2013).

Thus, when randomised control trials (RCTs) were published it was demonstrated that PGT-A in fact gave no advantage, or even reduced success rates (Mastenbroek *et al.* 2011). This was controversial at the time and hotly contended by initial pioneers of the treatment who continued to evidence improved success rates (Munne *et al.* 2007). However, it did lead to many changes in practice, most notably the introduction of comprehensive chromosome screening (CCS) and drove implementation of trophectoderm biopsy. The impact of biopsy at different stages of development is discussed in detail in section 1.3.

The rationale for PGT-A remained sound, and so a second generation of testing involving CCS and trophectoderm biopsy aimed to overcome the technical issues of FISH and the biological issue of mosaicism in the cleavage stage embryo. Multiple CCS methods have been applied to PGT-A including metaphase (Wilton et al. 2001) and array based comparative genomic hybridisation (CGH) (Yang *et al.* 2012), quantitative fluorescent PCR (Treff *et al.* 2012, SNP arrays (Treff *et al.* 2010), and most recently next generation sequencing (NGS) (Fiorentino 2014b). Initially aCGH proved the most widely adopted CCS platform, but has more recently been superseded by NGS, as discussed in section 1.1.2, NGS is discussed in more detail in section 1.2.2.

There are now a number of prospective and retrospective studies, randomised control trials (RCTs) and meta-analyses supporting the use of PGT-A in clinical practice as a means of increasing implantation rates, reducing miscarriage rates, and reducing time to pregnancy (Dahdouh *et al.* 2015, Lee *et al.* 2015, Coates *et al.* 2017, Friedenthal *et al.* 2018, Munne *et al.* 2019). A recent retrospective study employing trophectoderm biopsy, vitrification, NGS and single embryo transfer, reports implantation rates of 80-86%, live birth rates per embryo transfer of 60-73% and a clinical miscarriage rate <10% in patients of advanced maternal age (>37 years of age) (Vinals Gonzalez *et al.* 2019). A similar prospective study reports implantation rates of 80% and clinical pregnancy rates of 66% (Gorodeckaja *et al.* 2020).

1.1.4 Follow-up data of children born following PGT

The European Society of Human Reproduction (ESHRE) PGT consortium is part of the ESHRE reproductive genetics special interest group, focussing on collecting prospective and retrospective data on PGT cycles performed worldwide, and producing consensus guidelines for all elements of the PGT process to promote best practice. Part of their best practice recommendations include short and longer term follow up of any children born as a result of PGT (Carvalho *et al.* 2020).

Children born following ART have been reported as being at increased risk of some adverse perinatal outcomes when compared with natural conceptions (Fauser *et al.* 2014). Reassuringly, studies collating information on children born following PGT do not demonstrate any increase in risks of congenital malformation or adverse perinatal outcomes as a result of the increased manipulations the embryo undergoes (Sunkara *et al.* 2017, Heijligers *et al.* 2018).

1.1.5 Regulation of PGT

Since the inception of PGT there has been continual technical advance, increasing cycle numbers, and an increasing number of indications. Application of testing and embryo selection has always raised socioethical concerns – including the "slippery slope to eugenics" – and the rights or status of the embryo (Baertschi 2008, Bayevsky 2016). As such, the usage of PGT is often subject to regulation or even legal boundaries or restrictions. However, this varies widely across countries, creating environments ranging from very permissive to a complete ban (Ginoza & Isasi 2019),

Countries which have legislative frameworks include Austria, Belgium, Canada, France, Germany, India, Italy, the Netherlands, South Korea, Switzerland, and the United Kingdom, The laws in these countries vary widely, from outright bans of certain treatments to establishing a legislative framework of permissible usage. Other countries rely on self-regulation via guidelines that are not legally binding, including Australia, Brazil, Israel, Japan, Singapore and the USA. Other countries remain with no legal framework and no guidelines from governing, regulatory, or professional bodies (Ginoza and Isasi 2019). One area in which disparity is particularly noted is in non-medical sex selection. While the majority of countries mentioned previously legislatively prohibit this, some only prohibit by guidelines, some allow in limited circumstances, while it is allowed in the USA.

In the UK regulation of PGT comes under the remit of the Human Fertilisation and Embryology Authority (HFEA). Currently, they have licensed over 200 monogenic conditions for PGT-M. When an IVF clinic wishes to offer PGT-M for a condition not yet licensed, they must submit an application to the HFEA providing details of the

condition. The HFEA must ensure legal criteria are met when considering new conditions, mainly that there is a significant risk of a serious medical condition in any children, taking into account criteria including:

- Penetrance and variability
- Age of onset
- Symptoms

Their decision is based on the most severe presentation on the condition and IVF centres are then required to satisfy themselves that their patients fit the legal requirements for treatment. Licensing for HLA matching is done on a named patient basis and requires the support of a clinician treating the child requiring bone marrow or stem cell transplant, for example a paediatric haematologist or oncologist. The HFEA will additionally take into account:

- The degree of suffering associated with the existing child's condition
- The speed of degeneration in progressive disorders
- The extent of any intellectual impairment
- The prognosis of the existing child, considering all treatment options available
- The availability of alternative sources of tissue for treating the existing child, now and in the future
- The availability of effective therapy for the existing child, now and in the future

The differences in oversight means that in addition to offering treatment in line with local regulatory requirements and accrediting or licensing bodies, IVF clinics should have their own guidelines for staff to follow. This may include which conditions to offer PGT-M for and consider criteria similar to those previously discussed.

For all PGT cases the clinic may also wish to take into consideration the reliability of the test available (including accreditation status of the testing laboratory), likelihood of success, and safety (including any medical contraindications where an individual is affected by the condition for which testing is being performed). The clinic should also be able to provide or access appropriate counselling for couples seeking treatment including:

- Genetic risk assessment
- Reproductive options
- IVF related counselling
- PGT counselling

1.2 Current technologies for PGT diagnoses

At present, most PGT laboratories employ at least three technologies in order to make a diagnosis from embryonic material: One for the whole genome amplification of DNA from the biopsy one for detection of full and segmental chromosome gains and losses (PGT-A and PGT-SR) and one for detection of monogenic disease (PGT-M). The ideal would be to have a single test validated for all PGT. Currently, if a patient wishes to have PGT-M and also ensure their embryos are chromosomally normal, they have to undergo – and pay for – two separate tests. However, the advent of WGA technology means that both tests can be performed on amplified DNA from the original trophectoderm biopsy. SNP arrays and NGS both hold promise for providing a universal test platform.

WGA is the precursor step to both SNP arrays and NGS. WGA enables the production of several micrograms of DNA from a single cell, A large number of WGA protocols

have been published all with their own advantages and disadvantages (Zheng *et al.* 2011). Given this, the optimal WGA method of choice is essentially dictated by the intended downstream application.

The first PCR based WGA method, including primer extension amplification (PEP) and degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR) had issues with incomplete genome coverage and amplification bias (Zheng *et al.* 2011). An alternative, multiple displacement amplification (MDA), based on isothermal strand displacement was developed. MDA involves random exonuclease-resistant primers annealing to the denatured target DNA. DNA polymerase, such as Phi29, elongates the primers in an isothermal reaction and additional priming events can occur on each displaced strand leading to a network of branched DNA strands over 10 kb. The proofreading activity of the Phi29 polymerase, reduces the error rate compared to PEP and DOP-PCR, but the non-linear amplification can result in uneven genomic coverage (Spits *et al.* 2006).

Subsequent WGA methods have combined MDA with PCR amplification, including the Rubicon PicoPLEX/Illumina Sureplex system. This involves DNA fragmentation and a pre-amplification MDA reaction using hybrid primers, followed by PCR (Langmore 2002).

MDA remains the preferred method of WGA for applications like Karyomapping, while many NGS platforms employ the PicoPLEX/SurePlex system.

1.2.1 Single nucleotide polymorphism (SNP) arrays and Karyomapping

Karyomapping works on the same linkage-based principle as multiplex PCR and haplotyping. However, using high-density arrays allows genotyping of hundreds of thousands of SNPs across the genome in a single reaction, cutting the workload and time involved in test optimisation

SNPs are biallelic and the two possible alleles are identified as A (representing the nucleotide A or T) or B (representing the nucleotide G or C) (Illumina 2015). Thus, three genotypes are possible: homozygous AA or BB, and heterozygous AB. The method of SNP genotyping is dependent on the platform used, with methods including hybridization to SNP allele−specific probes or single-base extension reactions. However, the general principle is that following scanning of the array, the SNP genotypes are called based on total fluorescence and the ratio of hybridisation intensities for each allele (A and B). Typically, 20-40% of SNPs genome wide are expected to be informative (Illumina 2015), which can still equate to tens or hundreds of informative markers in the region of interest. For a SNP to be informative one parent must be heterozygous and the other homozygous. The informative allele from the heterozygous parent is used to set phase in the biopsy sample against the reference i.e. if they both inherited or both did not inherit the informative allele, they inherited the same chromosome from that parent.

SNP genotype data from parents, reference (usually an affected child or carrier grandparents) and biopsy samples is processed via software, producing Karyomaps, with phasing relative to the reference (Figure 1.2). A consequence of relative phasing is that recombination events in the reference cause a change of phase in all

corresponding embryos, meaning all embryos inheriting that allele would appear to have and identical crossover.

Figure 1.2 illustrates PGT-M for a genetic mutation at 16p13.3. In the case of a recessive genetic condition where the reference was an affected child, embryo 8 would be affected, embryos 1 and 4 would be paternal carriers and embryo 10 would be a maternal carrier. In the case of a paternally inherited dominant condition, where the reference was an affected child, embryos 1, 4 and 8 would be affected and embryo 10 would be unaffected. The detailed haploblock chart shows the SNP calling along chromosome 16 for embryo 4.

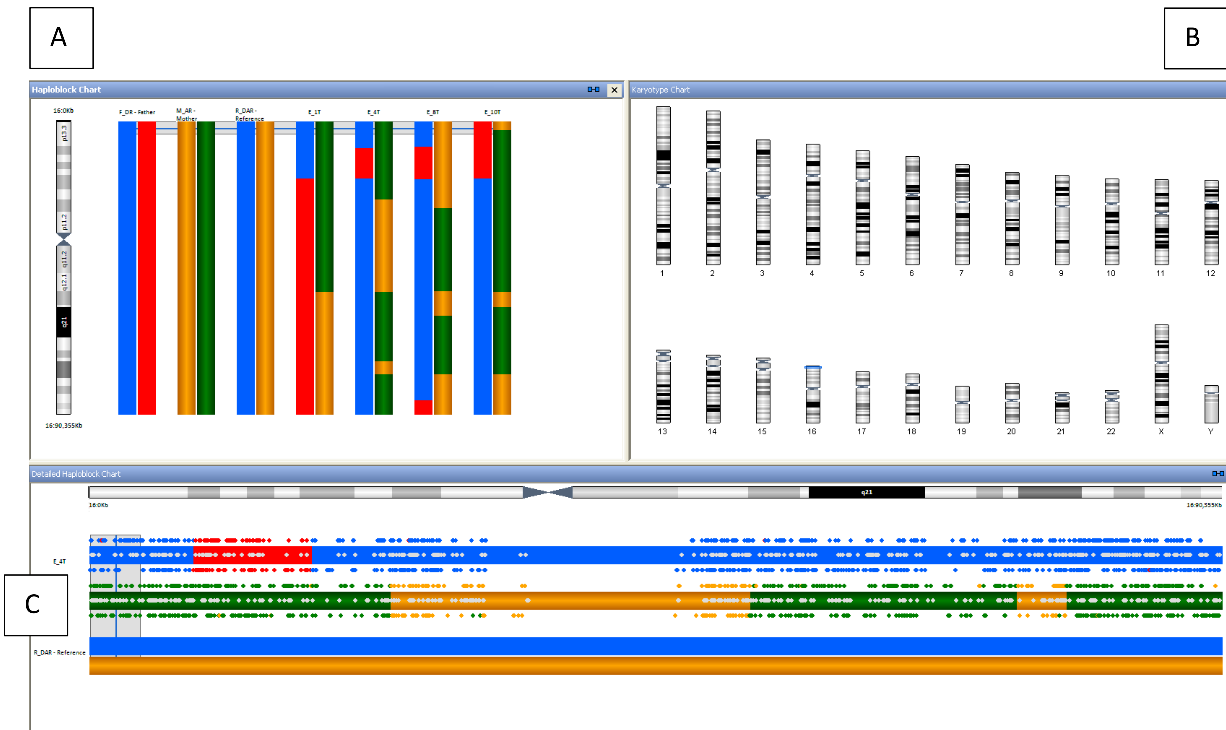

Figure 1.2. Software analysis of Karyomapping data (Illumina). Image of Bluefuse software analysis provided by CooperGenomics. A. Haploblock chart displaying the karyomaps of chromosome 16 for the parents, affected child (reference) and embryonic samples 1T, 4T, 8T and 10T. The region of interest (16p13.3) is indicated by a grey band, and a blue band marks the mutation.

B. Karyotype chart. Displays the mutation being followed as a blue bar across the region of interest on chromosome 16.

C. Detailed haploblock chart. Displaying the karyopmap of chromosome 16 for embryonic sample 4T at the level of the key and non-key SNPs against the reference sample. Sample 4T has the same paternal haplotype in the region of interest, but a different maternal haplotype. If the mutation in question was dominant and paternally inherited, embryo 4T would be affected. If the mutation was recessive, or maternal dominant, embryo 4T would not be affected.

If using Karyomapping as a universal method for monogenic disease and chromosome copy number analysis, trisomies of meiotic origin, can be identified by key SNPs phasing randomly and generating interchanging haploblocks along the length of the chromosome. Conversely, monosomies or deletions can be identified by the absence of either chromosome from one parent (Handyside 2013). This approach of copy number analysis based on genotype also allows the identification of uniparental heterodisomy parent of origin of meiotic errors (Natesan *et al.* 2014). However, sequence identical mitotic errors would not be identified using the described technology and would require an alternative quantitative approach (Treff *et al.* 2010b).

1.2.2 Next generation sequencing (NGS)

Sequencing refers to the determination of the order of nucleotides in a specific sequence. This involves exposure of a single stranded DNA template to dNTPs and determining the order in which the dNTPs are incorporated into the complementary strand. What made NGS revolutionary compared to earlier sequencing methods was the unique indexing of samples, allowing multiple samples to be pooled and processed on a single sequencing cell. The addition of barcodes to sample DNA allows software to identify each original sample individually from the data produced by the single sequencing cell.

The most commonly applied NGS platforms for PGT-A and PGT-SR are the Illumina VeriSeq and Ion Torrent systems. The Illumina Veriseq protocol employs sequencing by synthesis technology whereby fluorescently labelled chain-terminating nucleotides are incorporated during sequencing, allowing determination of the order of nucleotide incorporation using specialised optical detection (Fiorentino *et al.* 2014b, Victor *et al.*

2020). The Ion Torrent platform uses a completely different approach, detecting changes in pH caused by a release of protons at nucleotide incorporation releases protons, with the changing in pH of the surrounding solution being proportional to the number of incorporated nucleotides (Wells *et al.* 2014, Victor *et al.* 2020).

Sequenced fragments are aligned to a reference genome and read depth compared across regions, with trisomy or monosomy resulting in a greater or lower read depth respectively (Handyside 2013, Yin X *et al.* 2013, Illumina 2020). Software coverts this information into a visual chart (Figure 1.3).

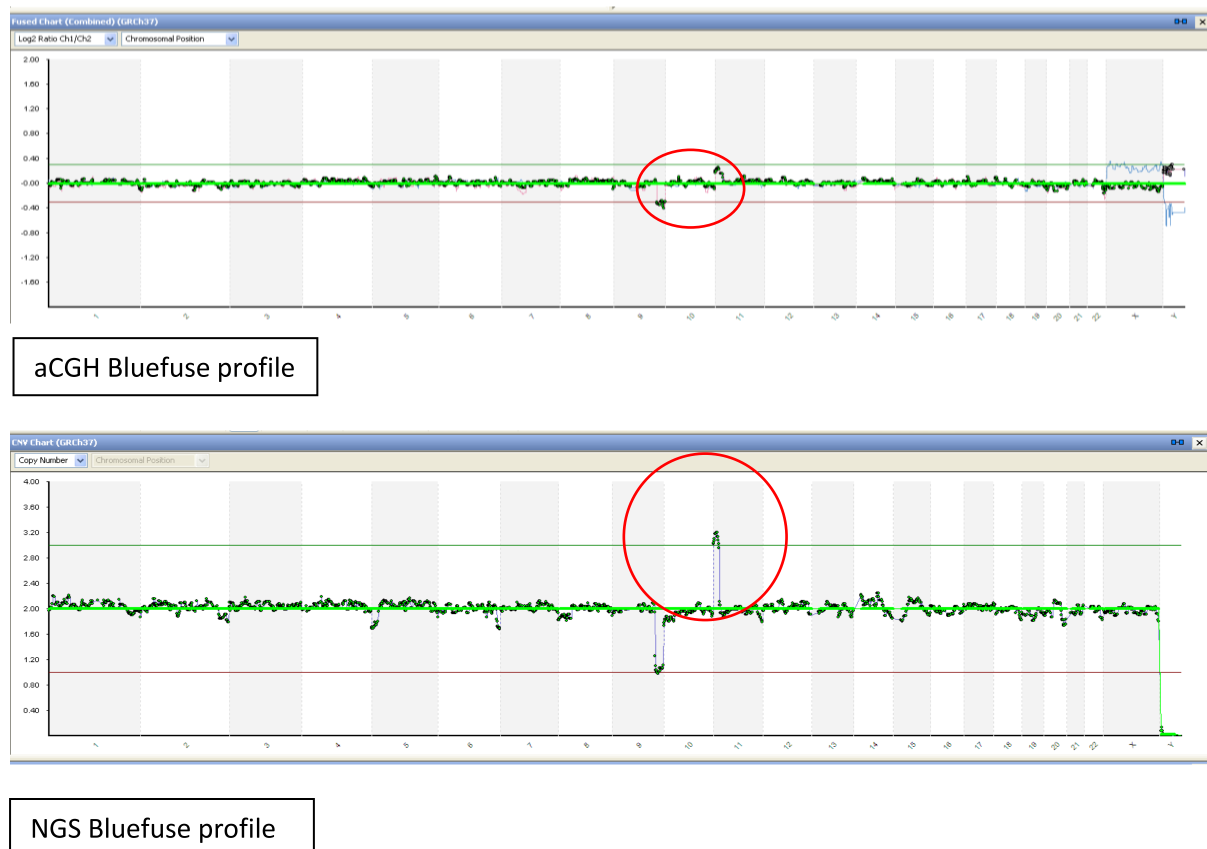

Figure 1.3. Bluefuse software charts displaying results for PGT-SR for a reciprocal translocation between the long arm chromosome 9 and the short arm of chromosome 11. The top image shows the chart generated when analysis is performed on the sample via aCGH while the bottom image shows the chart generated following analysis of the same trophectoderm sample via NGS On both charts, the X axis

displays the chromosome number from 1-22 and then the sex chromosomes, X and Y. Both profiles show a loss on 9q and a gain on 11p, related to an unbalanced form of the translocation being present.

The aCGH chart is a fused chart displaying the postprocessed results that have been normalised to correct for spatial intensity, dye-related effects, and combined replicates, and plots the sample against a male and female reference. The Y axis on the aCGH chart is a log ratio representing chromosome copy number, generated from the ratio of fluorescence between co-hybridised test and reference samples. The NGS chart is a CNV chart where the data is plotted using true copy number data, and thus the y-axis is chromosome copy number.

NGS may ultimately provide a universal test for PGT. The use of NGS for PGT-M could remove the need for family specific optimizations requiring reference individuals (e.g. affected children). While linkage analysis – be it STRs or SNP arrays – is recommended to guard against ADO and contaminations, applications of NGS in PGT-M have reported 0% ADO over multiple heterozygous sites (Kubikova *et al.* 2018). It is possible to sequence the gene of interest and closely linked SNPs, allowing the identification of any mutation. The NGS platform in itself allows for processing of multiple samples at a time, increasing throughput in combination with possible automation.

1.3 The embryology laboratory

Since the introduction of PGT into assisted reproductive medicine, many changes have occurred within the embryology laboratory. There has been change from single

cell biopsy approaches (polar body or blastomere) to multiple cell biopsy (trophectoderm biopsy), enabled by improvements in embryo culture, and from fresh embryo transfer on days 5 or 6 to vitrification and frozen embryo replacement.

Figure 1.4. Typical embryology laboratory set up for biopsy including microscope, ICSI holdings and syringes, laser, and PC with laser and biopsy software. Image courtesy of CooperSurgical Fertility and Genomics Solutions.

Regardless of the stage at which biopsy is performed, all laboratories will require the same general set up; an inverted microscope with a micromanipulator and laser, with visual display on a computer monitor or screen (Figure 1.4)

1.3.1 Polar body biopsy

This approach benefits from the fact that no essential cellular material is removed for testing, but can only be used for maternally inherited genetic conditions and will not detect post meiotic chromosomal errors. The biggest advantage of polar body biopsy is that the ploidy of polar bodies I and II accurately predicts the ploidy of the zygote as shown through ESHRE's ESTEEM trial (Geraedts *et al.* 2011). It is often used where there are legal or ethical contraindications to embryo biopsy. For an accurate result both polar bodies must be removed and tested – either sequentially or simultaneously, the advantage of the former being the greater ease in differentiating them. The timing of the removal of the second polar body is important in terms of the completion of anaphase II, complete cleavage from the oocyte (reducing the risk of the presence of spindle remnants in the cytoplasmic bridge and removal of chromatids from the oocyte), and maximising amplification rates (Magli *et al.* 2011). The process can be labour intensive, especially if the biopsies are performed sequentially, and given that not all oocytes will fertilise.

If performing sequential biopsy, the first polar body should be removed 36-42hrs post ovulation triggering via human chorionic gonadotrophin (hCG) (Verklinsky *et al.* 1990). The first polar body is removed and then the oocytes are inseminated using intracytoplasmic sperm injection (Figure 1.5). The second polar body can then be removed from fertilised oocytes at least 9hrs post ICSI (Magli *et al.* 2011) using the same procedure and same zona breach. Where simultaneous biopsy of both biopsies is performed, the same procedures are followed and biopsy is performed at least 9-22hrs post ICSI (Verlinsky *et al.* 1997).

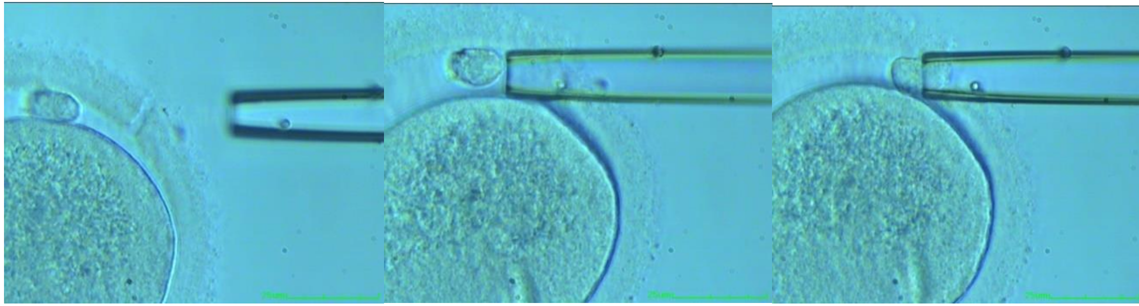

Figure 1.5. first Polar body biopsy with polar body in 12 o'clock position and zona breach at 1-2 o'clock. With the oocyte and polar body on the same plane of focus, the biopsy needle can be inserted through the breach with light suction used to remove the polar body. Image courtesy of CARE Fertility.

1.3.2 Cleavage stage biopsy

Historically, cleavage stage was the most common embryo biopsy approach (De Ryke *et al.* 2015) but has likely now been surpassed by trophectoderm biopsy. The technique is less labour intensive than polar body biopsy as only embryos that have reached the requisite cell number, and are of sufficient quality, are biopsied. Using older PGT technologies, it allowed the return of results for fresh embryo transfer at the blastocyst stage. Newer technologies require embryo freezing and clinics are moving towards frozen transfers to optimise outcomes (Coates *et al.* 2017). The biopsy is normally performed on the morning of day 3 but this may vary according to laboratory procedures. Criteria should be in place for which embryos are considered suitable for biopsy, with those having less than 5 cells, multinucleation/anucleation, or high levels of fragmentation, potentially being excluded. Moves away from cleavage stage biopsy mainly related to concerns relating to a possible reduction of implantation potential (Scott et al. 2013) and false positive/negative results relating to mosaicism (Baart *et al.* 2006, Hansen *et al.* 2009). Cleavage stage biopsy can be used to test for maternal and paternally inherited conditions, as well as meiotic and mitotic errors, but may miss

some mitotic errors depending on the stage they arise at, or the cell selected for testing. Embryos should be biopsied in individual drops of buffered Ca^{2+}/Mg^{2+} free media, which disrupts cell-to-cell adhesion and allows the easy removal of a single blastomere. The blastomere selected for biopsy should be of average size and have a visible nucleus. Ideally only a single blastomere biopsy be removed, as the removal of additional cells impacts on the embryo's implantation potential (Goosens *et al.* 2008). The most common practice it to aspirate the chosen blastomere with a biopsy pipette (Figure 1.6). The blastomere may also be removed by extrusion (where the blastomere is squeezed through the opening in the zona by pushing against the exterior of the embryo with the biopsy pipette) or displacement (where media is gently injected into the embryo to displace the blastomere through the zona breach).

Figure 1.6. Blastomere biopsy with selected blastomere and zona breach at around 3 o'clock. With the zona and blastomere on the same plane of focus, the biopsy needle can be inserted through the breach with light suction used to remove the blastomere. Image courtesy of CARE Fertility.

1.3.3 Morula biopsy

Morula biopsy is a more recent approach to embryo biopsy. The theory is that multiple cells can be taken late on day 3 or on day 4, and testing can be performed to allow fresh transfer. On the face of current evidence, fresh transfer, especially if late on day

5 or on day 6, may not provide the best chance of pregnancy for the patient, but is sometimes necessitated by reproductive health tourism, where the patient has travelled to another country for access to the treatment.

The morula has to be placed into buffered Ca^{2+}/Mg^{2+} free media to de-compact the embryo, making the individual blastomeres visible and reducing cell-cell adhesion. When the individual blastomeres are visible, biopsy can proceed as per cleavage stage, but with multiple cells taken. The embryo will recompact once it is returned to culture. Data is still limited on this approach, but early studies and case reports suggest this to be a feasible approach and that embryo development is not affected (Zakharova *et al.* 2014, Orvieto *et al.* 2020)

1.3.4 Trophectoderm biopsy

Trophectoderm biopsy was first reported in 1990 and as clinically applied in 2004 (Dokras *et al.* 1990, De Boer *et al.* 2004) but is now widely adopted. The procedure is proposed to be safer than cleavage stage biopsy in that it is reported to have little to no impact on implantation (Scott *et al.* 2013), while the cells removed are destined to form placental rather than foetal tissue. Additionally, although multiple cells are removed, improving the robustness of testing performed, they form a smaller proportion of the cellular volume of the embryo. Trophectoderm biopsy can be used for testing in all the same scenarios as cleavage stage and may pick up additional mitotic errors occurring post day 3. Mosaicism still exists at the blastocyst stage, but technologies like NGS allow samples to be categorised as aneuploid, euploid or mosaic. However, this requires limits for each of these categorisations to be set, and to an extent it supposes that the levels of aneuploidy within the sampled cells is

represented in the rest of the embryo. Nonetheless, some clinics are adopting the transfer of mosaic embryos in the absence of the availability of euploids, and achieving livebirths, (Greco *et al.* 2015, Munne *et al.* 2016) in the absence of definitive guidelines from professional bodies. While trophectoderm biopsy may initially appear the least labour intensive of the biopsy approaches, given the lower number of items being biopsied, the fact that embryos will reach blastocyst at different times over day 5, 6 and 7 can make the biopsy logistically difficult for the laboratory in the initial stages of offering the procedure. The additional need to also vitrify each biopsied blastocyst is also a consideration.

Blastocysts can be biopsied once they have cavitated and the inner cell mass (ICM) and trophectoderm can be differentiated. It is common practice to hatch the embryos on day 3 or late on day 4 (when it may be possible to visualise the ICM) to encourage herniation of the trophectoderm and make the biopsy procedure easier on day 5 or 6 (McArthur et al. 2005). However, it is also possible to hatch the embryo at the time of biopsy (Capalbo *et al.* 2015).

The biopsy can be performed using laser or mechanical approaches, or a combination of both (Figure 1.7)

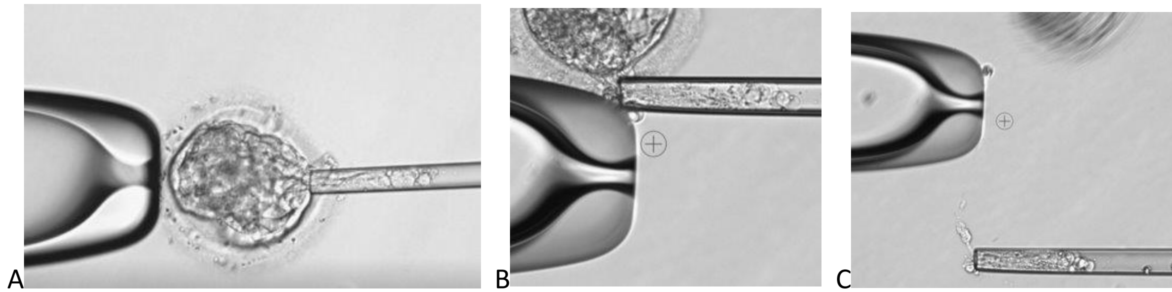

Figure 1.7. Biopsy of trophectoderm cells using laser and mechanical approaches. Images courtesy of CooperSurgical Fertility Solutions.

A. Between 5 and 10 trophectoderm cells are aspirated into the biopsy pipette. The laser is fired at a low pulse length a maximum of 3 times to create a weakness between trophectoderm cells.
B. The embryo is released from the holding pipette and the holding pipette and biopsy pipettes are brought corner to corner, into alignment on the same plane of focus.
C. The holding and biopsy pipettes are pushed against each other, causing the biopsy pipette to “flick” across the front of the holding pipette. In conjunction with the weakness generated between cells by the laser use, this mechanical motion will detach the cells in the biopsy pipette from the body of the embryo.

1.4 Adverse outcomes and misdiagnosis

A misdiagnosis is generally considered to have occurred when conception occurs, but the foetus or child born does not genetically match the PGT result. The true incidence of misdiagnosis in PGT is difficult to gauge. Many will go undetected, where a pregnancy or livebirth does not occur, where there are no adverse indications (i.e. unaffected vs. carrier), or where embryos are discarded without confirmatory diagnosis. Conversely, misdiagnosis may be incorrectly presumed in place of a natural conception. The ESHRE PGD consortium aims to collect and publish such data,

although not all clinics offering the treatment report to the consortium. Between 1997 and 2010 they have reported 21 misdiagnoses via FISH for PGT and 13 via PCR based approaches for PGT-M. This represents less than a 0.5% risk of misdiagnosis and is almost certainly lower than the actual figure (Harper *et al.* 2011, Moutou *et al.* 2014, De Ryke *et al.* 2015).

The causes of misdiagnosis can be categorised as human error, technical, or biological. In addition to this the cause may be intrinsic (a known phenomenon or limitation) or extrinsic (introduced to the process) (Wilton *et al.* 2009). Some potential examples are outlined in Table 1.1 along with possible preventive action. Further to the cause of a misdiagnosis it is important to identify the specific root cause to implement effective corrective action to prevent recurrence. For example, human errors may result from inadequate training, inadequate staff levels, or a failure of the SOP to fully or correctly describe the procedure.

Cause of Incident	Error Type	Potential Preventive Action
Natural conception	Human	Patient information and consents state to avoid unprotected intercourse
Mislabeled sample	Human	Robust labelling system
Misidentified samples	Human	Robust witnessing procedures
Misinterpreted report	Human	Identification suitable individuals to communicate results and appropriate training and witnessing
Transfer of incorrect embryo	Human	Robust witnessing procedures
Use of incorrect reagents	Human	Appropriate training and
Haploid cells	Intrinsic/biological	Use of linked markers or SNP array
Polyploid cells	Intrinsic/biological	Use of linked markers or SNP array
Chromosomal mosaicism	Intrinsic/biological	Biopsy of multiple cells
Test failure	Extrinsic/technical	Pre-clinical validation. Whole genome amplification allows repeat testing
Maternal contamination	Extrinsic/technical	Removal of all cumulus cells prior to biopsy
Paternal contamination	Extrinsic/technical	Use of ICSI to prevent extraneous bound sperm
Operator contamination	Extrinsic/technical	Appropriate training, personal protective equipment a lab model rules
Allele drop out	Intrinsic/technical	Multiple cell biopsy. Multiple linked markers
Incorrect setting of genetic phase	Human	Appropriate training and competency
Uniparental disomy	Intrinsic/biological	Use of linked markers or SNP arrays

Table 1.1 Summary of potential causes of adverse incidents in PGT and preventive actions. Adapted from Wilton *et al.* 2009.

Quality management is an essential part preimplantation genetic testing. Identification and analysis of the likelihood and seriousness of risk and the implementation of preventive action is a necessity given the potential level of severity of adverse incidents in this field. Robust staff training and competency assessment and confirmatory diagnosis of untransferred embryos are vital parts of the embryology aspect of PGT-M and PGT-A programmes.

1.5 Aneuploidy in gametes and embryos

Chromosome abnormalities arise in gametes and embryos through errors in the processes of disjunction and segregation. The purpose of meiosis is to generate haploid cells from diploid precursors and allow recombination to ensure genetic

variation in the resultant gametes (Figure 1.8) Gametes are produced following two rounds of meiosis; meiosis I and meiosis II. In spermatogenesis, the four resulting haploid cells are spermatids, which mature into spermatozoa, while oocytes, the result is a single mature ovum and the polar bodies.

In male gametogenesis, meiosis does not commence until puberty, and mature sperm are continually produced. In females however, prophase I is underway by the 8 months gestation (Gardner *et al.* 2012). Meiosis may be on hold then for up to five decades, which contributes to maternal age-related aneuploidy (Figure 1.9)

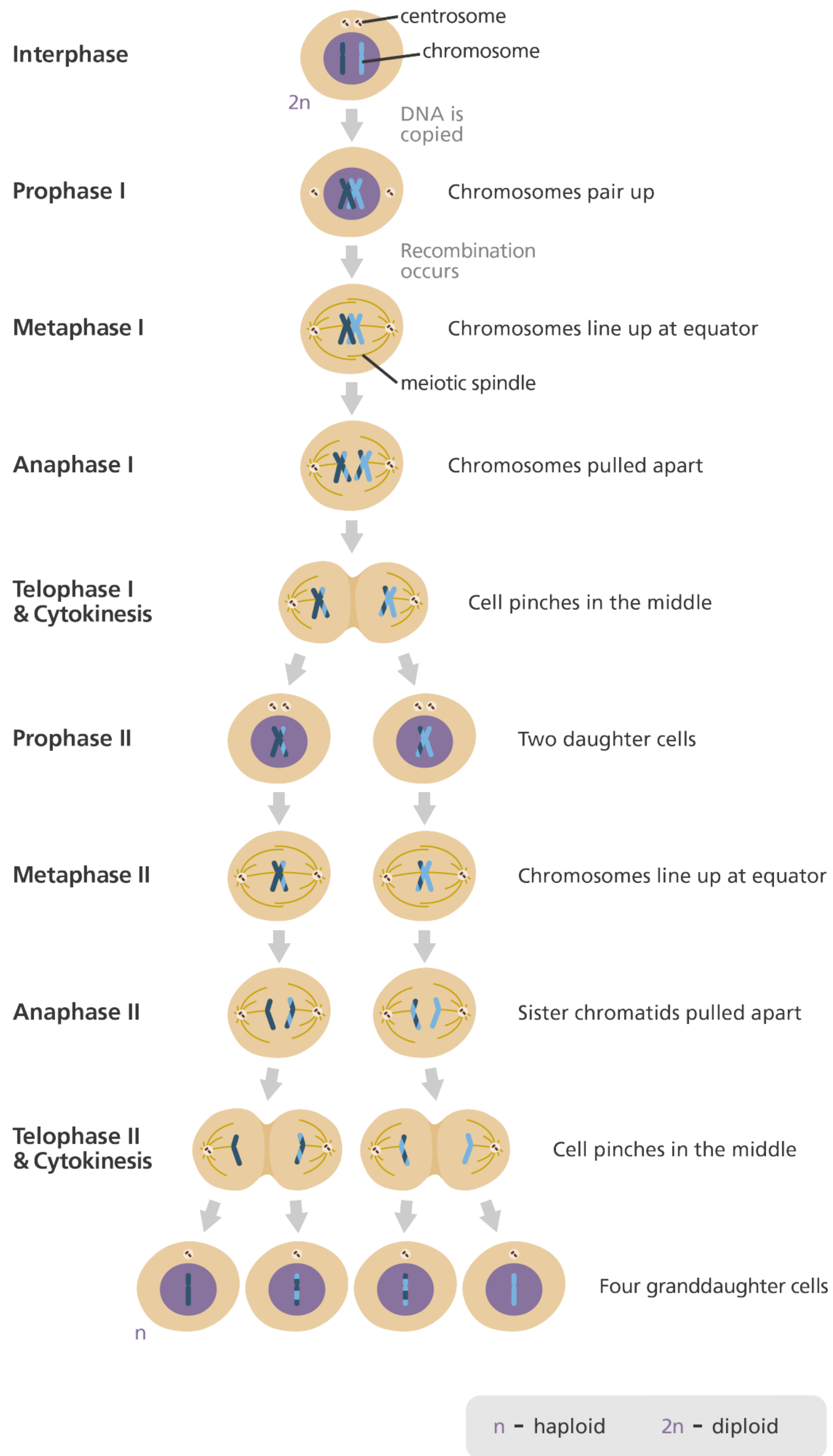

Figure 1.8 Diagram of meiosis I and II, illustrating recombination and the reduction division of diploid to haploid status. Image from Yourgenome.org.

In spermatogenesis, four genetically unique spermatozoa are generated from one primordial germ cell. In oogenesis, a single mature ovum is produced along with the first polar body (which technically performs a further division) and the second poplar body.

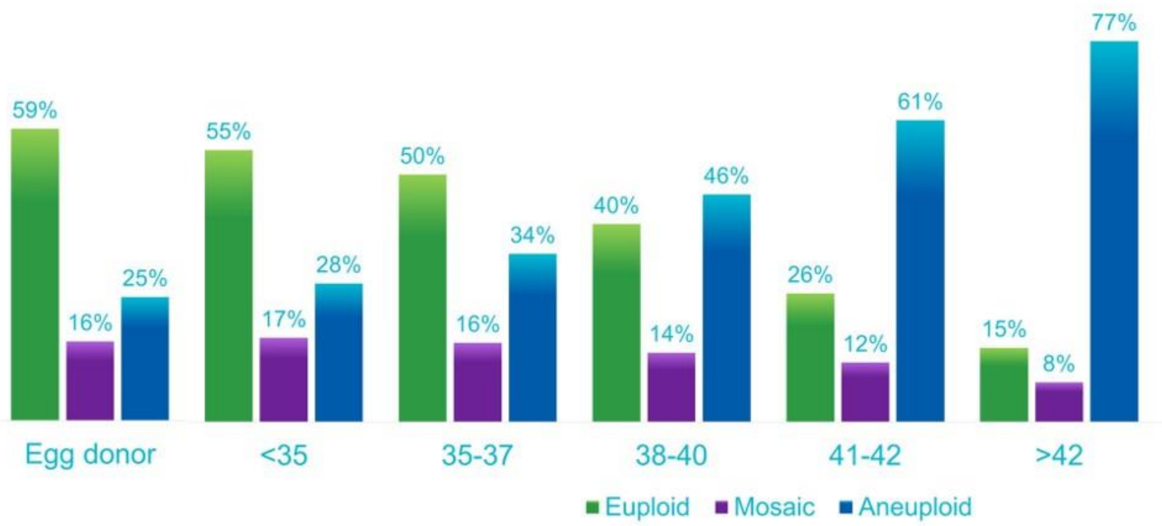

Figure 1.9. Data collected by Cooper Genomics showing the percentage of euploid, mosaic, and aneuploid embryos by maternal age, n=105316 total embryos November 2019 – April 2021 (egg donor n=8890, <35 n=27844, 35-37 n=23864, 38-40 n=23656, 41-42 n=11960, >42 n=8890). Image courtesy of Cooper Genomics.

Mitotic cell division involves a single cell division resulting in two identical diploid daughter cells (Figure 1.10). While it consists of the same stages as a single round of meiosis, there is no recombination. While an error in meiosis will result in an aneuploid gamete and an embryo with a constitutional aneuploidy, an error in mitosis only affects the daughter cells involved in the division and the lineage they will go on to create. This is what results in mosaicism in cleavage stage embryos, where there is the presence of more than one cell line in the embryo, with different chromosomal status.

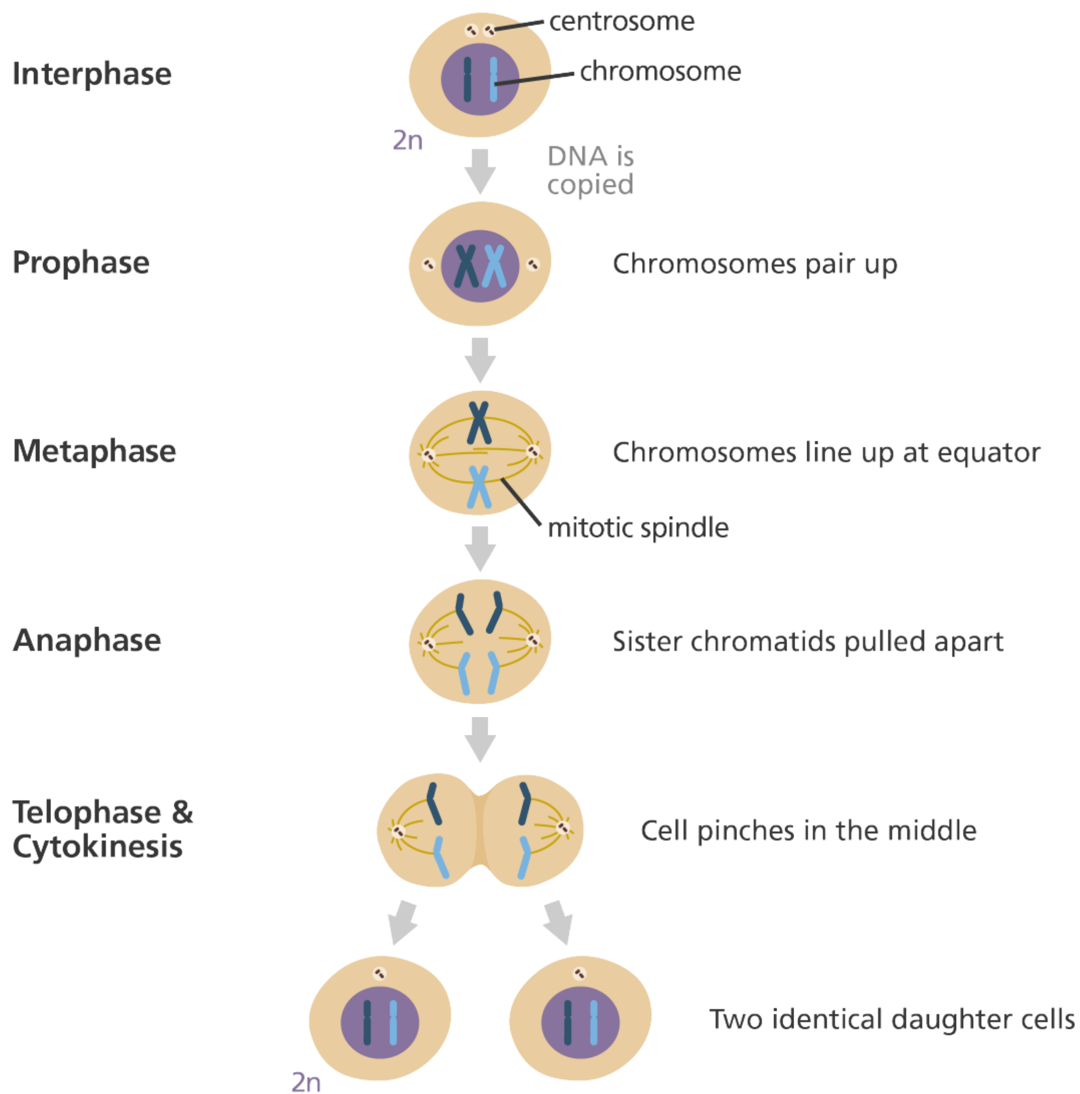

Figure 1.10. Diagram of mitosis, illustrating the production of two genetically identical daughter cells. Yourgenome.org.

1.6 Origin and behaviour of structural chromosome rearrangements

Structural chromosome rearrangements form when double-strand breaks occur and there is a failure in the DNA repair mechanisms, for instance, a failure of homologous recombination (Kurahashi H *et al.* 2009). De novo rearrangements can then be passed

on and inherited through generations as constitutional abnormalities. One family study has seen a reciprocal translocation traced back to an ancestor born in 1752 through nine generations (Koskinen *et al.* 1993).

The frequency of identification of specific translocations within the population reveals that certain breakpoints and combinations are over-represented. This is particularly marked in the non-random frequencies of the involvement of the acrocentric chromosomes in Robertsonian translocations. While, in theory, a homologous or heterologous Robertsonian translocation can form between any two of the five pairs of acrocentric chromosomes (chromosomes 13, 14, 15, 21and 22), the (13;14) Robertsonian translocation accounts for 75% of identified Robertsonian translocations (Therman *et al.* 1989). Nearly all (13;14) and (14;21) Robertsonian translocations share identical breakpoints, with others displaying a high variability in breakpoint location, pointing to a specific and recurring mechanism for the two most commonly occurring fusions (Page *et al.* 1996). In reciprocal translocations, most are unique to a specific family, but some rearrangements between highly homologous regions are seen in greater frequencies, with hot spots occurring at 11q23, 17q11, and 22q11, and leading to recurrent translocations t(11;22) and t(17;22) (Kurahasi *et al.* 2010).

If the size of the segment or segments involved in a structural chromosome rearrangement is small enough, it may be possible for chromosomes to still pair via heteroysnapsis and segregate as per normal meiosis. However, for most rearrangements, chromosomes will attempt to align homologous segments, resulting in abnormal meiotic configurations (McKinley Gardner 2012). In reciprocal translocations, a quadrivalent is formed with 5 theoretical segregation modes and 16

possible outcomes, all producing unbalanced gametes apart from the normal and the balanced gametes resulting from alternate segregation (Figure 1.11). Some of the monosomic and trisomic outcomes have only ever been observed via PGT-SR as opposed to in prenatal testing (Gardner *et al.* 2012). However, this does not necessarily represent the proportion of balanced:unbalanced gametes, which can vary widely.

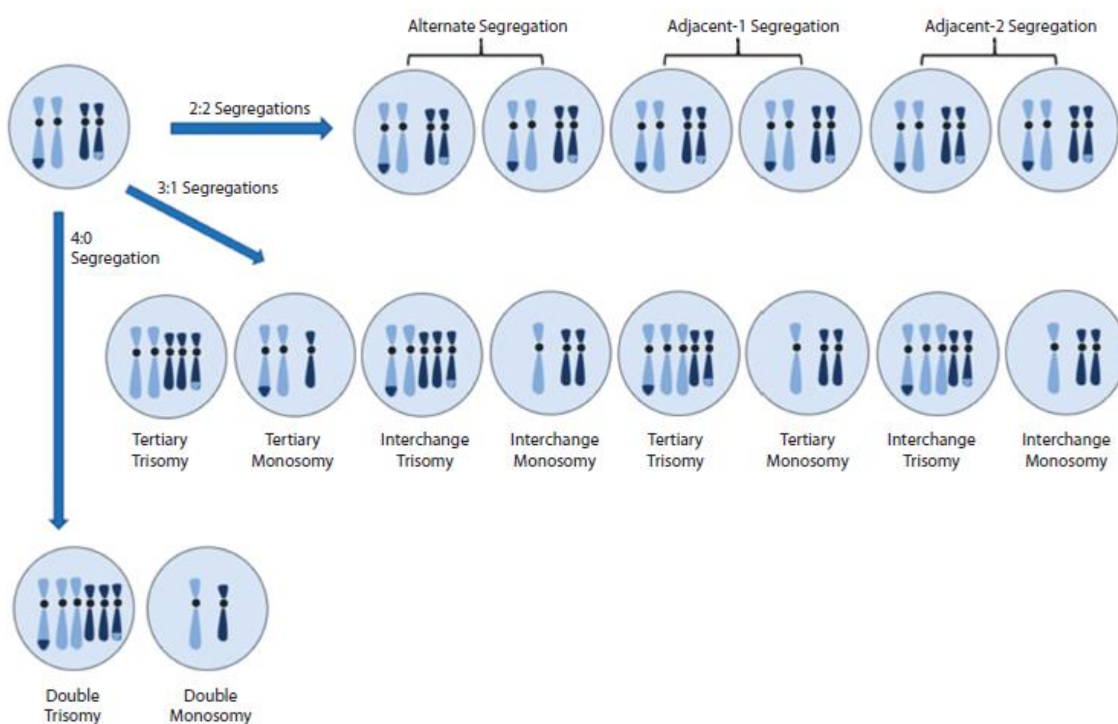

Figure 1.11 Segregation modes of a reciprocal translocation (Ogur and Griffin 2020).
2:2 segregation – two chromosomes go to each daughter cell from the meiotic quadrivalent. Alternate segregation produces euploid or balanced gametes, whereas adjacent-1 and adjacent-2 segregations will produce aneuploid gametes.
3:1 segregation – three chromosomes go to one daughter cell and one to the other. Tertiary trisomy occurs where the two normal chromosomes and one of the translocated chromosomes go to one daughter cell, whereas interchange trisomy occurs when the two translocated chromosomes and one of the normal chromosomes

segregate together. Tertiary monosomy relates to the segregation of one of the translocated chromosomes to a daughter cell, while interchange monosomy occurs with segregation of a single normal chromosome to a daughter cell.
4:0 segregation – complete non-disjunction where both the two normal and two translocated chromosomes segregate to the same daughter cell, assigned as double trisomy, and none segregate to the other daughter cell, generating double monosomy.

In Robertsonian translocation a trivalent is formed at meiosis, with three theoretical segregation modes and balanced or euploid gametes produced via alternate segregation (Figure 1.12) (Gardner *et al.* 2012). Again, this does not necessarily represent the real life proportion of balanced:unbalanced gametes. Additionally, the frequency of unbalanced gametes is lower in male Robertsonian translocation carriers than in female carriers, attributed to the stringency of cell cycle checkpoint mechanisms (Hunt 2020).

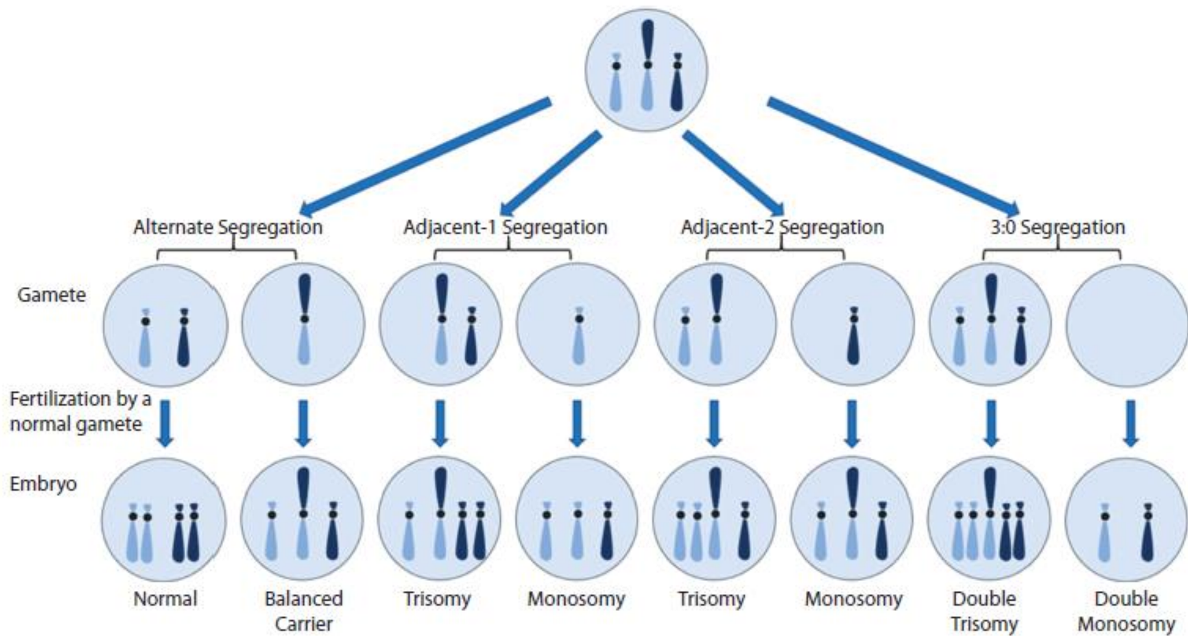

Figure 1.12 Segregation modes of Robertsonian translocations (Ogur and Griffin 2020). The segregation modes are as described in Figure 1.11, with the important differentiator that a trivalent as opposed to a quadrivalent is formed, meaning that instead of 3:1 and 4:0 segregations, there is 3:0 segregation.

1.7 Perspectives

While PGT is becoming more and more commonplace throughout the world, one of the challenges is the increased distance (both physically, and in terms of communication) between the embryology lab and the diagnostic lab. In the early days of PGT, both embryology and diagnosis were performed on the same site. The advent of service laboratories however has been one of the reasons why PGT has become so widespread. Nonetheless, it has become increasingly important that the embryology contribution is of sufficiently good quality to provide adequate material for the diagnostic lab, and also that practice does not impact on the implantation potential of the embryos. That is, successfully isolating 5-10 viable cells is essential, making sure they are definitely in the tube and that they are stored correctly to reach the

diagnostic lab in a good condition for diagnosis, is a modern challenge of PGT. Moreover, if the embryology is substandard, culture, blastulation rate, biopsy technique, warming rates - then there could be the best genetic testing lab in the world but there will still be poor PGT outcomes. With this in mind, there is a great need for a set of embryological guidelines of best practice to embryology labs, from the point of view of diagnostics labs.

As pointed out in Table 1.1 and discussed further in chapter 2, ICSI is usually insisted upon by certain clinics to minimise the chances of sperm contamination returning an incorrect result. To the best of my knowledge this has not been tested empirically, however. Similarly, as pointed out in section 2.5.4 and discussed further in chapters 4 and 5, a "no-result" is always a possibility however the extent to which it is an issue since NGS has not been investigated. In that regard re-biopsy is often considered an alternative strategy for when a no-result is returned, however the extent to which this is genuinely a viable option for patients in light of the outcomes when it is performed remains to be determined.

Finally, in section 1.6 and chapter 8, the issue of PGT-SR is covered. To date however a detailed analysis of outcomes, chromosome by chromosome, and by arrangement type, has not been performed. Going forward, this is important for genetic counsellors wishing to advise and empower patients carrying balanced chromosome rearrangements about their future reproductive choices.

1.8 Specific aims of the thesis

With the above perspectives in mind, the specific aims of this thesis were as follows:

- To liaise with diagnostic labs, embryology labs and medical affairs to create a unique set of guidelines for embryology labs wishing to use diagnostic services (chapter 2)
- To test the hypothesis that intracytoplasmic sperm injection is necessary as a preventive measure against paternal cell contamination in preimplantation genetic testing. Hence to ask if it is necessary for all patients undertaking PGT– even those with proven fertility – to have to undergo and pay additional fees for ICSI (chapter 3)
- To provide proof of principle that re-biopsy is, technically, a viable strategy when no result is obtained (chapter 4)
- To examine the "no result rate" in a leading group of UK PGT diagnostic laboratories since the introduction of next generation sequencing (NGS) to examine how much it varies between referring ART clinics, and assess whether it is a viable alternative for most patients (chapter 5)
- To perform a retrospective analysis of over 1,800 PGT-SR samples (479 cycles of 5 years) in order to:
  - Provide the biggest dataset to date on PGT-SR outcomes using CCS to date
  - Test the hypothesis that the level and nature of structural chromosomal rearrangements is correlated to age, sex and time of biopsy.
  - Test the hypothesis that an interchromosomal effect exists in this dataset
  - Provide a set of practical guidelines for genetic counsellors to advise patients on their likelihood of having euploid embryos for transfer based on the type

of chromosome rearrangement, sex of the carrier of the rearrangement, maternal age, and any other factors that may be implicated (chapter 6)

- To provide novel insight into the mechanism of twinning as a result of a PGT case (chapter 7)

2 Optimisation of embryology practice and embryo biopsy in preimplantation genetic testing

2.1. Chapter summary

The purpose of this chapter was to identify factors in the IVF laboratory that will impact on PGT success rates in the diagnostic laboratory, and to describe best practice where solid evidence and consensus exists. Since the development of preimplantation genetic testing (PGT), there have been numerous advances in both the embryology and diagnostic laboratories. This review and subsequent set of guidelines aims to examine the current status of embryology procedures that have enabled current PGT approaches, but also impact on clinical outcomes. While focus tends to be on embryo biopsy techniques, it is important to recognise and mitigate the differences in workflow between PGT cycles and standard cycles which may impact on culture conditions, and to ensure that vitrification and warming procedures are optimised. In identifying and evaluating the specified areas where PGT cycles deviate from standard cycles, this chapter evaluates and assesses the required changes to practice and whether consensus of best practice exists in published literature.

2.2 Introduction

Since the introduction of PGT into assisted reproductive medicine there have been exponential improvements and innovations in the field of genetic diagnostics (Handyside *et al.* 1990, Handyside 2013). This has seen PGT-A move from the testing of limited numbers of chromosomes to 24 chromosome testing, and from a binary aneuploid or euploid diagnosis to incorporate degrees of mosaicism (Fishel *et al.* 2009, Munne *et al.* 2016). In PGT-M, we have moved from lengthy and labour-intensive

custom work-up and testing procedures to the ability to check hundreds of genetic markers in a single reaction (Natesan *et al.* 2014). Within the embryology laboratory change has also occurred. There has been movement from single cell biopsy approaches (polar body or blastomere) to multiple cell biopsy (trophectoderm biopsy), enabled by improvements in embryos culture, and from fresh embryo transfer on days 5 or 6 to vitrification and frozen embryo replacement (De Boer *et al.* 2004, Coll *et al.* 2018). In identifying and evaluating the specified areas where PGT cycles deviate from standard cycles, we aim to evaluate and assess required changes to practice and whether consensus of best practice exists in published literature.

The feasibility of performing trophectoderm biopsy in human embryos was demonstrated in 1990 by Dokras *et al.* (1990). Their approached involved mechanical hatching at the blastocyst stage, creating a slit of less than a quarter of the diameter of the blastocyst, with herniation usually occurring within 18-24hrs. Biopsy was then performed when the size of the herniation was equal to the diameter of the blastocyst, and was performed mechanically by rubbing a glass micropipette across the narrowest point (just outside the zona) against the bottom of the dish. When De Boer et al. reported the first livebirth following trophectoderm biopsy and PGT (De Boer *et al.* 2002) their procedure involved laser assisted hatching on day 3 or 4 of culture, creating a 25-30mn opening, then culture to blastocyst. On the day of biopsy, the herniating trophectoderm was positioned at 3 o'clock and pulled gently away from the blastocyst while three to five laser shots were used, aimed at cell junctions, to detach the biopsy sample (McArthur *et al.* 2005).

Trophectoderm biopsy is increasingly the preferred method of biopsy for all forms of PGT (Van Montfoort *et al.* 2020), enabled by advances in embryo culture techniques which have increased blastocyst formation rates. It confers a number of advantages, the most obvious being that time and resources are not used biopsying and testing embryos that have not reached the blastocyst stage and will not be capable of implantation. This approach can optimise the cost of the procedure, something that is ultimately passed on to the patient (De Boer *et al.* 2004). The biopsy of multiple cells both reduces the risk of test failure and increases diagnostic accuracy (Forman *et al.* 2012), and at the blastocyst stage, allows certainty that cells are not being taken from part of the embryo that will develop into the foetus, i.e. the inner cell mass (ICM) (De Boer *et al.* 2004). In the era of molecular cytogenetic testing (PGT-A), the biopsy of multiple cells also enables reporting of levels of chromosome mosaicism (Mamas *et al.* 2012, PGDIS 2016), and due to the advanced stage of embryo development, will be informative for maternal, paternal and mitotic errors.

As pointed out in section 1.3, there has been a transition from cleavage stage to trophectoderm biopsy. Many labs have reported fewer embryos to biopsy but a higher euploidy rate (Coll *et al.* 2018) suggesting a selection for euploid embryos around blastulation. It has been held that the process of trophectoderm biopsy has negligible impact on implantation (Scott *et al.* 2013), but this continues to be examined in terms of a number of factors, including embryo quality, number of cells biopsied, biopsy technique, and the biopsy practitioner. Additionally, along with the advantages that trophectoderm biopsy confers, adoption of this approach may also require changes in laboratory workflow, given the later stage of development. It also requires a high blastocyst development rate (otherwise there will be few embryos on which to work),

an optimized culture system and excellent vitrification program. If none of these are in places then PGT outcomes will inevitably be negatively impacted.

2.3 The embryo culture environment

The first steps in a successful PGT cycle with trophectoderm biopsy are the same as a standard cycle – oocyte retrieval, sperm preparation, insemination, and embryo culture. Whilst there is no need to deviate from standard laboratory practice, it may be sensible to ensure that PGT oocyte retrievals and inseminations are the first to be performed, to maximise the time for blastocyst development. Dependent on clinic protocol and the requirements of the PGT laboratory, it should be possible to inseminate via IVF or ICSI.

Retrospective analyses (Feldman *et al.* 2017, Sahin *et al.* 2018, Palmerola *et al.* 2019) and prospective studies (De Munck *et al.* 2020) demonstrate no difference between IVF and ICSI PGT patients in terms of aneuploidy rates, contamination rates, parental origin of aneuploidy, or parental origin of uniparental disomy, indicating no additional risk factors stemming from IVF insemination. Further prospective studies of a size in line with Feldman's retrospective study (927cyles, 5093 biopsied blastocysts) would add further weight to the conclusions. Many professional guidelines still recommend ICSI as the preferred means of insemination in PGT cases (Kokalli *et al.* 2020, Practice committees of ASRM & SART 2012). Whilst ASRM and SART acknowledge a lack of data supporting this in practice (Practice committees of ASRM & SART 2012), ESHRE states ICSI to be the preferable method of insemination for PGT cases in order to minimise the risk of both maternal contamination from residual cumulus cells, and paternal contamination from surplus cells attached to the zona pellucida (Kokalli *et al.*

2020). However, the risk of maternal contamination remains with ICSI, and embryologists should be rigorous when it comes to the removal of cumulus cells at denudation or fertilization check. Conversely, bound sperm can be more easily observed and avoided by the embryologist performing the biopsy, and even in large numbers, sperm are unlikely to amplify due to the fundamental nature of their DNA structure (Lynch *et al.* 2019 – see also chapter 3). Testing methodologies involving SNP analyses will detect extra-embryonic contamination, both maternal and paternal in origin (Illumina 2015), but this may still render the embryo unsuitable for clinical use, and so strict embryology procedures for avoidance of contamination remain vital regardless of the method of insemination. Empirical evidence for this is covered in the next chapter.

One area of the embryology laboratory that does bear further scrutiny is the embryo culture system. While the culture system may not impact directly on aneuploidy rates – though could foreseeably contribute to mosaicism given it is generated during embryonic mitosis – it does impact on embryo metabolism and ultimately implantation and livebirth rates. As previously stated, poor blastocyst formation rates or poor blastocyst quality will negatively impact PGT outcomes. Improvements in culture systems have enabled most laboratories to generate good numbers of high-quality blastocysts, increasing the need for objective selection methods like PGT. However, even if the laboratory is satisfied with their blastocyst culture system prior to the introduction of trophectoderm biopsy, they must consider how the process itself will disrupt the culture system, how it will differ from standard cycles, and how these differences can be minimized or mitigated. Hidden weaknesses in the culture system will be exposed by increased manipulations in a PGT cycle. Given that blastocyst

development is dynamic and embryos within a cohort may be ready to biopsy at different time or on different days, time-lapse is useful for monitoring development rather than having to remove embryos from culture multiple times to check development. If time-lapse incubation is not available, the laboratory should have a minimal number of set time points each day to check development, based on knowledge of their culture system, and when they usually expect to see full blastocysts.

It is unknown if any specific formulation of culture media leads to better outcomes in standard treatment cycles (Swain *et al.* 2016). Similarly, available studies cannot categorically determine if sequential or single step media is preferable (Swain *et al.* 2016). However, it can be said that the latter contributes to less work for the embryologist and contributes to less interruption in the culture system, an important factor in stability and quality (Swain *et al.* 2016).

It is important to realise a culture system is much more than just culture media and relies on stability of temperature, pH and osmolality, the key being to minimise stresses to the gametes and embryos. Stability at the optimum temperature is required inside and outside of the incubator and is dependent on factors such as drop size, oil overlay volume, and dish type. As such it is important that labs validate how long different dishes used can be out of the incubator in different work areas before dropping to a critical threshold in terms of these parameters (Cohen *et al.* 2020).

Given the need to keep the environment in the dish within defined parameters and the time taken to perform the procedure, trophectoderm biopsy is often performed one

blastocyst at a time, and so the number of openings of the incubator will increase; with each time the culture dish is removed to put an embryo in to a biopsy dish, and again when an embryo is returned to culture. This may be overcome somewhat by having a "handling incubator" to reduce the impact on other dishes and other patients, or simply using multiple chambers across a benchtop incubator, which have much quicker recovery rate in terms of temperature and gas atmosphere (Swain 2014). Further, the welfare of additional embryos within culture dishes needs to be considered each time they are removed from the incubator, and so it is sensible to split a cohort of embryos across a number of culture dishes. Single embryo culture is necessary from the point of biopsy onwards, for sample identification and the chain of custody leading to results and may also be considered prior to this.

2.4 Assisted hatching

Assisted hatching is generally considered a necessary part of the biopsy process for direct access to and aspiration and separation of trophectoderm cells. In most culture systems, it is not expected for all embryos that have blastulated to begin the hatching process in vitro. Therefore, the question is when hatching should be performed and using which method.

There are 3 main methods by which assisted hatching of the zona pellucida is undertaken; mechanical, chemical, and laser assisted (Balaban *et al.* 2002). Laser is currently the most widely used (Kokkalli *et al.* 2020) and is the most standardised and reproducible. Laser hatching is less operator dependent, less time consuming and easier to learn. Mechanical hatching was the first method applied to assisted hatching but is used to a lesser extent for biopsy. Human studies and data have shown the

laser to be safe (Kanyo & Konc 2004, Taylor *et al.* 2010) while raising concerns about possible toxicity and impact on embryo viability when employing chemical hatching. (Jones *et al.* 2006, Geber *et al.* 2011). Taylor *et al* (Taylor *et al.* 2010) used a pulse length of 0.604-1.010mS, correlating to a hole size of 10.5-16.5nm to perform assisted hatching and remove blastomeres on day 3 of development. Subsequent blastocyst quality between the three groups and a control group was equivalent in terms of percentage and quality. Current commercially available lasers are non-contact lasers operate at a wavelength of 1460-1480nm, far away from the DNA absorption peak of 260nm, and power of 100-400mW (Davidson et al. 2019, Vitrolife 2021). Kanyo and Konc (Kanyo & Konc, 2004) reported no evidence of increased incidence of chromosomal aberrations or congenital malformations in children born after assisted hatching. Similar large scale studies following children born following PGT have also found no increased risk of congenital malformations, adverse perinatal outcome (Sunkara *et al.* 2017 Heijligers *et al.* 2018) or childhood neurodevelopment (Kuiper *et al.* 2018, Heijligers *et al.* 2019).

Historically, hatching was performed on day 3 of development with trophectoderm biopsy performed from day 5 (McArthur *et al.* 2005). The rationale was that this would encourage herniation of the trophectoderm and aid the biopsy procedure, possibly in part due to historically poorer general blastocyst formation and development, prior to modern developments in culture systems. Because of the risk of the ICM hatching, many moved to hatching late on day 4, where there was potentially a higher chance of identifying and hatching opposite the ICM. There is a concern that this approach encourages herniation of the trophectoderm from blastocysts earlier in development, and with fewer cells in the trophectoderm, and that biopsy may be performed too early

and have a greater impact on implantation potential (Wininger *et al.* 2019, Singh *et al.* 2019). Additionally, hatching prior to expansion means that while trophectoderm herniates, the zona remains at the same thickness as day 3 or 4 in development, which may impede natural hatching and implantation (Wininger *et al.* 2019). To overcome these concerns, some groups have switched to hatching on the morning of biopsy when the embryo is expanded, and returning to the incubator to allow herniation, or hatching and biopsying expanded blastocysts simultaneously (Capalbo *et al.* 2016). The latter is the only approach that avoids an additional disturbance to the culture system, and so if hatching prior to biopsy is undertaken, it is important to validate the impact on the culture dish and how this can be undertaken minimising stress to the embryo. Regardless of the day of hatching, most recent papers quote creating a zona breach of approximately 10-12μm (Capalbo *et al.* 2014, Whitney 2016). Unfortunately, the majority of papers do not quote the size of zona breach that is created, the number of laser pulses used, or the size of the laser hole generated by the laser pulse length used. Some papers give information on laser model and pulse length used but not the hole size generated. Given the variables between laboratories (use of thermal plates, dish type, media volume, ambient environment), pulse length is not reliable to generate the same hole size if replicated, and so reporting of the actual hole size generated would be useful for comparisons between protocols.

Rubino *et al.* (2020) showed improvement in post warming survival rates, pregnancy rates and implantation rate when moving from day 3 hatching to a protocol where hatching and biopsy were performed sequentially and then a quarter of the zona was removed prior to vitrification. This could be due to a change in the stage of the blastocyst at time of biopsy, the impact of the disruption to the culture system at day

3, or the removal of a larger portion of the zona, and so does not necessarily show that performing hatching sequentially at the time of biopsy is superior to hatching prior to biopsy where all aspects of the culture system are validated. In disagreement with protocols and results from the vast majority of teams performing trophectoderm biopsy and PGT, Singh *et al.* (2019) suggests that the biopsy of non-physiologically hatching blastocysts damages them in ways that are not reflected in ongoing development, but leads to poorer clinical outcomes, and that laboratories should wait until natural hatching occurs before performing trophectoderm biopsy.

2.5 The biopsy procedure

Rather than arbitrarily selecting the number of embryos to place in the biopsy or the maximum time for the completion of the biopsy procedure, it is important to validate how long the environment within the biopsy dish remains stable (Cohen *et al.* 2020). This will depend on the type of dish being used, the type of buffer in the media, the size of the drops, the volume of the media used, the volume of the oil overlay, and whether the prepared dish has been allowed to equilibrate prior to the biopsy procedure. As with embryo culture, the key is stability and minimising stress on the embryo. Increased stress on embryos can potentially be inferred if performance indicators begin to fall.

Throughout biopsy and tubing procedures, all embryo movements require an appropriate witness and witness events must be recorded. Manual witnessing will be mandatory for some movements, for example, where more than one embryo is placed in the biopsy dish; in this case we are not simply matching dish to dish or dish to tube,

but also that the embryo/sample identifier matches culture drop to culture drop or culture drop to tube.

The first detailed descriptions of clinically applied trophectoderm biopsy involved assisted hatching via laser on day 3 or day 4, creating a zona breach of 25-30μm. Embryos were cultured to day 5; those with expanded hatching trophectoderm cells were biopsied and the remainder were cultured for a further 8-24hrs to be rechecked. A 30μm biopsy pipette was used to aspirate 5-6 trophectoderm cells, and the laser was fired 3-5 times, at a reduced pulse length, at cell junctions. The biopsy pipette was pulled away from the embryo as the laser was fired, allowing the cells to detach (De Boer *et al.* 2004, McArthur *at al* 2005). This approach has been modified by instead performing hatching at the time of biopsy (Capalbo *et al.* 2014) or by using a mixture of laser and mechanical methods to detach the trophectoderm cells (Whitney *et al.* 2016). In terms of the biopsy procedure and separation of the trophectoderm cells, two methods encompass the approaches of most groups, and the main differences pertain to when or if hatching is performed, as discussed in the previous section (Kokkalli *et al.* 2020).

2.5.1 Laser and stretch

The blastocyst is gently aspirated on the holding pipette with the hatching trophectoderm cells/opening of the zona pellucida opposite from the holding pipette, preferably working in a horizontal line, and with the ICM in clear view. The trophectoderm cells are gently aspirated into the biopsy pipette, holding the embryo securely to create tension. The laser is fired at the thinnest portion of the aspirated

cells, directed at cell junctions, until the sample separates. (McArthur *et al.* 2005, Capalbo *et al.* 2014)

2.5.2 Laser and flick

The blastocyst is gently aspirated on the holding pipette with the hatching trophectoderm cells/opening of the zona pellucida opposite from the holding pipette, preferably working in a horizontal line. The trophectoderm cells are gently aspirated into the biopsy pipette and then the aspiration pressure is neutralised. The laser is at cell junctions along the outside of the opening of the biopsy pipette. The embryo is then released from the holding pipette. The biopsy pipette is positioned above the holding pipette, ensuring they are on the same plane of focus. The biopsy pipette is moved to the edge of the holding pipette and they are pushed against each other, causing the biopsy pipette to flick across the front of the holding pipette, detaching the trophectoderm sample, which should remain in place in the biopsy pipette (Whitney *et al.* 2016).

2.5.3 Cell lysis and damage

Guidance on laser assisted biopsy warns against over-use of the laser as a potential source of cell damage and partial destruction of cellular DNA (PGDIS 2016). As with assisted hatching, papers usually reference the make and model of laser use, but much less frequently refer to the pulse length used, the hole size this generates, or the number of times the laser is fired. Kelk et al. (2017) demonstrated no impact to DNA profiles with up to 5 shots of the laser in the range commonly used to perform trophectoderm biopsy. However, the H1 human embryonic stem cells used may have been more robust than human trophectoderm cells and were not placed under the

same stresses i.e. aspiration, stretching, as trophectoderm cells during biopsy. Therefore, it is still possible that stresses on the trophectoderm cells such as increased laser exposure, multiple flicking, scraping on the holding pipette or overstretching could lead to cellular damage that could impact on PGT data, especially where cell lysis is observed. Poor quality samples will lead to lower quality/noisier data that is more difficult to interpret and could lead to overcalling of mosaic changes where data analysis is manual rather than via validated algorithms (Whitney 2018). Some groups suggest that using the laser and flick method is recommended (Whitney J2018, Herrero Grass 2019) on the basis that laser and stretch produces more mosaic profiles, but this has not been replicated by all groups investigating the impact of the biopsy method (Benavent *et al.* 2019). Mechanical biopsy via the flicking method on days 5 or 6 (following laser hatching on day 4) has shown similar amplification rates and clinical pregnancy outcomes to blastocysts undergoing biopsy via the "laser and flick", but blastocysts in the latter group demonstrated significantly better survival rates on warming post vitrification (Armstrong 2020).

2.5.4 Blastocyst quality and number of cells biopsied

When performing biopsy there needs to be a balance between maximising diagnostic accuracy, minimising the failed amplification and "no result" rate and, obviously, impact on the implantation potential of the blastocyst. As such, the number of trophectoderm cells taken at biopsy may be vital, especially given that trophectoderm function is increasingly recognised as vital to implantation and sustained pregnancy (Ahlstrom *et al.* 2011, Honnma *et al.* 2012). Where chromosome mosaicism is reported, cell number is also vital in terms of this being a meaningful measure, and as such a minimum sample size of 5 cells has previously been recommended (PGDIS 2016). If

we accept that cell loss through cryopreservation (El Toukhy *et al.* 2003) and removal more than a single blastomere at cleavage stage (De Vos *et al.* 2009) impacts on implantation, it is logical to assume that removing too high a number of cells relative to the total number of cells within the trophectoderm could impact on continued development and implantation.

Neal *et al.* (2016) demonstrated biopsies with the highest relative DNA content – estimated at 15-20 cells – were associated with lower live birth rates after single embryo transfer. Comparing genetic testing technologies requiring different cells numbers has also appeared to show biopsying an average of 10 cells negatively affects embryo implantation when compared to biopsying an average of 5 cells (Guzman *et al.* 2019). Similarly, despite employing FISH as the analysis method, another study demonstrated that diagnostic efficiency was maximised when a minimum of 6 cells were biopsied, but that there was a trend of decreasing implantation rates with increasing cell number where trophectoderm was graded B or C according to the Gardner score (Zhang *et al.* 2016). The Gardner score is a widely used method of blastocyst scoring, where a grade of A to C (with A being best) is assigned to the trophectoderm and inner cell mass separately, and a number from 1-6 (with 6 being most advanced) is assigned based on the stage of expansion. CGH was performed on products of conception following miscarriage, ruling out the role of aneuploidy, and further seeming to indicate the interaction between trophectoderm and endometrium as a factor.

Factors influencing the “no result rate” post biopsy – a combination of both amplification failure and poor-quality test data – have been demonstrated to include

the centre performing the treatment cycle and biopsy, day of biopsy, and number of cells biopsied, but not morphological quality of the blastocyst (Cimadomo *et al.* 2018). With testing via qPCR, diagnostic efficiency was maximised when a minimum of 8 cells were biopsied (Cimadomo *et al.* 2018). It is common for centres to set criteria for which embryos are suitable to biopsy, basing this on the day of development, stage of development and embryo morphology, given this normally associates with vitrification/warming survival rates and clinical outcomes in standard cycles. However, poorer quality blastocysts can be euploid and do result in clinical pregnancy and live birth (Cimadomo *et al.* 2019). Poor quality blastocysts do show higher aneuploidy rates and it is not certain when euploid if they have the same potential as good quality blastocysts, but patients of advanced maternal age particularly benefit from their inclusion in testing. The culture to and biopsy of day 7 embryos has also proved controversial but again, it has been demonstrated that while they have lower euploid rates, they may have similar sustained implantation rated to day 5 and day 6 blastocysts (Tiegs *et al.* 2019, Hernandez-Nieto *et al.* 2019). Faster and slower growing embryos have been shown to have similar aneuploidy rates (Capalbo *et al.* 2014) and so, while it is important that the blastocyst has expanded to the point where the inner cell mass and trophectoderm can be clearly distinguished prior to biopsy, the specific time point at which the biopsy is performed can be left open to include embryos reaching the requisite stage on day 5, 6 or 7.

2.5.5 Re-biopsy

With the “no result rate” varying between clinics and testing laboratories, depending on patient circumstance, re-biopsy and retesting may be requested rather than disposing of the embryo or transferring an embryo of unknown genetic status. Given

that the in the majority of PGT cycles embryos are vitrified post biopsy, this requires warming, followed by a second round of trophectoderm biopsy and vitrification. If the embryo proves to be genetically suitable for use, it then must be warmed for a second time. Understandably there was initially scepticism surrounding how this would impact on the viability of the embryo and if it was fair to put the patient through this, but initial data seemed remarkably positive. Embryo survival on warming, the return of results, and the genetic status (e.g. euploid vs. aneuploid) is similar to first biopsy data (Cimadomo 2018), so in terms of getting a result and being suitable for clinical use, it is a worthwhile process for the patient. Multiple studies have demonstrated that re-biopsied embryos are as likely to yield a result, and be suitable for transfer as embryos being biopsied for the first time (Zhange *et al.* 2014, Howard *et al.* 2018, Neal *et al.* 2019). When these embryos are used clinically, similar clinical outcomes have been observed, including gestational age at delivery, and birth weight, as embryos that have only been through one round of biopsy, vitrification, and warming (Tyler *et al.* 2014, Zhange *et al.* 2014, Neal *et al.* 2018, Neal *et al.* 2019). However, overall numbers of embryos transferred remains very low compared to those that have been through a single round of biopsy, vitrification and warming, and so data should continue to be collected and analysed. Re-biopsy is covered in more detail in chapters 4 and 5.

2.6 Sample Preparation and Tubing

Few publications on PGT have any extensive discussion on the preparation and tubing of trophectoderm samples despite the importance of this stage with respect to PGT results. If the sample degenerates, sticks inside the pipette, sticks to the side of the PCR tube, or is not placed into the tube, it will result in failed amplification or poor-quality data. Additionally, the amplification reaction is a balanced chemical reaction,

and so if too little buffer, too much buffer, or the incorrect buffer (including too much carry over of culture media) is used, this will again impact on the data produced. Post biopsy, it is useful to release the embryo in a different part of the drop to the biopsy sample, to avoid pipetting the sample when moving the embryo back to the culture dish. It is also not advisable to place the biopsy sample close to the edge of the drop, as this will make it more difficult to identify using a standard stereo microscope. Given the nature of trophectoderm biopsy samples, it is recommended to use a separate pipette for each sample to avoid carry over contamination (Kokkali *et al.* 2020). For the same reason, where negative controls from drops of wash buffer are collected, this should be done prior to handling of the trophectoderm sample, or with a different pipette.

Many specifics on the tubing procedure will depend on the genetic testing service used; type of buffer, volume of buffer, conditions for storage. However, good standard practise is to wash the biopsy sample through multiple drops of the buffer provided, avoiding carry over of culture media or oil before placing it in the PCR tube (Kokkali *et al.* 2020). The dispensing of the biopsy sample into the PCR tube can be visualised with a stereo microscope during pipetting. Care should be taken not to expel bubbles, as this could displace the sample from the buffer and result in it sitting at the meniscus and potentially adhering to the side of the tube. It is good practice to check the pipette in a drop of buffer post tubing to confirm that the sample is not still present in the pipette or has been drawn back in by capillary action when withdrawing the pipette from the PCR tube – Aoyama and Kato report 2 incidences in 149 procedures of recovering and reloading biopsy samples in this way (Aoyama & Kato K 2020).

2.7 Vitrification and warming

Numerous studies have now shown that the use of vitrification and frozen embryo replacement in PGT cycles does not negatively impact clinical outcomes, and in some situations results in improvements (Coates *et al.* 2017, Coll *et al.* 2018). Vitrification is necessary due to the processing time of the molecular technologies used for testing, and the fact that this testing now tends to be performed in dedicated centralised PGT laboratories, requiring sample transportation to be factored in to processing times. The main concern attached to frozen embryo transfer in PGT cycles is the loss of euploid or genetically suitable embryos. Loss rates do not appear to be increased in biopsied embryos (Coll *et al.* 2018).

Vitrification and warming is now a key part of PGT cycles but few papers reporting PGT outcomes give details on the vitrification and warming protocols used. Whether the embryo is vitrified while collapsed or expanded, the time point post biopsy when it is performed, if hatching is performed on warming, how long embryos are cultured prior to transfer, and whether they need to re-expand prior to transfer are all factors that could impact on clinical outcomes.

In a study where blastocyst were given up to 6hrs post biopsy to re-expand prior to vitrification, higher implantation and ongoing pregnancy rates were seen from blastocysts that had at least partially re-expanded in that time frame (Chen *et al.* 2017). While statistical significance was not shown, there was a trend toward better outcomes when embryos were cultured for ≥3hrs post biopsy prior to vitrification. However, these results could be due to the laboratories normal practice being validated and optimised with respect to vitrifying expanded blastocysts rather than collapsed ones. As such,

Maggiulli (2019) conversely recommends vitrifying within 30 minutes of biopsy, while the blastocyst remains collapsed from the procedure, and performs artificial collapsing for non PGT cycles.

Aside from vitrification protocols, it is essential that embryos are vitrified individually, where manual witnessing may be required to confirm the identification of a specific embryo in a culture dish. Cryo devices should be clearly labelled with the embryo identifier, and the use of different colours can be a useful extra visual identifier when it comes to selecting a genetically suitable embryo for warming and transfer.

2.8 Discussion

Despite the increasing use of PGT in recent years, variability in terms of clinical outcomes remain, leading to questions on the efficacy of the treatment itself. However, there is wide variability in terms of how centres approach the embryology aspects of PGT cycles in all aspects of the process; culture, insemination, assisted hatching, biopsy, tubing, vitrification and warming. Just as we see variations in clinical outcomes for standard cycles between centres, it stands to reason we will see the same for PGT cycles. It may be that the increased manipulations in PGT cycles expose weaknesses in embryology practice that have not previously been evident, and therefore outcomes for some centres do not show the improvements they expect.

Very little literature on PGT thoroughly covers all elements of the embryology processes, as outlined, and as such it is difficult to identify where there is common practice, or widely divergent practice. Consensus seems to exist in terms of stage of development, days of biopsy, and number of cells to biopsy, but disparity remains with

respect to assisted hatching, the inclusion of poorer quality blastocysts, biopsy technique, and vitrification protocols. It would be of benefit to have more information on how embryos were cultured prior to biopsy and how the laser was used at hatching and biopsy.

The main guidance, therefore, that can be given to laboratories undertaking or embarking on offering PGT cycles must be to perform thorough risk assessments and identify where there are divergences from standard practice and how this might impact on clinical outcome. Then action can be taken to mitigate this where necessary without making unvalidated changes to laboratory procedures. Current literature does not allow the identification of definitive best practice and allows for divergence in practice between or within laboratories without necessarily having a detrimental impact. Specific recommendations and assurances that can be made to common concerns are:

- Insemination via IVF or ICSI is acceptable dependent on amplification methods used by the genetics laboratory.
- Assisted hatching on day 3 or 4 or culture or at time of biopsy is acceptable.
- Laser assisted or mechanical biopsy is acceptable, and the focus should instead be on minimising cell lysis in both the embryo AND the biopsy sample.
- Poorer quality blastocysts can be biopsied, vitrified, warmed and transferred and contribute to overall success rates.
- Day 7 blastocysts can be biopsied, vitrified, warmed and transferred and contribute to overall success rates.
- Aim to biopsy between 5 and 10 trophectoderm cells from the blastocyst.
- Rebiopsy is an acceptable option for blastocysts with no PGT result.

- Vitrification can be performed after biopsy when the blastocysts are collapsed, or once they have re-expanded. This should be performed in line with your standard practice of whether embryos are artificially collapsed before vitrification.

3 Intracytoplasmic sperm injection is not necessary as a preventive measure against paternal cell contamination in preimplantation genetic testing

This work is taken from the following manuscript

Lynch, C., Cater, E., Charitou, M., Forbes, H., Griffin, D. & Gordon, T. 2019. Intracytoplasmic sperm injection is not necessary as a preventive measure against paternal contamination in preimplantation genetic testing. Reproductive BioMedicine Online. 39. e24-e25. 10.1016/j.rbmo.2019.04.051.

My own contribution was design of the study protocol, including the embryology elements, and collection and analysis of the data.

3.1 Chapter summary

The purpose of this chapter is to investigate if it is necessary, as per much professional guidance, for all patients undertaking PGT– even those with proven fertility – to have to undergo and pay additional fees for ICSI. ICSI is widely recommended for patients undergoing preimplantation genetic testing (PGT), but are sperm cells a potential source of paternal cell contamination in PGT? Semen samples were obtained from 5 normozoospermic male patients consenting to research. From each sample 1, 2, 4, 8 and 10 sperm were collected in PCR tubes and underwent whole genome amplification. None of the 25 samples submitted – a total of 125 sperm – showed evidence of DNA amplification. Paternal cell contamination resulting from using conventional in vitro fertilisation (IVF) as the insemination method is a negligible risk

in PGT. Therefore, intracytoplasmic sperm injection (ICSI), in the absence of male factor infertility, is an unnecessary intervention for PGT patients.

3.2 Introduction

Preimplantation genetic testing (PGT) has rapidly developed in terms of scope and technology since the first applications (Handyside *et al.* 1990, Handyside *et al.* 1992, Munne *et al.* 1993, Munne *et al.* 1998). Originally introduced as a treatment for couples at risk of having a child with a hereditary genetic condition, it is now most commonly applied as a test to select chromosomally normal embryos most likely to implant and result in a successful pregnancy.

The sensitivity and specificity of preimplantation genetic tests have increased with advances in technologies and changes in gamete, zygote and embryo biopsy practices. Both the IVF laboratory and genetic testing laboratory strive to reduce the risk of misdiagnosis – both benign and adverse. Misdiagnosis and adverse outcomes remain a concern for the IVF laboratory, the genetic testing laboratory, and the patient. Many published guidelines and recommendations (Wilton *et al.* 2009, Practice Committee ASRM 2012) were not written in relation to current approaches to testing and so should be examined and risk assessed with respect to the biopsy practice and technology employed, and the needs of the patient undergoing treatment. These recommendations have included the use of intracytoplasmic sperm injection (ICSI) for insemination for all amplification-based testing to reduce the risk of paternal contamination from extraneous sperm bound to the zona pellucida, or non-decondensed sperm within blastomeres, based on theoretical risk assessment. The vast majority of PGT is now performed via amplification-based techniques, beginning

with whole genome amplification of the submitted samples. Therefore, it is important to examine the need for this additional and expensive intervention for such a large number of patients that could undergo and achieve fertilisation via conventional in vitro fertilisation (IVF).

3.3 Materials and methods

The study follows the principles of the Declaration of Helsinki, and all samples and data were anonymised. 5 normospermic male patients were selected at the time of semen analysis who had consented to the use of their sperm in research. The semen sample was prepared as per the IVF laboratory standard operating procedure for sperm preparation for IVF/ICSI insemination.

2ml of semen was layered onto a HEPES and bicarbonate buffered ready-to use density gradient (55%/80%) of silane coated colloid silica particles (SupraSperm, CooperSurgical) in a conical centrifuge tube (Falcon). The tube was centrifuged at 300g for 10 minutes, separating motile sperm from extraneous cells and seminal plasma. The motile sperm form a pellet at the bottom of the tube which is removed and washed in bicarbonate buffered IVF culture media (Fertilcult IVF Medium, Fertipro) by centrifuging at 300g for 5 minutes. Following the washing step, the supernatant was removed from the tube, leaving 0.5ml sperm stock.

For each sample an ICSI dish (Falcon) was prepared with a drop of clinical grade polyvinylpyrrolidone (PVP) (CooperSurgical), a drop of 3-(N-morpholino) propanesulfonic acid (MOPS) buffered media (GMOPS, Vitrolife), and 5 x 2µl drops of biopsy buffer (CooperGenomics), overlaid with mineral oil (FertiPro). A small

amount of the sperm stock was placed in the PVP to reduce motility and individual sperm cells were caught and immobilised using an ICSI micropipette (Humagen, CooperSurgical). The individual sperm were washed in the GMOPS media and transferred to the biopsy buffer so that each drop contained 1, 2, 4, 8 and 10 sperm respectively. Each biopsy buffer drop was pipetted and transferred in its entirety to a separate 0.2ml PCR tube (CooperGenomics).

All 25 samples were submitted to the genetic testing laboratory as blinded research samples. The genetics laboratory was not made aware of the cell type or number of cells submitted and they were processed as standard. The samples underwent whole genome amplification (WGA) (SurePlex, Illumina) according to the genetic testing laboratory's standard operating procedure. A negative control (reagents only) and positive control of 2µl 30pg/µl female genomic DNA (SureRef, Illumina) were included in the amplification reaction. Cell lysis and pre-amplification steps were performed in the pre-amplification area of the laboratory with dedicated equipment and personal protective equipment. The final amplification step was performed in the general area of the laboratory, producing 90µl amplified product for each sample

DNA amplification was assessed in the genetics lab via gel electrophoresis for the 25 submitted samples, WGA positive control, and WGA negative control. A gel electrophoresis positive control (previous successfully amplified sample) and negative control (Millipore water) were included. The gel box is filled with 1XTAE buffer and the pre-cast 1% agarose gel with ethidium bromide added. 5µl WGA product or control is mixed with 1.1µl running buffer and 5µl is pipetted into the corresponding well of the gel. The gel is run at 100V until there is clear colour separation of the loading dye and

is then visualised on a UV light box. The presence of an illuminated band or smear indicates the presence of amplified DNA.

3.4 Results

Positive presence of an illuminated band or smear demonstrated the presence of amplified DNA in the WGA positive control and the gel positive control, as expected. The WGA negative control and gel negative control did not show evidence of the presence of amplified DNA, as expected. There were 25 submitted samples from 5 separate patients containing 1, 2, 4, 8 and 10 sperm respectively. None of the samples containing sperm demonstrated the presence of amplified DNA.

3.5 Discussion

Despite the fact that ICSI is widely recommended for patients undergoing PGT as a preventive measure against paternal cell contamination, sperm DNA failed to amplify when subjected to the same protocol used to amplify DNA from polar bodies, blastomeres, and trophectoderm cells. This data demonstrates that paternal contamination is a very low risk in PGT, as even if sperm cells are accidentally transferred to the sample tube, it is highly unlikely that they will amplify, or do so to the extent that it will affect the result obtained. This is due to the fundamentally different way in which sperm DNA is packaged, making it inaccessible in the whole genome amplification reaction without additional steps to effectively decondense and isolate the DNA (Jiang *et al.* 2005, Patassini *et al.* 2013).

During spermiogenesis haploid sperm DNA is packaged into a compact and inactive nucleus. Transition proteins and protamines are present and responsible for chromatin

condensation, and after ejaculation chromatin stability is further increased by the seminal plasm. When a sperm enters an oocyte, the nucleus remains highly condensed and inactive. The ooplasm contains a sperm decondensation factor, and only on fusion of the sperm and oocyte membranes does the sperm DNA becomes accessible, as histones in the ooplasm replace the protamines, allowing chromatin decondensation, pronucleus formation, DNA replication, and entry into the mitotic phase (Ward 2010).

When performing PGT, paternal contamination has historically been deemed a risk factor based on the possibility of amplification of extraneous bound sperm or decondensed sperm remaining in a blastomere (Wilton *et al.* 2009, Harton *et al.* 2011). This has led to recommendations to perform ICSI for all amplification-based testing methods. However, as discussed, the inactive and condensed state of the sperm DNA is very different to the nuclear DNA present in other cell types, leading to the question of whether paternal contamination via sperm is a high enough risk factor to exclude the use of IVF. Successful amplification of DNA by whole genome amplification first requires release of DNA. Given the packaging of sperm DNA, it is much more difficult to isolate, and therefore does not amplify under the conditions used for embryo derived cells in amplification based PGT. Although this study was only conducted using SurePlex WGA amplification, the results may be similar – no or impaired amplification – for other whole genome amplification methods, given it is the fundamental structure and storage of sperm DNA which prohibits its amplification. Likewise, any non-decondensed sperm within blastomeres resulting from polyspermy would be expected to yield the same result. In these instances, a sperm would have fertilised the oocyte, and the oocyte's mechanism to block polyspermy would have failed, resulting in

additional sperm breaching the zona and entering the ooplasm. However, normally these cases would be identified by additional pronuclei at fertilisation check and excluded from treatment as polyploid. If an additional sperm were to breach the zona and enter the ooplasm but fail to decondense, the DNA would remain as an inactive nucleus in the sperm head and fail to amplify for the reasons previously stated.

Over 15 years of data collection by the ESHRE PGD consortium 12 misdiagnoses – where the foetus or baby do not have the genetic status indicated by PGT - have been reported from 12 500 cycles (De Rycke *at el* 2017). Given that most clinics reporting to the consortium follow their published guidelines, less than 1% of cycles underwent fertilisation via IVF (De Ryke *et al.* 2017). However, it is unclear if any of these cycles were involved in misdiagnosis events, and no misdiagnoses were attributed to paternal cell contamination. A literature search has failed to identify any reports of misdiagnosis or contamination attributable to paternal cell contamination via intact sperm cells. Retrospective analysis (Feldman *et al.* 2017, Sahin *et al.* 2018, Palmerola *et al.* 2019) and prospective studies (De Munck *et al.* 2020) demonstrate no difference between IVF and ICSI PGT patients in terms of contamination rates, parental origin of aneuploidy, or parental origin of uniparental disomy, indicating no additional risk factors stemming from IVF insemination.

While ESHRE, ASRM and SART recommends the use of ICSI for all amplification based PGT cases (Kokalli *et al.* 2020, Practice committees of ASRM & SART 2012), ASRM and SART acknowledge a lack of data supporting this in practice. Recently published ESHRE good practice recommendations (Kokkali *et al.* 2020) state ICSI to be the preferable method of insemination for PGT case in order to minimise the risk of

both maternal contamination from residual cumulus cells, and paternal contamination from surplus cells attached to the zona pellucida. However, the risk of maternal contamination remains with ICSI, and embryologists should be rigorous when it comes to the removal of cumulus cells at denudation or fertilization check. Prior guidelines from ESHRE have recommended ICSI to reduce the chance of paternal contamination from bound sperm or non-decondensed sperm with in blastomeres (Harton *et al.* 2010) and so the addition of warnings of maternal cell contamination is vital.

Thus, performing ICSI for patients undergoing PGT solely as a method to reduce contamination and misdiagnosis risk, may be unnecessary. While ICSI is an incredibly helpful intervention in patients with male factor infertility and certain oocyte issues, for patients not requiring it, it introduces a risk of oocyte damage and degeneration, additional pipetting of oocytes, and increased time outside the optimal incubator environment. The cost of the ICSI procedure is also a factor that may contribute to patients opting not to undergo PGT. While ICSI can be suggested to maximise fertilisation, improved outcomes have not been seen in patients with low oocyte numbers (Sfontouris *et al.* 2015, Practice committees of ASRM & SART 2012) or for routine use where male infertility is not present (Practice committees of ASRM & SART 2012, Bukulmez *et al.* 2000). ICSI has been shown to be associated with an increased risk of congenital birth defects (Lacamara *et al.* 2017) but it is generally agreed this is attributable to a combination of factors including the underlying causes of infertility in couples seeking treatment and other factors associated with IVF/ICSI procedures rather than solely the ICSI micromanipulation procedure. Conversely, (Palmerola 2019) it has been indicated that IVF may generate a higher proportion of mosaic, complex mosaic and complex aneuploid embryos than ICSI, although the underlying

basis of this is unclear and has not been noted in any similar studies (Feldman *et al.* 2017, Sahin *et al.* 2018, De Munck *et al.* 2020).

While professional guidelines continue to recommend ICSI for amplification based PGT it is understandable that many clinics continue to operate on this basis. However, given the additional cost and lack of clear benefit in the absence of male factor infertility, it seems reasonable for clinics and genetic testing laboratories to risk assess their cases, based on the likelihood of sperm contamination and amplification versus the severity of the outcome were it to occur, and decide their clinical policy on which insemination method is required for individual testing indications.

4. Investigating the technical feasibility of successive biopsy, vitrification and warming in PGT

A study adapted and expanded from:

Lynch C, Jenner L, Campbell A, Gordon A, Griffin D. 2017. Live birth following two rounds of trophectoderm biopsy, vitrification, and warming: Assessment of the efficacy of retesting PGD and PGS embryos: Presented at Fertility 2017, Newcastle.

4.1 Chapter summary

The current best practice and most common approach for preimplantation genetic testing (PGT) is trophectoderm biopsy at the blastocyst stage, followed by vitrification while awaiting genetic test results (Kokkali *et al.* 2020). While the majority of samples will yield a result and allow embryos to be assigned as suitable for patient use or not, it can be expected that – depending on the technology being employed – some samples will fail to return a result. Recent data collection by the ESHRE PGT consortium reports amplification rates of 91% from 254,820 samples (De Ryke *et al.* 2015) and as such the Vienna consensus sets 90% amplification as a competency level and 95% as a benchmark (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine 2017).

The question then is what to do with embryos of unknown genetic status. Depending on the indication for testing, some patients may undergo transfer of such embryos, but other patients may be prohibited from, or unwilling to do so. Any attempt at retesting would require warming, re-biopsy and re-vitrification, with a further round of warming

prior to transfer should samples return a result and be assigned suitable for patient use. Limited literature reported implantation rates of 50%, despite the multiple manipulations. Here, a similar approach was applied to re-biopsy and retesting in 55 embryos, obtaining results in 93% of submitted samples, and with 25% embryos assigned as suitable for patient use. Six embryos were transferred resulting in two livebirths, one clinical miscarriage, one preclinical miscarriage, and two negative tests. This study provides proof of principle for the re-biopsy approach.

4.2 Introduction

As pointed out in section 1.1.3 and discussed in greater detail in chapter 2, current best practice for PGT is considered to be trophectoderm biopsy at the biopsy stage, followed by vitrification while awaiting the return of results. Embryos which are considered suitable for patient use on the basis of genetic results can then be warmed and used in a frozen embryo replacement cycle. Despite the fact that trophectoderm biopsy provides more material for testing in comparison to previous polar body and cleavage stage biopsy approaches, it is still expected that some samples will fail to generate a result (De Ryke *et al.* 2015, ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine 2017). It also may not be possible to release results for a submitted sample if it does not meet the genetic laboratories sample acceptance criteria, or if contamination is detected in the course of the testing. In these instances, re-biopsy and retesting is a theoretical option, but would require blastocysts to be warmed, re-biopsied, re-vitrified, and potentially re-warmed if a result is returned indicating they are genetically suitable for patient use. Literature on the approach is very limited, making patient counselling and informed consent incredibly difficult.

The majority of patients undergoing PGT-M are either prohibited from using embryos of unknown genetic status following testing, or are unwilling to do so. This is due to a known heritable risk of 25-50% that any resulting child would be affected by the genetic condition being tested for. Patients undergoing PGT-SR find themselves in a similar situation, knowing that less than only around 30% of their embryos will be chromosomally normal (Zhang *et al.* 2016, Wang *et al.* 2019), and unwilling to put themselves at risk of further miscarriage. For patients undergoing PGT-M and PGT-SR then, embryos without a result and of unknown genetic status are treated the same as embryos which are affected/aneuploid, and re-biopsy and retesting is the only strategy that may enable them to use these embryos.

Conversely patients undergoing PGT-A to increase the chance of treatment success can be given the option of having an embryo of unknown genetic status post testing transferred. This can be a personal decision for the patient, and depending on their reproductive history, some will be as opposed to this as PGT-M or PGT-SR patients would be. However, this does represent a fundamental conceptual difference between PGT-M, PGT-SR and PGT-A with the latter treatment applied to infertile patients hoping to improve embryo selection and increase their chances of success, while the former two treatments are often applied to fertile patients not wishing to conceive naturally, with a defined heritable risk to pregnancy or children.

At the time of writing, a single publication by Zhang *et al.* (2014) provided the largest cohort of 10 single embryo transfers of re-biopsied and re-vitrified embryos, with a 50% implantation rate, equivalent to their standard PGT-A results. To the best of my knowledge however there was no further published studies corroborating this, and

thus the need for further proof of principle investigations to establish whether re-biopsy was a viable strategy.

4.3 Materials and Methods

Blastocysts underwent trophectoderm biopsy according to standard laboratory operating procedures, and as described in section 2.5.1. Vitrification was performed when blastocysts re-expanded post biopsy, using the cryotop closed vitrification device and Kitazato vitrification media, according to validated product insert instructions.

PGT-M and PGT-SR patients were given the option of re-biopsy and retesting of no result embryos. Promising initial data saw this extended to PGT-A patients. Rebiopsy was performed to the same operating procedure as the original biopsy and the original opening in the zona was used.

A total of 55 blastocysts were warmed using the Kitazato warming media, according to validated product insert instructions, with 100% survival. Following re-expansion, blastocysts underwent a second biopsy using the same procedure as the initial biopsy, with 100% survival. Re-vitrification was performed when blastocysts re-expanded post biopsy, using the cryotop closed vitrification device and Kitazato vitrification media, according to validated product insert instructions.

Tubed trophectoderm biopsy samples were submitted to the Genesis Genetics (now Cooper) laboratory, Nottingham.

The samples underwent whole genome amplification (WGA) (SurePlex, Illumina) according to the genetic testing laboratory's standard operating procedure. A negative control (reagents only) and positive control of 2µl 30pg/µl female genomic DNA (SureRef, Illumina) were included in the amplification reaction. Cell lysis and pre-amplification steps were performed in the pre-amplification area of the laboratory with dedicated equipment and personal protective equipment. The final amplification step was performed in the general area of the laboratory, producing 90µl amplified product for each sample

DNA amplification was assessed in the genetics lab via gel electrophoresis for the 25 submitted samples, WGA positive control, and WGA negative control. A gel electrophoresis positive control (previous successfully amplified sample) and negative control (Millipore water) were included. The gel box is filled with 1XTAE buffer and the pre-cast 1% agarose gel with ethidium bromide added. 5µl WGA product or control is mixed with 1.1µl running buffer and 5µl is pipetted into the corresponding well of the gel. The gel is run at 100V until there is clear colour separation of the loading dye and is then visualised on a UV light box. The presence of an illuminated band or smear indicates the presence of amplified DNA. Samples displaying amplified DNA were further processed via karyomapping or NGS.

An aliquot of amplified samples for PGT-M were sent to the Genesis Genetics (Cooper) laboratory Detroit, Michigan to undergo Karyomapping, with GTC files imported and analysed via the Karyomapping module of Bluefuse software (Illumina) at the Genesis Genetics Nottingham laboratory.

Amplified samples for PGT-A underwent next generation sequencing according to the Illumina Veriseq protocol (Illumina 2020) at the Genesis Genetics Nottingham laboratory, with data analysis via the CytoChip module of BlueFuse software (Illumina). No re-biopsy and retesting was performed for PGT-SR. Results were reported to the referring clinic as standard and feedback was requested on embryo usage and clinical outcomes.

4.4 Results

In this study, warming and a second round of biopsy followed by re-vitrification was performed for 55 embryos.

The most common reason for retesting in PGT-A was a failure of DNA amplification from the initial biopsy (73%), followed by failure of initial test results to meet QC criteria (24%) (Figure 4.1). In PGT-M both these factors occurred at the same incidence (47%) (Figure 4.2).

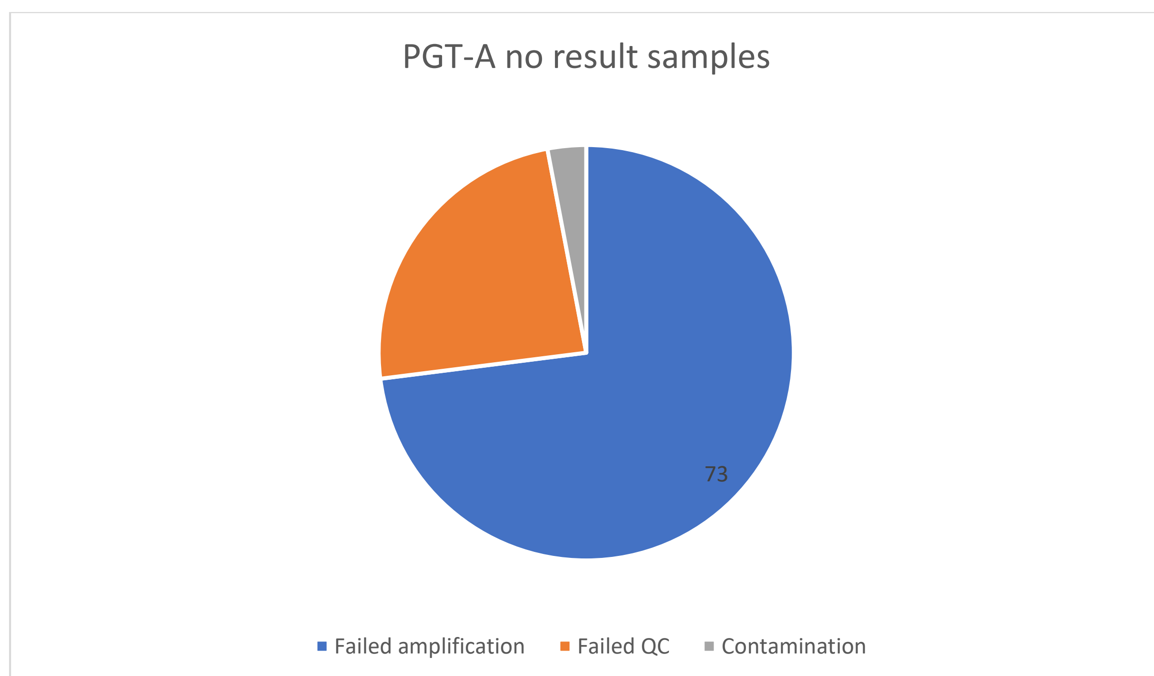

Figure 4.1. Chart illustrating the reasons for PGT-A samples failing to yield a result

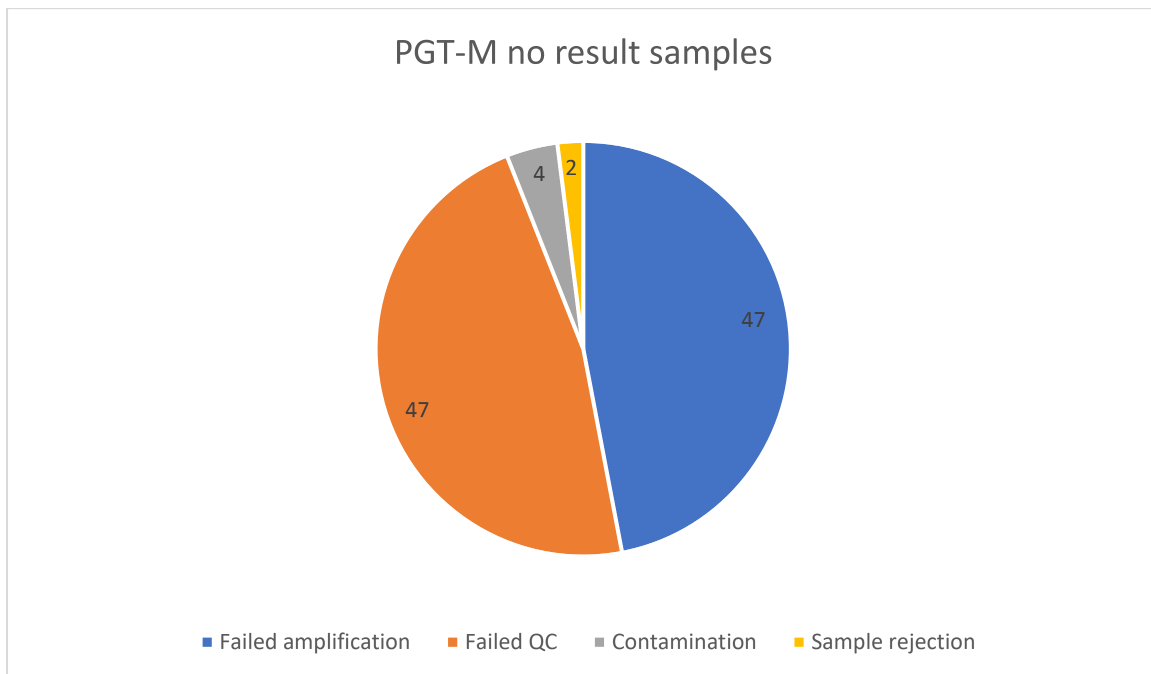

Figure 4.2. Chart illustrating the reason for PGT-M samples failing to yield a result.

Results were obtained from 93% of samples, with 25% of those yielding results classed as suitable for patient use. Six of the suitable retested embryos were transferred resulting in two livebirths. There was also a clinical miscarriage a preclinical miscarriage, and two negative tests (Figure 4.3).

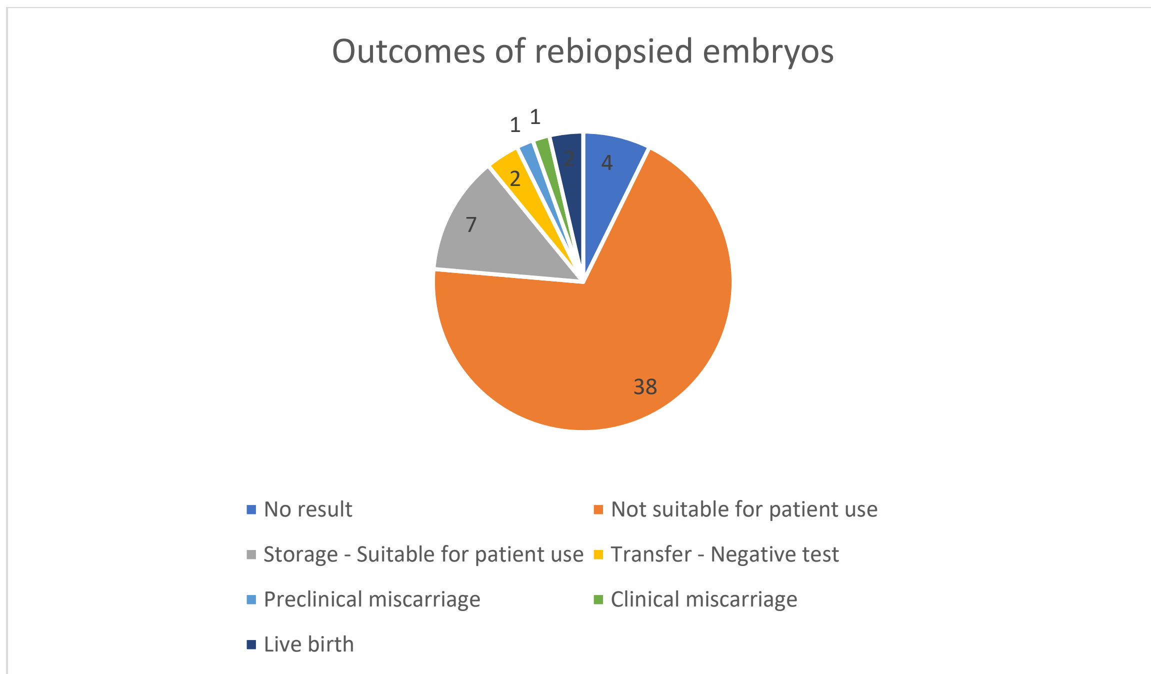

Figure 4.3. Chart illustrating the fate and outcome of re-biopsied no result embryos

4.5 Discussion

At the time of writing, this was only the second study to demonstrate the feasibility of a re-biopsy strategy for patients with no result embryos they were either unwilling or unable to use in treatment.

The available data demonstrates the efficacy of the procedure in terms of generating a genetic result and a proportion of those results being unaffected/euploid. Additionally, it supports the other published study (Zhang *et al.* 2010) in demonstrating that embryos that have been through two rounds of biopsy, vitrification, and warming can retain the potential to successfully implant and result in a healthy livebirth.

This study provided sufficient proof of principle for the following chapter in which, inspired by this study, re-biopsy and retesting was offered more widely to ART clinics and patients. The data collected in the following chapter seeks to examine the efficacy of the approach in terms of parameters already tested, but using a larger data set, and

also comparing clinical outcomes with patients who declined re-biopsy and opted to undergo transfer of embryos of unknown genetic status.

5. Analysis of no result rates and rebiopsy outcomes in PGT-A cycles undergoing trophectoderm biopsy and next generation sequencing

5.1 Chapter summary

As pointed out in section 1.2, PGT-A cycles involving trophectoderm biopsy and next generation sequencing are becoming more routine in clinical practice. However, clinics often work in isolation from one another, meaning they do not know how some of their performance indicators compare with other clinics offering the same treatment. As a reference laboratory processing NGS samples for clinics across Europe, Asia and Australia the CooperGenomics group have collated anonymised data demonstrating varying performance across clinics in terms of no result rates in PGT-A cycles.

The purpose of this chapter was to examine the no result rate in the CooperGenomics UK laboratories since the introduction of next generation sequencing (NGS) in 2015. Specifically, the question addressed was how much no result rates vary between referring ART clinics, and to follow-up the fate of no result embryos following NGS testing. The ultimate aim was to estimate the likely prospects for re-biopsy.

The fate of no result embryos was followed up and compared with clinical outcomes in re-biopsied embryos to those transferred without results. While there is a trend to improved livebirth rate in the re-biopsy group, implantation rates are below those expected in PGT-A cycles, and miscarriages were higher than expected. Thus, while re-biopsy will give a patient the genetic information on their embryos, this needs to be balanced with the potential impact on clinical outcomes while more data is generated.

5.2 Introduction

Approaches to PGT-A have evolved since the first clinical cases involving polar body or cleavage stage biopsy, with testing methodologies that would enable fresh embryo transfer (Munne *et al.* 2003). The advent of more sophisticated and expensive molecular cytogenetic testing platforms has seen testing move to specialist laboratories, with clinics undertaking IVF treatment and embryo biopsy, then sending biopsy samples to reference laboratories for testing. In tandem with this, IVF laboratories have moved towards biopsy of trophectoderm at the blastocyst stage, necessitating embryos to be frozen while awaiting PGT-A results (Kokkali *et al.* 2020).

With any testing platform used, there is always a chance that a sample will not yield a result. Even IVF laboratories with significant experience in the field display variation when it comes to no result rate (Yang *et al.* 2015, Fiorentino 2014, Capalbo *et al.* 2015). In PGT-A via NGS, a no result can arise from two main scenarios; either no DNA is detected following the whole genome amplification procedure, or the DNA amplifies and is processed via NGS, but data is of poor quality and does not meet quality control (QC) reporting criteria. A number of factors in the IVF or genetics laboratory can contribute to the incidence of either of these outcomes (Cimadomo *et al.* 2018).

Depending on patient circumstance, including whether they have any genetically suitable embryos for transfer, re-biopsy and retesting may be requested rather than disposing a no result embryo (or transferring an embryo of unknown genetic status). With cycles involving trophectoderm biopsy and vitrification, this requires the blastocyst to be warmed, re-biopsied, and then re-vitrified. If the embryo proves to be

genetically suitable for use, it then must be re-warmed prior to embryo transfer. There are therefore many stages at which this process can fail; at warming, re-biopsy, or rewarming for transfer (Parriego *et al.* 2018, Neal *et al.* 2019).

Understandably, there was initially scepticism surrounding how this would impact on the viability of the embryo and if it was fair to put the patient through the stress of waiting for a re-biopsy result. Embryo survival on warming, the return of results, and the genetic status (e.g. euploid vs. aneuploid) is similar to first biopsy data, so in terms of getting a result and being suitable for clinical use, it is a worthwhile process for the patient. Multiple studies have demonstrated that re-biopsied embryos are as likely to yield a result, and be suitable for transfer as embryos being biopsied for the first time (Zhang et al. 2014, Howard *et al.* 2018, Neal *et al.* 2019). When these embryos are used clinically, initial studies demonstrated similar clinical outcomes to single biopsy and vitrification cycles, including gestational age at delivery, and birth weight (Tyler *et al.* 2014, Zhange *et al.* 2014, Neal *et al.* 2018) but more recent data urges more caution, with comparatively poorer clinical outcomes. (Parriego *et al.* 2018, Neal *et al.* 2019, De Vos et al. 2020).

To the best of my knowledge, there have been, hitherto, no large-scale studies that have addressed inter-centre variation in no result rates and subsequently followed them up. Such information is essential in order to inform clinics whether re-biopsy is a practicable strategy going forward.

5.3 Materials and Methods

Data was collected from PGT-A cycles performed at the Nottingham and London CooperGenomics laboratories between January 2015 and December 2019. All cases involved analysis of trophectoderm samples via NGS, where the guidance given to clinics is to biopsy 5-10 cells.

Biopsy samples are tubed using biopsy kits provided by CooperGenomics, which include 0.2ml DNA/RNA free PCR tubes and PBS based buffer. Trophectoderm samples underwent whole genome amplification (WGA) (SurePlex, Illumina) according to the genetic testing laboratory's standard operating procedure and DNA amplification was assessed by gel electrophoresis.

Next generation sequencing was performed, using the Illumina Veriseq protocol, on biopsy samples displaying positive DNA amplification. Standard dilutions of WGA products underwent tagmentation, amplification and indexing, and cleanup, before undergoing library normalisation. The samples were then pooled and loaded on the sequencer. Sequencing data was processed via Bluefuse software and analysed manually by a minimum of two scientists.

Analysis was undertaken to compare the data generated by the laboratories to map how sample numbers and no result rates had changed since the introduction of the assay. No result rates were split by failed amplification, and failed QC.

No result rates were also compiled and anonymised for referring IVF clinics for the period January 2015 – November 2018. These were compared across clinics based

on sample volume, with clinics split into three groups; >500 samples, 100-499 samples, and <100 samples.

Clinics were contacted for follow up data on embryos which had yielded no result. They were asked to confirm if the embryo had been re-biopsied, stored as a no result for patient use, donated to training or research, or undergone disposal. For embryos undergoing re-biopsy they were asked if the embryo had survived warming and subsequent re-biopsy. They were asked to confirm if an embryo had been used for transfer, and if so if it had survived warming. The clinic was also asked to provide clinical follow up including initial pregnancy test result, whether a foetal heart had been identified, whether miscarriage had occurred, or whether the pregnancy was ongoing and had resulted in a livebirth.

The two tailed Fishers exact test was used for statistical analysis due to the variation and smaller number of cycles and samples in some groups. Statistical significance was reached when the p value was less than 0.05.

5.4 Results

5.4.1 No result rates 2015-2019

In the 4yr period of data collection, the number of samples processed via NGS has increased from 350 to 9507.The failed amplification rate has fallen from 6.8% in 2015 to 1.8% in 2019. The proportion of samples failing QC has risen in the same time period from 1.4% to 3.3%. However, in combination, there are significantly more embryos with results reported in 2019 (p=0.0139) (Figure 5.1).

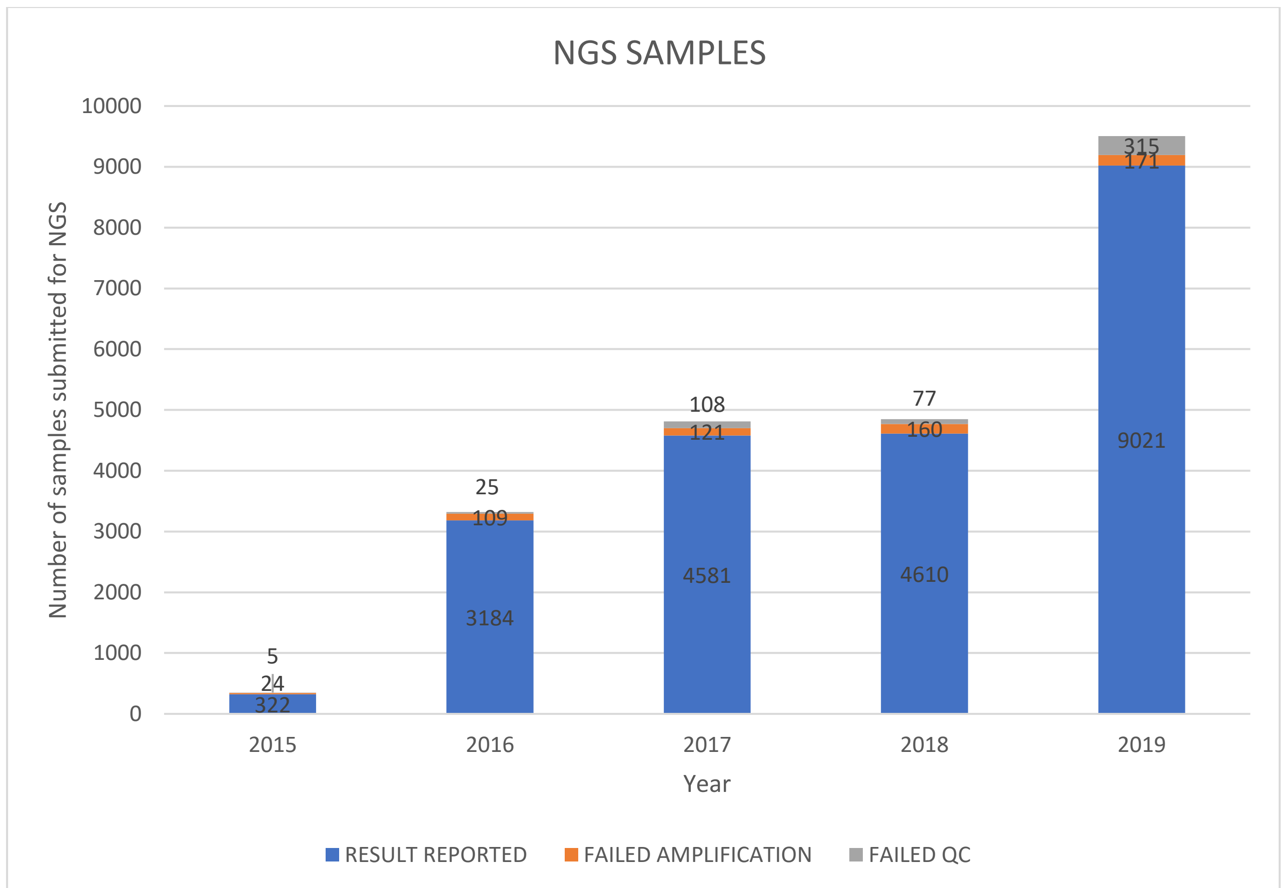

Figure 5.1 Graph showing samples processed via NGS in CooperGenomics UK laboratories by year and result status

5.4.2 Centres submitting over 500 samples

Nine centres submitted over 500 samples each between January 2015 and November 2018, ranging from 512 to 2633 (Figure 5.2). The failed amplification rate ranged from 0.0% to 2.2%, while the QC failure rate ranged from 0.5% to 5.3%. Clinic A had the highest overall no result rate of 7.4%, and clinic F the lowest at 0.6%, the difference between the clinics being statistically significant (p=0.0001).

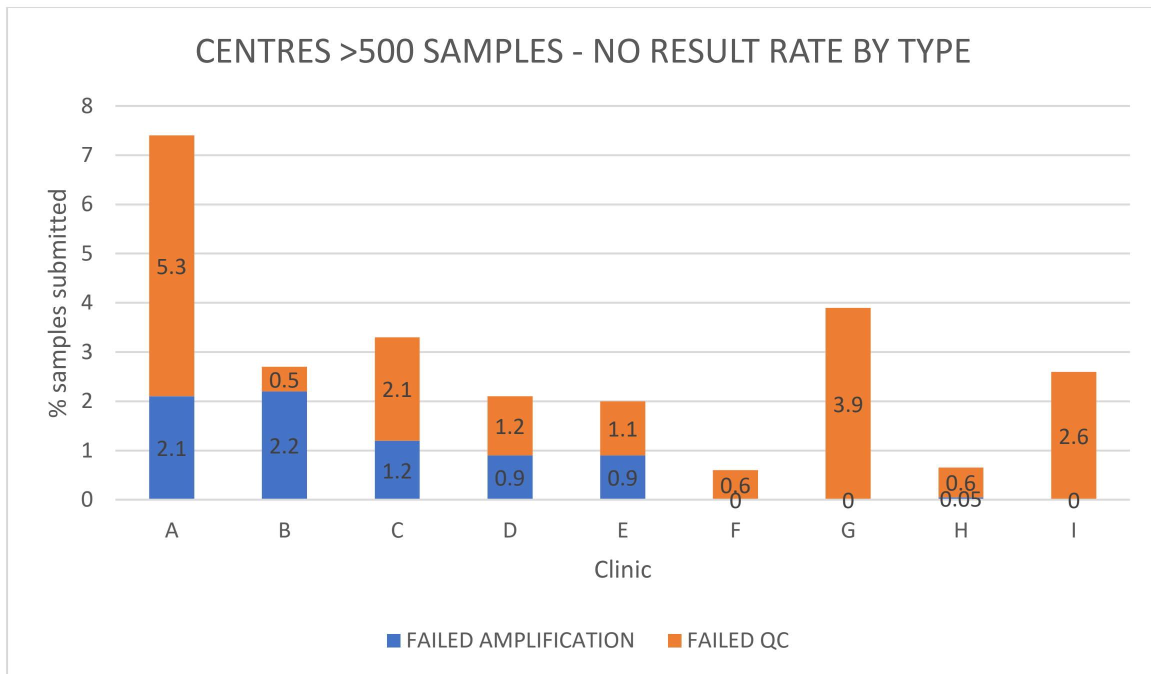

Figure 5.2 Graph showing the % no result rate by type in clinics that submitted >500 samples.

5.4.3 Centres submitting 100-499 samples

Ten centres submitted between 100 and 499 samples each between January 2015 and November 2018, ranging from 110 to 303 (Figure 5.3). The failed amplification rate ranged from 0.0% to 3.9%, while the QC failure rate ranged from 0.0% to 3.7%%. Clinic N had the highest overall no result rate of 5.8%, and clinic J the lowest at 1.1%, the difference between the clinics being statistically significant (p=0.0265).

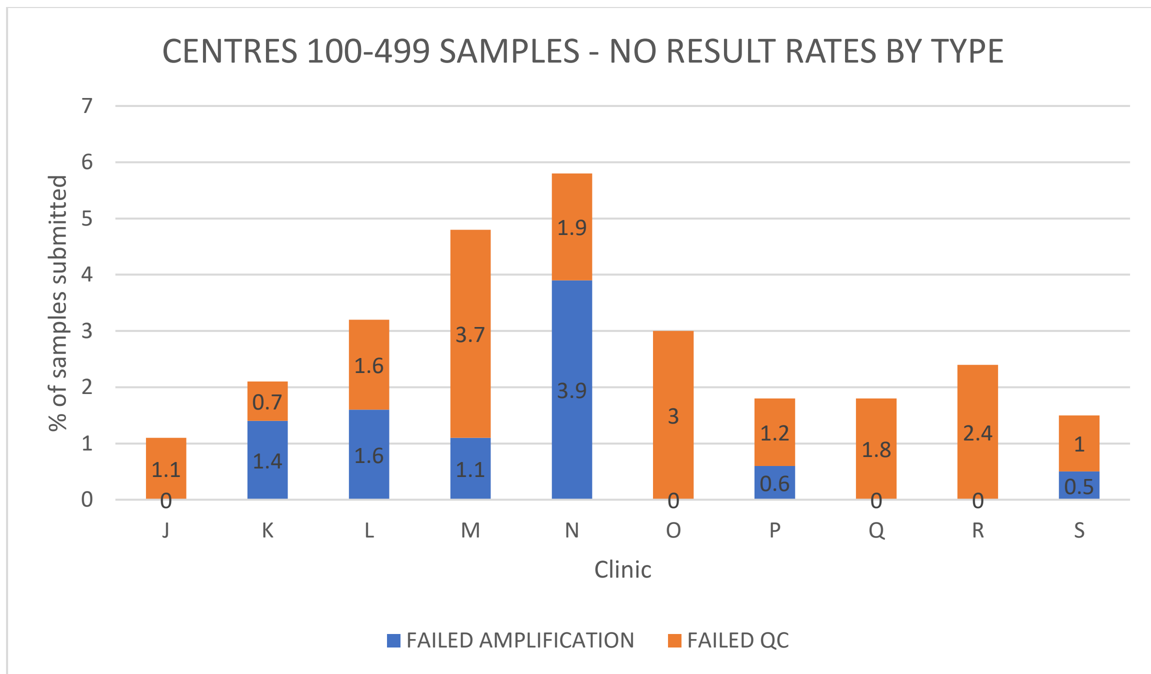

Figure 5.3 Graph showing the % no result rate by type in clinics that submitted 100-499 samples.

5.4.4 Centres submitting less than 100 samples

Eleven centres submitted less than 100 samples each between 2015 and 2018, ranging from 9 to 82 (Figure 5.4). The failed amplification rate ranged from 0.0% to 7.5%, while the QC failure rate ranged from 1.3%% to 13.3%. Clinic W had the highest overall no result rate of 13.3%, and clinic CC the lowest at 2.4%, but the difference was not statistically significant (p=0.1118).

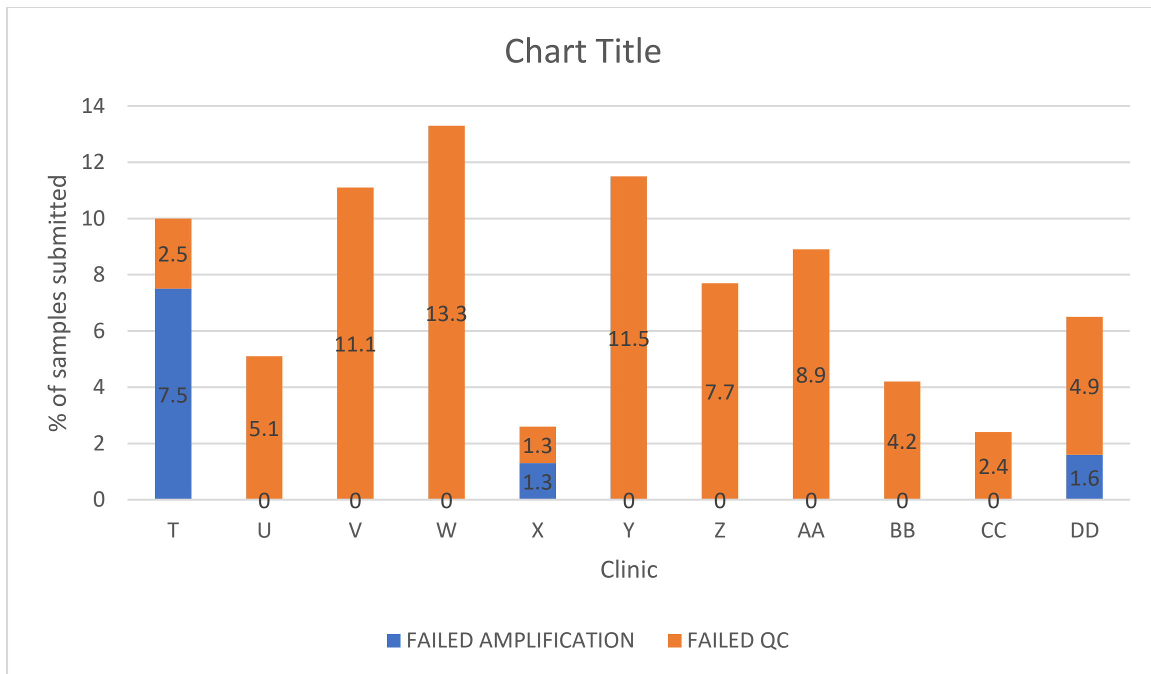

Figure 5.4 Graph showing samples the no result rate by type in clinics which submitted <100 samples.

5.4.5 Fate of no result embryos

Of the 554 no result embryos followed up, 231 (41.7%) underwent re-biopsy. Of those undergoing re-biopsy, 55.0% had embryos suitable for transfer in storage from the original cycle.

Of the 323 embryos that were not re-biopsied, 42 were used in treatment despite returning no result, 198 remained in storage for patient use, with the remainder disposed (Figure 5.5). In cycles where re-biopsy was not performed, 67.9% of patients had embryos suitable for transfer in storage from their original cycle

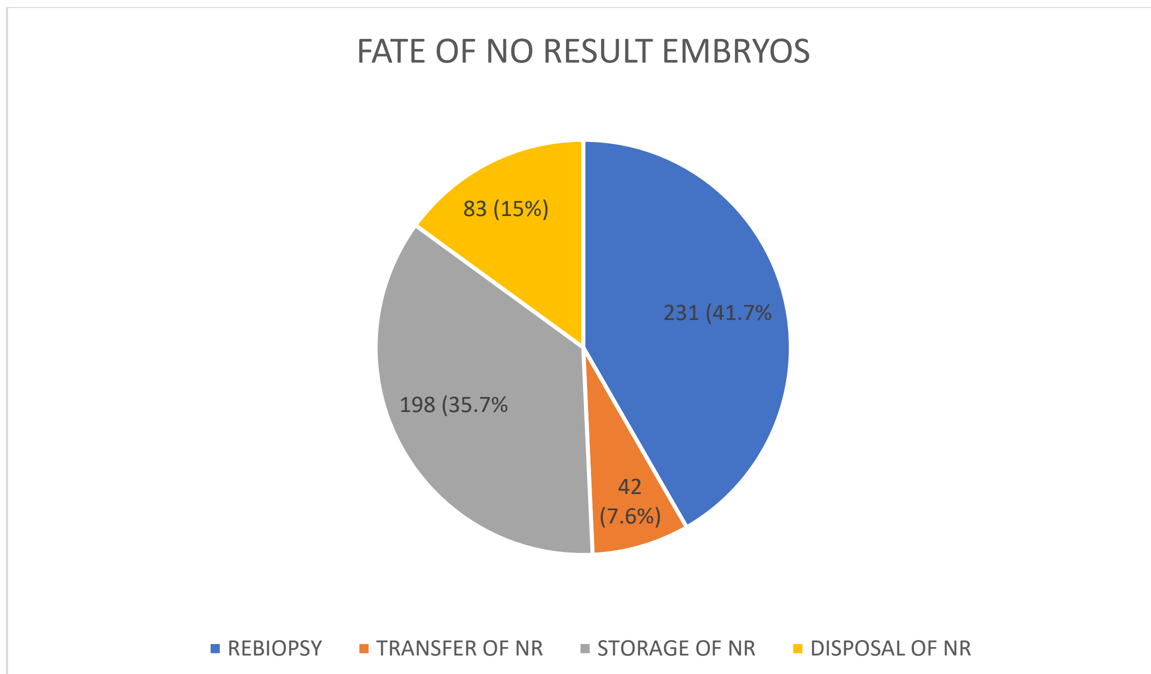

Figure 5.5. Chart illustrating the fate of embryos returning no result following NGS

5.4.6 Re-biopsied embryos

Of 231 embryos undergoing re-biopsy, 23 embryos did not survive warming (6.5% failed thaw) and re-biopsy (3.7% failed biopsy). Therefore,208 embryos successfully underwent re-biopsy and were re-vitrified and 192 embryos successfully returned a result (Figure 5.6).

The initial classification of no result – failed amplification or failed QC – had no impact on whether the embryo would yield a result on re-biopsy, (p=1.000). Of the embryos returning no result a second time, 60% had the same classification (failed amplification v failed QC) as the first time, indicating no specific recurring issue.

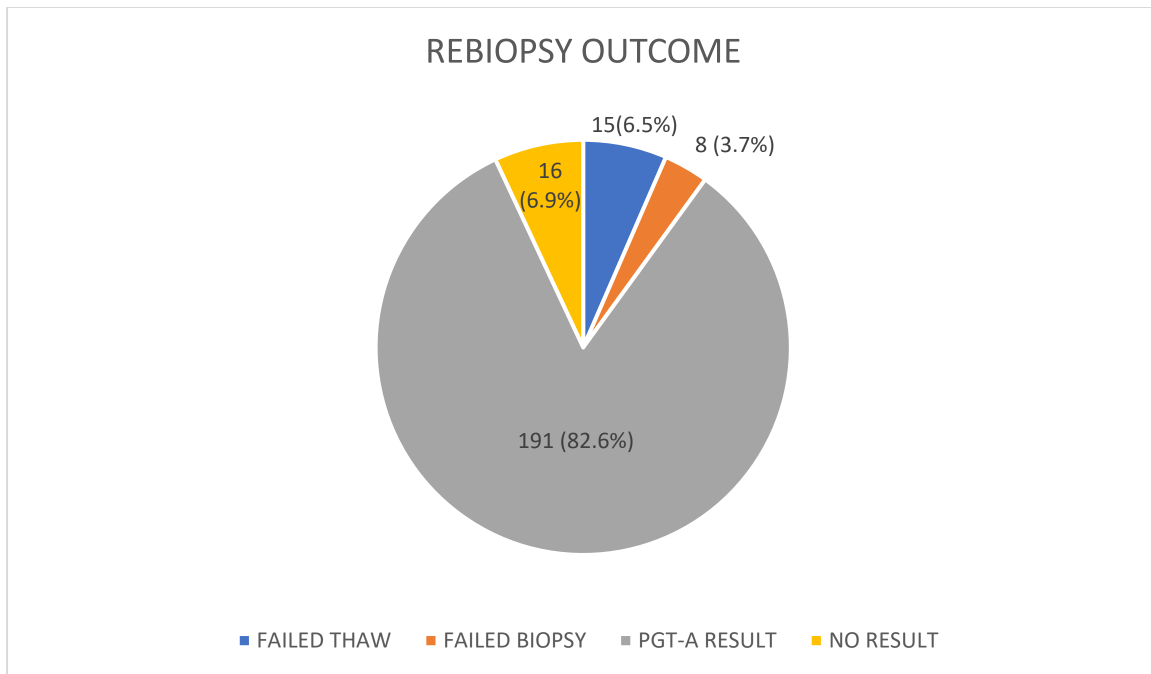

Figure 5.6 Chart illustrating the fate and NGS result of embryos undergoing re-biopsy

In total, 191 embryos yielded a result following rebiopsy and retesting. Of these, 61 embryos returned a euploid result (31.9%), 32 of which were used in treatment with 29 remaining in storage for patient use. There were also 4 (2.1%) mosaic embryos, which remain in storage for patient use, along with 9 of the 16 embryos which returned a second no result.

31 euploid embryos survived warming and were transferred (3.1% failed thaw). There were 19 positive tests (61.2%), leading to 11 livebirths and sustained implantations (35.5%). The preclinical miscarriage rate was 31.5% and the clinical miscarriage rate was 15.4.% (Figure 5.7).

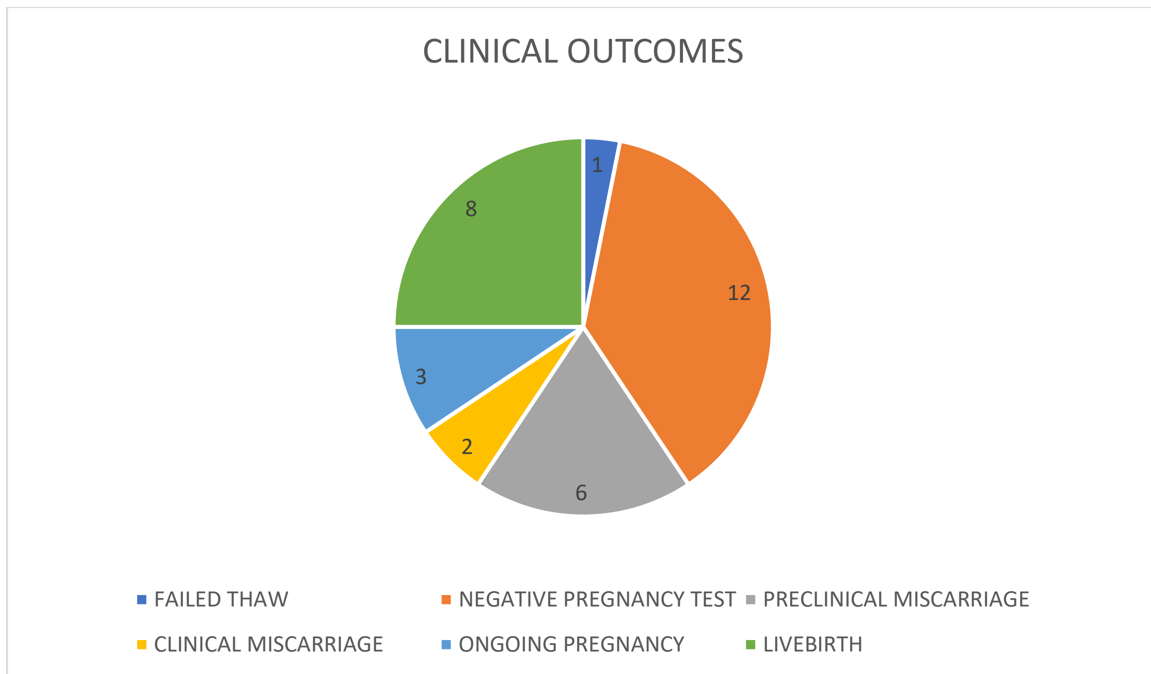

Figure 5.7 Chart illustrating the clinical outcomes of embryos which had undergone re-biopsy and NGS and returned a euploid result.

Of the 58 patients undergoing re-biopsy without any euploids in their initial cycle, 18 had a euploid embryo identified for future use.

5.4.7 Non re-biopsied embryos

Of the 323 embryos that were not re-biopsied, 42 were used in treatment and 198 remain in storage for patient use.

41 embryos survived warming and were transferred (2.4% failed thaw). There were 14 positive tests (34.1%), leading to 7 livebirths and sustained implantations (17.1%). The preclinical miscarriage rate was 21.4% and the clinical miscarriage rate was 36.4% (Figure 5.8).

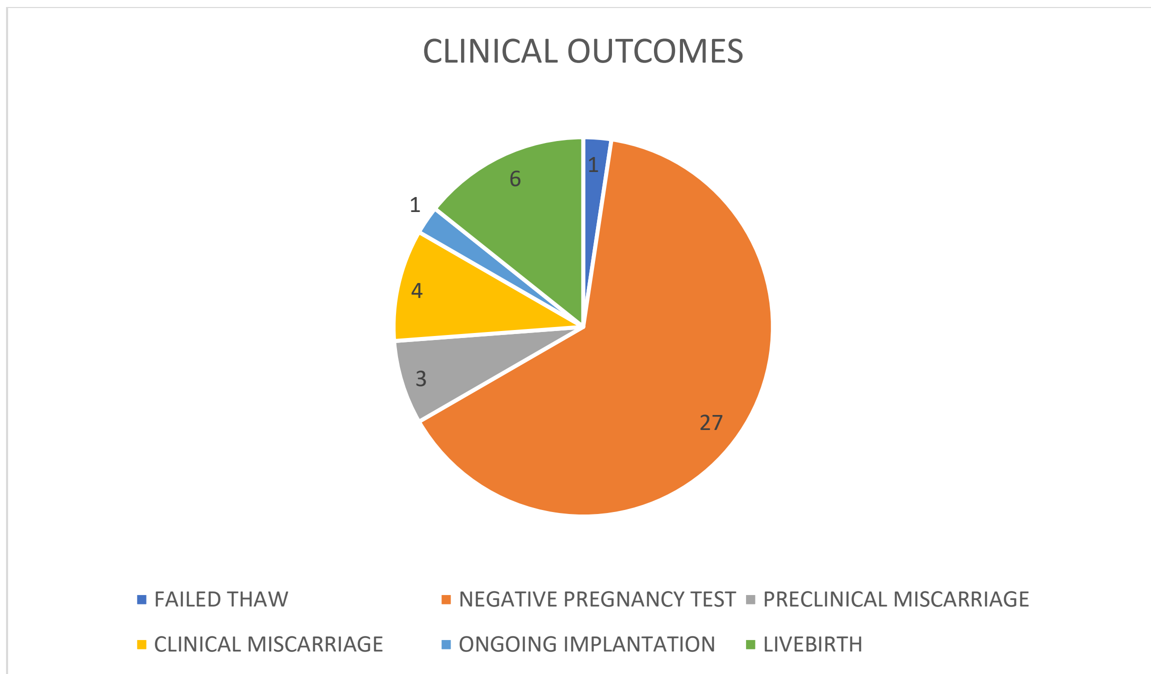

Figure 5.8. Chart illustrating the clinical outcomes of embryos which were transferred as no result without re-biopsy.

5.4.8 Comparison of clinical outcomes in biopsied and non re-biopsied embryos

Patients within the re-biopsy group had transfer of a euploid embryo which had undergone two rounds of biopsy, vitrification and warming, while those in the non re-biopsy group had transfer of an embryo of unknown genetic status which had undergone a single round of those procedures. The extra round of procedures does not seem to impact on embryo survival rates, with only 1 embryo in each group failing to survive warming.

Figure 5.9 compares clinical outcomes by % of the total number of embryos in each group. Following embryo transfer, there is a statistically higher chance of a negative test after transfer of a no result embryo (p=0.0341). The percentage of ongoing pregnancies and livebirths is 35.5% per transfer and 57.9% per positive pregnancy

test in the re-biopsy group, and 17.1% and 50% respectively in the non re-biopsy group. This illustrates a non-significant trend (p=0.1006) towards improved ongoing pregnancy and livebirth per embryo transfer in the re-biopsy group, with differences less marked between groups if analysed per positive test. The miscarriage rate in the re-biopsy group is 25.8% per embryo transfer and 42.0% per positive test, and 17.1% and 50.0% respectively in the non re-biopsy group. This data suggests that the biggest risk in the re-biopsy group is biochemical loss/preclinical miscarriage while in the non re-biopsy group it is a negative pregnancy test.

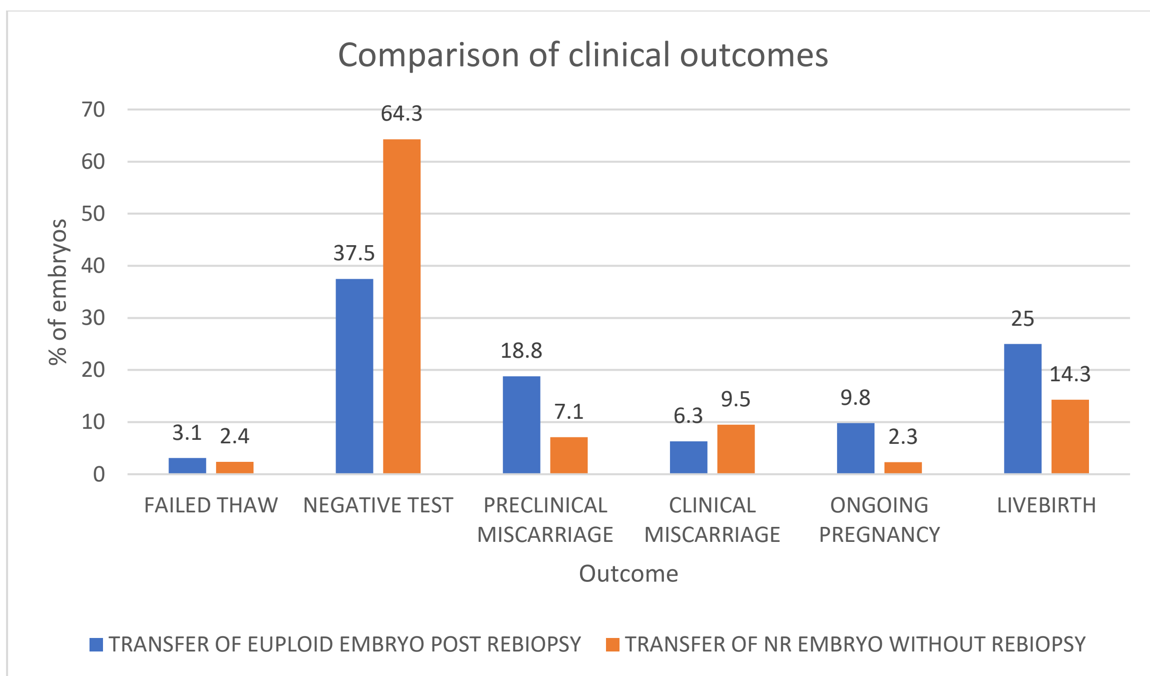

Figure 5.9 Chart illustrating the clinical outcomes by % in biopsied and no re-biopsied groups.

If we group the negative tests and biochemical losses together, this amounts to 58.1% and 73.2% per embryo transfer in the re-biopsy and non re-biopsy groups respectively. Whilst not significant (p=0.2124), the total preclinical losses in the non re-biopsy group are higher. Examining clinical miscarriage in isolation then, in the re-biopsy group this

is 6.4% per embryo transfer, 10.5% per positive test, and 15.4% per confirmed clinical pregnancy. In the non re-biopsy group, this is 9.8%, 28.6% and 36.4% respectively. Whilst statistical significance is not noted in terms of the higher clinical loss rate at each stage, the difference is greatest when examined by confirmed clinical pregnancies.

5.5 Discussion

Since NGS was introduced to the laboratory in 2015 there has been an exponential increase in the number of samples for processing, rising from 351 in the year of introduction to 9507 in the most recent full calendar year (2019). Reassuringly, the no result rate has significantly dropped in this time, from 8.2% in 2015, to 5.1% in 2019, due in part to scientists advancing competences in trophectoderm biopsy, cell tubing, and running the NGS assay.

There was a wide range in sample numbers submitted by clinics over the time period, ranging from 9 samples through to 2633. In the group of clinics submitting over 500 samples the no result rate ranged from 0.6% to 7.4%, and in the group submitting 100-499 samples it ranged from 1.1% to 5.8%. Both these differences proved to be statistically significant between the best and worst performing clinics, and shows that a gap in performance still exists between clinics that needs to be addressed.

Of the embryos not yielding a result, 41.6% underwent re-biopsy, with 35.8% remaining in storge, 15.0% undergoing disposal, and 7.6% used for transfer without retesting. The majority of embryos undergoing re-biopsy yielded a result (92.3%), pointing to a technical issue in the original cycle, either in the embryology or genetic

testing laboratory (Cimadomo *et al.* 2018, Neal *et al.* 2019). Of the 58 patients undergoing re-biopsy without any euploids in their initial cycle, 18 had a euploid embryo identified for future use. From this perspective, the process of re-biopsy is proven to clarify the genetic status of an embryo, add to a patient's stock of euploid embryos, and identify euploid embryos where none were found on the initial cycle.

The rate of livebirth/ongoing implantation in the re-biopsy group is 35.5% and 17.1% in the non re-biopsy group, illustrating a non-significant trend towards a higher chance of livebirth in the re-biopsy group. With groups reporting live birth rates reaching 73% (Vinals Gonzalez *et al.* 2019) and 66% (Gorodeckaja *et al.* 2020) in PGT-A cycles, concern must remain that the re-biopsy process is impacting on the ongoing potential of the embryo. However, this recent data may also reduce incidence of re-biopsy, given the success rates are inherently high.

This data suggests that the biggest risk in the re-biopsy group is biochemical loss/preclinical miscarriage while in the non re-biopsy group it is a negative pregnancy test. If we group the negative tests and biochemical losses together, this amounts to 58.1% and 73.2% per embryo transfer in the re-biopsy and non re-biopsy groups respectively. Whilst not significant (p=0.2124), the total preclinical losses in the non re-biopsy group are higher. Whilst statistical significance is not noted in terms of the higher clinical loss rate at each stage, the difference is greatest when examined by confirmed clinical pregnancies.

As well as increasing implantation, PGT-A also aims to reduce miscarriage. Whilst the incidence of positive tests and ongoing pregnancy and livebirth are significantly higher

in the re-biopsy group, the impact on miscarriage is less clear. The combined pre clinical and clinical miscarriage rate in the re-biopsy group is 25.8% per embryo transfer and 42.0% per positive test, and 17.1% and 50.0% respectively in the non re-biopsy group. Examining clinical miscarriage in isolation, in the re-biopsy group this is 6.4% per embryo transfer, 10.5% per positive test, and 15.4% per confirmed clinical pregnancy. In the non re-biopsy group, this is 9.8%, 28.6% and 36.4% respectively. While there was no significant difference in miscarriage rates, this demonstrates a trend towards lower clinical miscarriage rates in the re-biopsy group. In the re-biopsy group, biochemical loss/preclinical miscarriage is the limiting factor, while in the non re-biopsy group it is negative pregnancy test and clinical miscarriage. These clinical results for re-biopsied embryos are in alignment with other recently published studies looking at clinical outcomes in re-biopsied embryos (Parriego *et al.* 2018, Neal *et al.* 2019) but previous studies lack comparison with embryos transferred without re-biopsy and retesting.

The small number of embryo transfers prohibited examining clinical outcomes by clinic. Given the differences in no result rates between clinics, it may also be the case that clinical outcomes for re-biopsied embryos differ between clinics also. With 29 re-biopsied euploid embryos and 198 no result embryos remaining in storage, analysis of the data at this time can only be considered preliminary and must continue to be monitored. Re-biopsy will give patients the genetic information on their embryos, but the importance if this must be balanced with its potential impact on the embryo.

5.6 Conclusions

Returning to the core question of the ultimate utility of re-biopsy, the collective results herein presented provide a number of angles to address the question. First, the overall no result level is variable between clinics, suggesting that it is possible to get to levels of approaching zero percent, meaning that it would not be necessary at all. Second however, if it is applied, the vast majority return a result, and a significant number turn out to be euploid, which suggest it might be useful. Third, evidence presented here for the first time suggests that, if applied, it may increase the number of patients having transfer, and increase the chances of ongoing/pregnancy, whilst reducing the risk of clinical miscarriage, again pointing to its possible utility. As PGT-A success rates continue to improve, combining this with the best practice and, subsequent low no result rates of the best performing clinics, would mean the best outcome of no requirement for re-biopsy. However, more data is required to definitively show whether the impact of multiple procedures outweighs the benefit of selection via PGT-A, and if re-biopsy is universally preferable in terms of clinical outcomes compared with transfer of embryos of unknown genetic status.

6. To perform a retrospective analysis of over 1,800 PGT-SR samples (479 cycles over 5 years)

6.1 Chapter summary

Since the first cases of pre-implantation testing for structural rearrangements (PGT-SR) (Munne *et al.* 1998), clinical outcomes have been well characterised in subsequent years. As pointed out in section 1.1.2, studies and case reports have used varied approaches to both biopsy and cytogenetic testing (Ogur and Griffin 2020). Less well studied are the indications for testing; the types of structural chromosome rearrangements that result in referrals for PGT-SR, and what this can tell us about the relationship between these rearrangements and infertility and subfertility. Advances in embryology and molecular cytogenetics now see us test multiple trophectoderm cells via next generation sequencing (NGS). The information thus allows us to examine in much greater detail the cytogenetic profile of resultant embryos and investigate potential biological phenomenon such as mosaicism and inter-chromosomal effect (ICE). In addition, SNP arrays also allow testing for uniparental disomy (UPD) (Ogur and Griffin 2020). To date however, the number of cycles analysed have ranged from 149 to 4253 for FISH studies, 32 to 266 for SNP arrays, 17 to 50 for aCGH and 21 to 129 for NGS (Ogur and Griffin 2020). Thus, for the most widely used modalities for comprehensive chromosome screening, case numbers have been small.

Large data sets provide the opportunity to examine potential influencing factors such as sex, maternal age, day of biopsy and ICE. In this chapter, analysis of data comprising 479 PGT-SR cycles performed between April 2015 and March 2020, is presented, involving the analysis of 1814 trophectoderm samples for couples with a

variety of structural rearrangements. This is, to the best of my knowledge, the largest data set of this kind presented to date.

Data analysis indicates that the most important factor in whether a cycle will have embryos available for transfer is maternal age, and that in female Robertsonian translocation carriers, this will even impact of the proportion of unbalanced embryos. Reassuringly, there was no evidence of interchromosomal effect during meiosis or mitosis, with expected levels of incidental aneuploidy and mosaicism observed.

6.2 Introduction

Structural chromosome rearrangements are known to be implicated in subfertility and infertility and were first identified as a cause of recurrent pregnancy loss in 1962 (Schmid 1962). They usually involve the exchange of translocated segments between two chromosomes (reciprocal translocations) or fusion of two chromosomes (Robertsonian and dicentric translocations). They can also involve the transposition or inversion of a segment in a single chromosome (insertions and inversions), or the transposition of a segment from one chromosome to another (insertions).

Individuals who are carriers of balanced chromosome rearrangements are usually phenotypically normal unless a breakpoint involved leads to the disruption or disfunction of a gene. Some rearrangements carry a high risk of having a child affected by a chromosome disorder, some have a low risk of an unbalanced livebirth but will be associated with recurrent pregnancy loss, while some appear to be of no reproductive significance (Gardner *et al.* 2012). Because of this, it is difficult to ascertain their frequency in the general population. It is estimated than the frequency

of reciprocal translocations is 0.2% in newborns, with a similar frequency of Robertsonian translocations and even lower incidence of other structural rearrangements (Jacobs *et al.* 1992). Because of the relationship between chromosome rearrangements and infertility or subfertility, the frequency of carriers is higher within this population.

One study demonstrated the frequency of structural chromosome rearrangements to be approximately 9% in couples experiencing recurrent pregnancy loss, and 3% in those experiencing implantation failure (Stern *et al.* 1999). Another estimated that a translocation is present in 2.2% of couples after one miscarriage, 4.8% after two miscarriages, and 5.7% after three miscarriages (De Braekeleer & Dao 1990). Conversely, a study of 10,202 fertile men donating sperm identified only 7 Robertsonian translocations, 5 reciprocal translocations, and 9 inversions, with 4 of the inversions being common variants (Ravel *et al.* 2006).

The association of structural chromosome rearrangements with failed implantation and miscarriage results from the production of unbalanced gametes by carriers, and subsequent unbalanced aneuploid embryos (Gardner *et al.* 2012). Gamete karyotyping indicates that in male reciprocal translocation carriers 55% sperm are unbalanced (Benet *et al.* 2005), while in female carriers up to 70% of oocytes are unbalanced (Conn *et al.* 1999, Escudero *et al.* 2000). In male Roberstonian translocation carriers 10-20% sperm are unbalanced (Ogur *et al.* 2006), while in female carriers up to 42% of oocytes may be unbalanced (Munne *et al.* 2000), Additionally, male chromosome rearrangement carriers often present with reduced sperm counts (Mayeur *et al.* 2019). As such karyotyping now forms a routine part of

pre-treatment work up for couples presenting with implantation failure, recurrent pregnancy loss, and severe oligospermia. Couples where any type of chromosome abnormality is identified will be referred for genetic counselling, and those with balanced structural rearrangements will be given information on PGT-SR (see section 1.1.2). As described in the general introduction, PGT-SR allows the identification of euploid or balanced embryos for use in treatment. The aim is to increase the chances of implantation and livebirth, while decreasing the risk of miscarriage and time to pregnancy and livebirth.

PGT-SR was first performed in 1998 (Munne *et al.* 1998) using fluorescent in situ hybridisation (FISH) to test polar bodies for maternally inherited translocations. Subsequently, testing was performed on single blastomeres from cleavage stage embryos to allow the inclusion of testing for paternal carriers also (Munne *et al.* 2000b). High percentages of unbalanced embryos, variable clinical outcomes, and a continued risk of miscarriage led some to suggest that PGT-SR offered negligible advantages to carriers of chromosome rearrangements over natural conception, which in some studies was more expedient in terms of achieving a healthy livebirth (Scriven *et al.* 2013, Murugappan *et al.* 2015). Technical limitations of FISH when applied to single cell analysis impacted clinical outcomes, with concerns relating to issues with cell fixation, signal splitting, failed hybridisation, and the inability to examine additional chromosomes. Nonetheless, the number of cycles analysed by FISH have ranged from 149 to 4253 in individual studies. The advent of comprehensive chromosome screening (CCS) allowed the additional detection of aneuploidy in chromosomes not involved in the chromosome rearrangement, and has demonstrated that many embryos selected for clinical use after PGT-SR via FISH were aneuploid (Treff *et al.*

2010). Clinical pregnancy rates in PGT-SR via CCS are equivalent to PGT-A outcomes, with studies reporting clinical pregnancy rates up to 70% per embryo transfer (Morin *et al.* 2016). Indeed, since CCS, pregnancy rates have gone from 40% (when using FISH) to 70% when using CCS.

6.2.1 Comprehensive Chromosome Screening (CCS)

Comprehensive chromosome screening refers to methodologies providing information on the status of all 22 autosomes and both sex chromosomes. In terms of PGT-SR, this also normally means that a lengthy workup is not required prior to treatment, as was the case with FISH analysis. PGT-SR studies have been published using technologies including polymerase chain (PCR) based short tandem repeats (STR) (Fiorentino *et al.* 2010), SNP arrays (Treff *et al.* 2011), array based comparative genomic hybridisation (aCGH) (Alfarawati *et al.* 2011, Fiorentino *et al.* 2011), and most recently next generation sequencing (NGS) (Tan *et al.* 2014, Zhang *et al.* 2016). In addition to the advantages of comprehensive chromosome screening, these molecular cytogenetic technologies also involve computational and objective data analysis and are adaptable to automation and scaling up processing, increasing laboratory throughput and reducing the risk of errors. Number of cases performed in previous studies range from 32 to 266 for SNP arrays, from 17 to 50 for aCGH and from 21 to 129 for NGS. They are still therefore small and of limited use.

An important consideration in PGT-SR is the sensitivity of the testing platform used in terms of being able to detect the smallest imbalance that may result from an unbalanced form of the chromosome rearrangement. Validation of SNP arrays for PGT-SR demonstrated identification of segmental changes of 13.8 Mb (Treff *et al.*

2011), and initial NGS studies demonstrated similar capabilities (Fiorentino *et al.* 2014). Currently, NGS platforms and data pipelines are available allowing identification of segmental changes as small as 5 Mb (Bono *et al.* 2015, Blanca *et al.* 2018).

The major limitation of most CCS molecular cytogenetic techniques is the inability to reliably detect unbalanced derivatives from rearrangements that have breakpoints in the telomere or subtelomere (Morin *et al.* 2016). While FISH does provide coverage of these regions, the limitations of this approach remain as previously described and misdiagnoses have been reported (Van Echten-Arends *et al.* 2013). It should be noted that no misdiagnosis have been reported or published relating to NGS or SNP arrays . Before any case is accepted for PGT-SR it is important that the testing laboratory is able to examine breakpoints and the size of all potential imbalances to ensure this will be detectable by the platform in use.

6.2.2 Biopsy stage

In tandem with the advent of CCS technologies, improvements in embryo culture systems enabled most clinics to move towards biopsy at the blastocyst stage. Studies have demonstrated that this also has an impact on the proportion of euploid embryos. When cleavage stage biopsy has been employed for PGT-SR in cases of reciprocal translocations, the proportion of unbalanced embryos has been up to 82%, while studies involving trophectoderm biopsy and analysis see around 60% unbalanced embryos (Morin *et al.* 2016). Treff et al. identified a significantly higher proportion of unbalanced derivative in embryos which had arrested prior to blastulation, suggesting

some imbalances will also negatively impact very early embryo development (Treff *et al.* 2011).

6.2.3 Specific aims of study

With the above in mind, the purpose of this chapter was to collate 5yrs of PGT-SR data to understand how this compares to general population estimates on the frequency of specific structural chromosome rearrangements. Specifically to:

a. Provide the biggest dataset to date on PGT-SR outcomes using CCS to date
b. Test the hypothesis that the level and nature of structural chromosomal rearrangements is correlated to age, sex and day of biopsy.
c. Test the hypothesis that an interchromosomal effect (the hypothesis that parental chromosome differences, such as translocations or inversions, may increase the frequency of meiotic chromosome nondisjunction) exists in this dataset
d. Provide a set of practical guidelines for genetic counsellors to advise patients on their likelihood of having euploid embryos for transfer based on the type of chromosome rearrangement, sex of the carrier of the rearrangement, maternal age, and any other factors that may be implicated

6.3 Materials and Methods

Data was collected from PGT-SR cycles performed at the Nottingham and London CooperGenomics laboratories between April 2015 and March 2020. All cases involved analysis of trophectoderm samples via NGS, where the guidance given to clinics is to biopsy 5-10 cells.

Biopsy samples are tubed using biopsy kits provided by CooperGenomics, which include 0.2ml DNA/RNA free PCR tubes and PBS based buffer. Trophectoderm samples underwent whole genome amplification (WGA) (SurePlex, Illumina) according to the genetic testing laboratory's standard operating procedure and DNA amplification was assessed by gel electrophoresis.

Next generation sequencing was performed, using the Illumina Veriseq protocol, on biopsy samples displaying positive DNA amplification. Standard dilutions of WGA products underwent tagmentation, amplification and indexing, and cleanup, before undergoing library normalisation to equalise the concentration for multiplexing . The samples were then pooled and loaded on the sequencer. Sequencing data was either processed via Bluefuse software and analysed manually, or via an in-house validated data pipeline with automated calling via an in-house validated algorithm.

For the purposes of this study, embryos were classified as Euploid/Balanced, Euploid/Unbalanced, Aneuploid/Balanced, Aneuploid/Unbalanced, Mosaic/Balanced, or Mosaic/Unbalanced, to reflect the status of the embryo with respect to the chromosome rearrangement (balanced or unbalanced) and also in terms of aneuploidy in chromosomes not involved in the rearrangement (euploid, aneuploid or mosaic). Mosaic data was not reported in all cases due to clinic or patients' wishes. Where mosaicism was not reported, for ethical reasons we have used the data reported rather than perform a reanalysis. Day of biopsy – day 5, 6 or 7 – was assigned where possible. For the purposes of comparing day of biopsy, only cycles including samples biopsied over multiple days were included to remove bias of laboratory workflow practices.

Analysis was undertaken to compare the data generated by the different types of structural chromosome rearrangement. For each separate indication, data was analysed according to the sex of the carrier, maternal age, and day of biopsy. Paracentric and pericentric inversions were also compared. The proportion of incidental aneuploidy – aneuploidy in chromosomes not involved in the rearrangement – was examined by rearrangement type for evidence of inter chromosomal effect (ICE), as was the incidence of mosaicism. The number of cycles with at least one embryo available for transfer was also calculated in each group and sub-group.
Additionally, a small proportion of couples opted to undergo Karyomapping of euploid/balanced embryos to exclude uniparental disomy (UPD). This data is also presented and discussed.

The two tailed Fishers exact test was used for statistical analysis due to the variation and smaller number of cycles and samples in some groups. Statistical significance was reached when the p value was less than 0.05. Statistical analysis was not applied to the insertion, complex rearrangement, or dicentric translocation group due to small cycle and sample numbers.

6.4 Results

6.4.1 PGT-SR indications and list of parental karyotypes

A total of 479 PGT-SR cycles were included, involving the analysis of 1814 trophectoderm samples. Reciprocal and Robertsonian translocations comprised 92% of all cycles. Reciprocal translocations were in the majority, accounting for 67.4% of cycles, while Robertsonian translocations accounted for 25.1% (Figure 6.1). The

proportion of Robertsonian translocations compared to reciprocal translocations is lower than expected based on estimates of population incidence, which may be due to the lower risk of unbalanced gametes associated with these rearrangements.

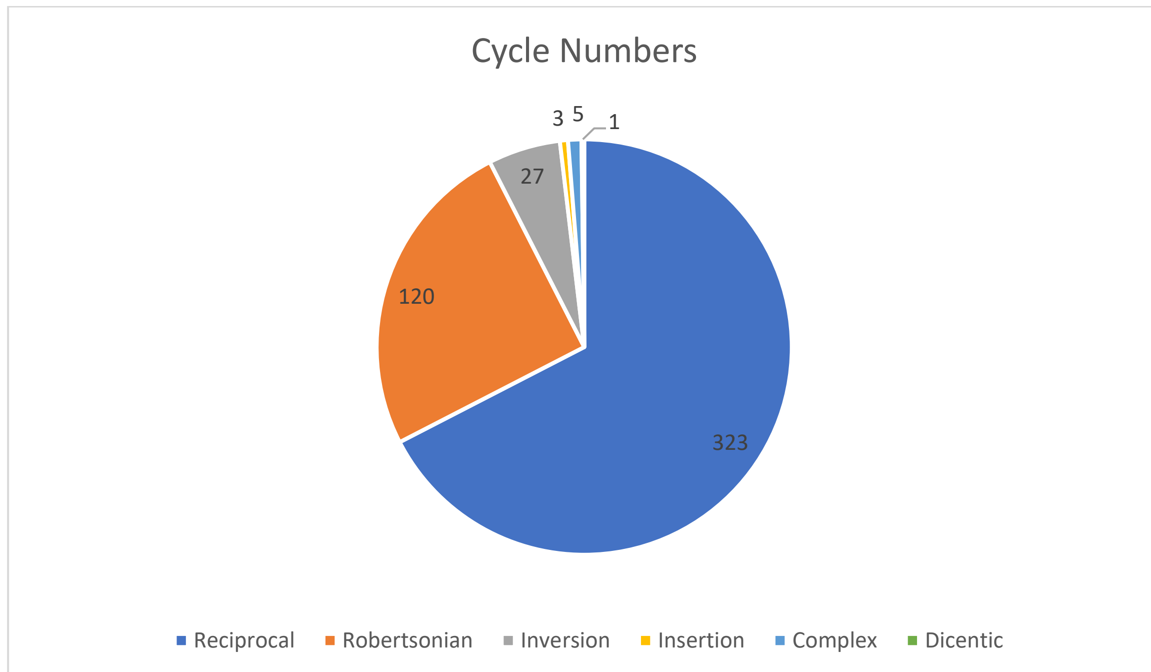

Figure 6.1. Chart illustrating the proportion of cycles by type of structural chromosome rearrangement

The same pattern was seen when looking at sample numbers, with reciprocal translocations accounting for 69.7% of samples and Robertsonian translocations accounting for 23.3% samples (Figure 6.2). The proportions of cycles:samples was comparable for the other indications also; inversions, insertions, complex rearrangements, and dicentric translocations. This indicates that none of the rearrangement types are exerting an undue influence on embryo development or blastocyst formation.

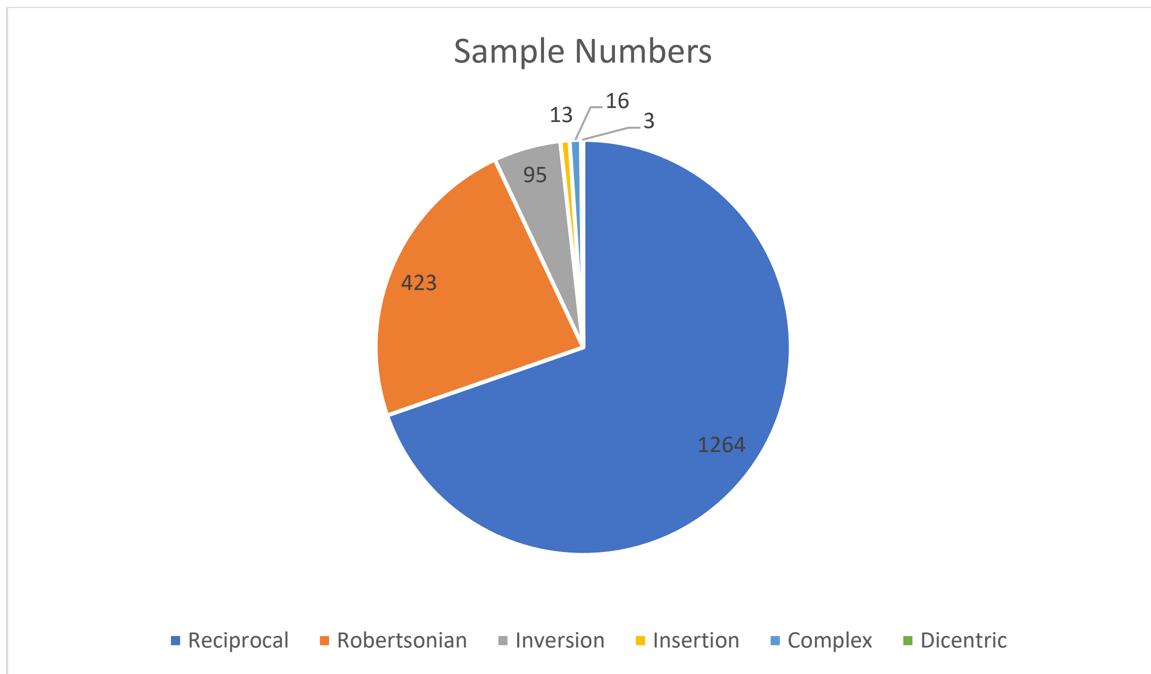

Figure 6.2. Chart illustrating the proportion of samples by type of structural chromosome rearrangement

The average and median maternal ages were the same for the reciprocal and Robertsonian translocation groups (Table 6.1). Average and median maternal ages were higher in the inversion group, possibly only being identified at a later stage given the low proportion of unbalanced samples. The cycle numbers are too low in the insertion, complex rearrangement, and dicentric translocation groups to draw any conclusions with respect to maternal age. However, the lower maternal age observed in the complex rearrangement group may be accurate; given the low percentage of cycles reaching transfer it could be expected that these rearrangements would be identified early.

	RECIPROCAL		ROBERTSONIAN		INVERSION		INSERTION		COMPLEX		DICENTRIC	
Average mat age	34.4		34.4		37.3		31.3		31.9		41.0	
Median mat age	35		35		39		32		32		41	
Cycle no.	323	67.4%	120	25.1%	27	5.6%	3	0.6%	5	1.2%	1	0.2%
Sample no.	1264	69.7%	423	23.3%	95	5.2%	13	0.7%	16	0.9%	3	0.2%
Euploid/Balanced	278	22.0%	139	32.9%	31	32.6%	2	15.4%	1	6.3%	0	0.0%
Unbalanced	726	57.4%	127	30.0%	6	6.3%	9	69.2%	9	56.3%	1	33.3%
Aneuploid	986	78.0%	284	67.1%	64	67.4%	11	84.6%	15	93.8%	3	100%
Aneuploid (excl unbalanced only)	643	50.1%	238	56.3%	63	66.3%	6	46.2%	12	75.0%	3	100%
Cycles with embryos for ET	178	55.1%	81	67.5%	16	59.3%	2	15.4%	1	6.3%	0	0.%

Table 6.1. Overview of chromosome rearrangement types and NGS results

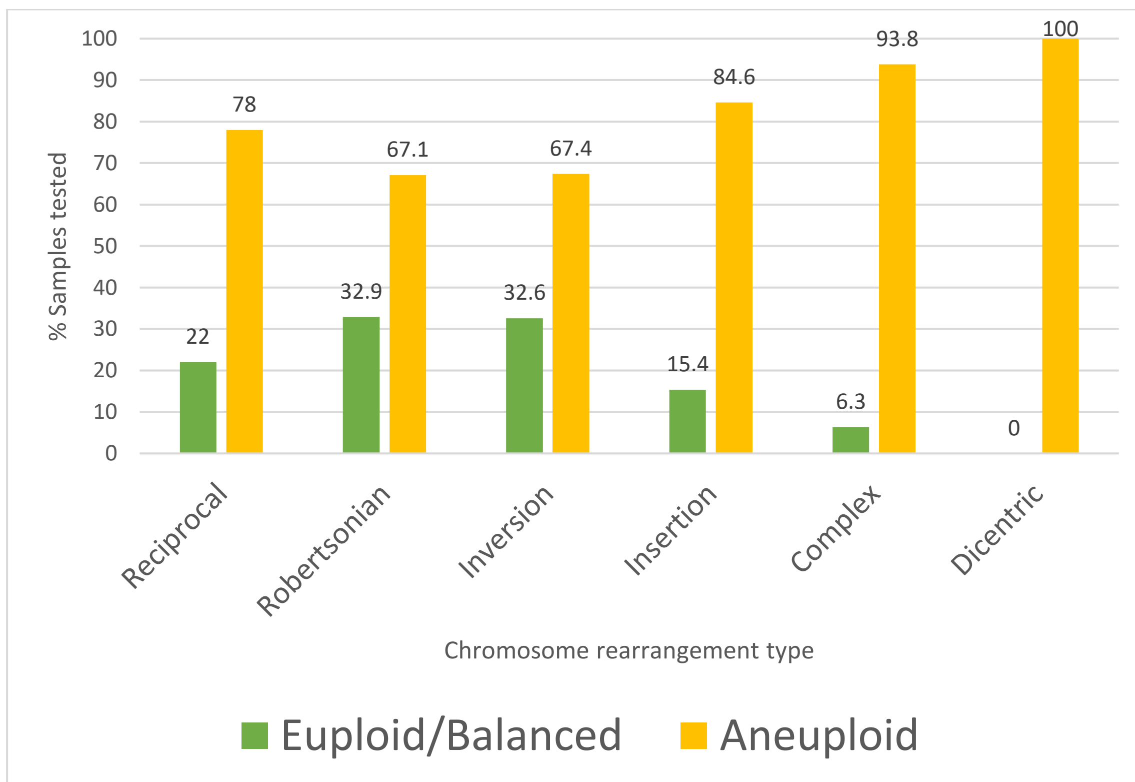

Figure 6.3. Graph illustrating proportion of normal vs abnormal embryos. For simplicity, any imbalance is referred to as “aneuploid.”

In comparing the data from the different indications, the proportion of Euploid/Balanced samples for reciprocal translocations was significantly lower euploid than the Robertsonian group (P<0.0001) and inversion group (p=0.0219). Correspondingly, looking at the proportion of unbalanced embryos, this was significantly higher in the reciprocal group than the Robertsonian group (p<0.0001), and significantly higher in the Robertsonian group than the inversion group (p<0.0001) (Figure 7.3 and Table 7.1).

There was no significant difference in the rate of incidental (unrelated to the chromosome rearrangement) aneuploidy between the reciprocal and Robertsonian group or between the Robertsonian group and the inversion group. However, the difference in incidental aneuploidy between the reciprocal group and the inversion group was significant (p=0.0039), with the inversion group having a higher proportion of aneuploid embryos, excluding unbalanced chromosome derivatives. This could be due to the higher average and median maternal in the inversion group as opposed to evidence of inter chromosomal effect.

The Robertsonian group had a significantly higher chance than the reciprocal group of having at least one embryo available for transfer (p=0.0225) but there was no statistical difference when comparing the reciprocal and the inversion group or the Robertsonian and the inversion group. Again, the lower proportion of cycles with at least one embryo for transfer in the inversion group could be a result of the increased maternal age and resultant increased incidental aneuploidy observed.

Finally, for each abnormality, the proportion of cases that led to an embryo transfer is given by rearrangement type (Figure 7.4). Over 50% of cases led to an embryo transfer for reciprocal translocations (55%), Robertsonian translocations (68%) and inversions (59%), whereas very few led to embryo transfer for insertions (15%), complex rearrangements (6%) and dicentrics (0%).

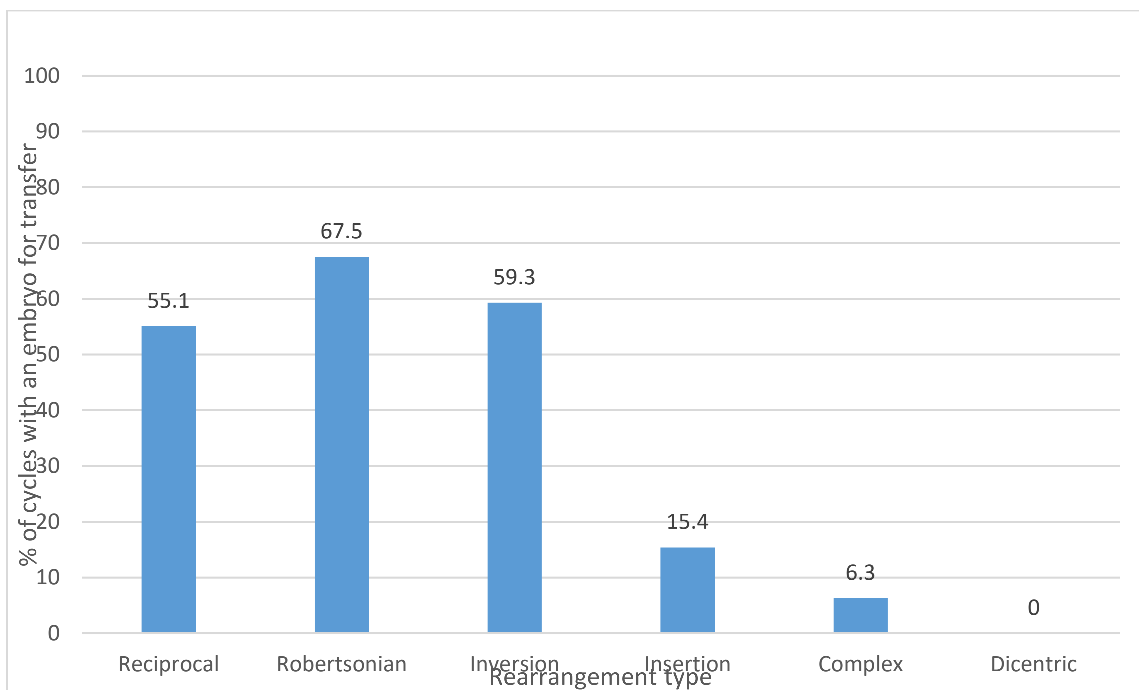

Figure 6.4 Graph illustrating proportion of PGT-SR cases that led to an embryo transfer, broken down by rearrangement type.

6.4.1.1. Reciprocal translocations

(1;10) (p36.1q24)	(11;22)(q23;q11.2)	(3;7)(p26.3;q31.33)
(1;10)(p13.3;p13)	(12;13)(q12;q22)	(3;8) (p21.1;p21.1)
(1;12)(q42.1;q21.2)	(12;15)(p11.2;p13)	(3;9)(q13.2;q21.2)
(1;12)(q42.1;q24.11)	(12;18) (p11.2;q21.1	(3;9)(q25.3;q13)
(1;14)(p36.22;q32.1)	(12;18) (q13.3;q21.1)	(4:17)(q25:q21.3)

(1;14)(q42.3;q31)	(12;20) (p12.2;p13)	(4;11) (q28.2;q23.3)
(1;16) (q43;q22.1)	(12;20) (q?12;p11.2)	(4;11)(q25;q11)
(1;16)(q10;q10)	(12;21)(p10;q10)	(4;11)(q27;q24)
(1;16)(q21;q22)	(13;18)(p10;p10)	(4;11)(q35.1;q23.3)
(1;19)(p10;q10)	(13;18)(q14.1;q23)	(4;12) (q31.1;q24.1)
(1;2) (p32;q33)	(14;16)(q13;q23)	(4;13)(p14;q34)
(1;2)(q32;p16)	(14;20) (q11.2;p11.21)	(4;14) (p15.1;p11.2)
(1;2)(q42.3;q13)	(15;17)(q11.2;p12)	(4;14) (q31.3;q32.1)
(1;20) (p34.1;q13.1)	(16;19)(p11.2;q13.1)	(4;14)(p15.1;p11.2)
(1;20)(p36.22;p11.21)	(16;22) (p13.3;q13.1)	(4;16)(q22.3;q22.3)
(1;20)(q21;p11.2)	(18;21) (p11.21;p11.2)	(4;17) (q27;q23.1)
(1;21)(q31.2;p12)	(2:17) (q21.1:q23.1)	(4;18) (q31.23;q21.2)
(1;22) (q42;q11.2)	(2;12) (p25.3;q23.3)	(4;20)(q13.2-p11.2)
(1;4) (p32.3;q33)	(2;14) (p24.2;q24.3)	(4;20)(q32;p12)
(1;4) (q12;q31.3)	(2;15) (p24.2;q26.1)	(4;5) (p16.1;q22)
(1;4) (q23.1;q31.3)	(2;15) (q33;q15)	(4;5) (q13;p15)
(1;4) (q24;q28)	(2;15)(q34;q15)	(1;6)(p13.3;q22)
(1;4) (q41;p15.32)	(2;16)(p25.3;q21)	(1;6)(q32.1;q25.2)
(1;4)(p36.21;p16.3)	(2;18) (q22;q21.3)	(1;9)(p10;q10)
(1;4)(q31;p12)	(2;20) (q13;p13)	(1;9)(p34.3;q22)
(1;5)(p36.1;q31.3)	(2;21)(q21.3;q21.1)	(10;13) (p12.2;q14.3)
(1;6) (q32;q25)	(2;22)(p24;q12)	(10;18)(q11.2;q21.1)
(10;20)(q26.1;p11.2)	(2;3)(p11.2;q27.3)	(10;20)(q26.1;p11.2)
(10;21) (p12.33;q21.2)	(2;4)(q21.3;p15.3)	(10;21) (p12.33;q21.2)
(11;12)(q13.2;q24.32)	(2;5)(q37.1;p15.1)	(11;12)(q13.2;q24.32)
(11;14)(q23;q24.1)	(2;6)(q14;p23)	(11;14)(q23;q24.1)
(11;15)(q24.1;p11.2)	(2;7) (p25.1;q32)	(11;15)(q24.1;p11.2)

(11;20)(q14;p12)

(11;21)(p11.2;p11.2)

(11;22) (q23.3;q11.2)

(11;22)(q23;q11)

(3;5) (q13.3;q35.3)

(3;5)(p13;q14)

(3;6)(p24.2;q11.2)

(3;7)(p26.1;q32.3)

(4;5)(p15;p13)

(4;5)(q27;q33)

(4;6)(q34.2;q24.3)

(4;7)(q25;p15)

(4;8)(p14;p21.3)

(4;8)(p15.1;q24.12)

(4;8)(q21. 1;p23.1)

(4;8)(q33;q21.2)

(4;9) (q32.3;q34.3)

(4;9)(q31.1;q21)

(5;10) (q34;p12.3)

(5;18)(p15.?2;q21.?2)

(5;20)(q23.1;q13.2)

(5;20)(q33.1-p11.2)

(5;6)(p15.1;p22.2)

(5;7) (q35.1;q32)

(5;7)(q23;q36)

(5;8)(p13;q13)

(9;14)(p24;q32.3)

(2;7) (q35; q31.3)

(2;7)(p25.1;q32)

(2;8)(p21;q22.1)

(2;8)(p24;p21)

(2;8)(q31; q21.2)

(3;11)(p21.3;q13.1)

(3;13)(p26.2;q12.3)

(3;14)(p25;q24)

(5;9)(q33.1;p13)

(6;10)(p21.1;q24.1)

(6;11)(p24;q24.2)

(6;12)(q23;p13)

(6;14) (q13;q13)

(6;14)(q23.3;q11.2)

(6;17) (p22.2;q22)

(6;17)(p21,2;q12)

(6;20)(q21;q12)

(6;20)(q27;q11.2)

(6;21) (q13;p12)

(6;22) (p23;q11.21)

(6;22)(p11.2;q13.31)

(6;22)(q12;q11.21)

(6;8)(q21.3;p23.1)

(6;9) (p23;q34)

(7;11) (q33;p15.1)

(7;11)(p13;p15.1)

(7;8)(p1;:q22.3)

(11;20)(q14;p12)

(11;21)(p11.2;p11.2)

(11;22) (q23.3;q11.2)

(11;22)(q23;q11)

(3;4)(p26;q28.2)

(3;4)(q26.2;q33)

(3;4)(q26.2;q35)

(3;17)(q26.2;q11.2)[12].46.XY[88]

(8;12) (p21.1;p13.3)

(8;12)(p11.2;q22)

(8;12)(p23.1;p13.1)

(8;14) (p21.3;q23)

(8;15) (q24.2;q24)

(8;15)(q21.2;p11.2)

(8;16)(p23.3;q11.2)

(8;16)(q22.3;q23.2)

(8;17)(p21;q24)

(9;10)(q22.?3;q26.?3)

(9;10)(q22.3;q26.1)

(9;11)(p22;q23.1)

(9;11)(q34.1)(q13.1)

(9;14)(p13;q11.2)

(7;13) (q32;q32)

(7;13)(p11.2;q12.1)

(7;13)(q32;q21.2)

(7;16)(p13;q11.2)

(9;20)(q34.2;q11.1)

(9;15)(q22;q21)	(7;8)(p13;q22.3)	(9;20)(q34.2;q11.1)
(9;15)(q32;q11.2)	(8;11) (q21.3;q24.3)	(X;11)(q26;q25)
(9;18)(q13;p11.2)	(8;11)(q24.1;q23.3)	(X;14)(q26.1;q32.3)
(9;18)(q13;p11.2)	(8;12) (p11.2;q22)	(Y;4)(q12;p15.32)

6.4.1.2 Inversions

(1) (q31q41)	(12)(p12/2p13)	(19)(p11/2q11/1)
(10)(q24q26)	(12)(q13-q24/1)	(3)(p21/3q23)
(11) (p13p15/3)	(17)(q23/1q25/1)	(6) (p22/2q23/3)
(12)(p11/2;q13)	(18)(p11/21q11/2)	inv(1)(q21/1q25/3)

inv(1)(p34/1q42)(19)/46,XY(14)

6.4.1.3 Insertions

(2;3)(q13;p24/3p25/3)

(9)(p24/1q21/2q22)

(12) (q15p11/23p12/3)

6.4.1.4 Complex rearrangements

(1;11;13)(q31;p14;q22),ins(2) (q23/3q32/2q35)

(7;13;9) (p22;q32;q21)

t(9;21)(p22;q21/2)ins(9;18)(p22/3;q21/1q23)t(9;18)(p22/3;q21/1))t(18;21)(q23;q21/2)

6.4.1.5 Dicentric translocation

Mos

45,XX,dic,(18;22)(p11/3;p11/2)[18]/46,XX[12]

6.4.2 Reciprocal translocations and chromosome size

A general trend can be seen associating chromosome size with frequency of involvement in reciprocal translocations, however certain chromosomes do not follow this pattern (Figure 6.5). Chromosome 4 was the chromosome most commonly implicated in reciprocal translocations, present in 10.5% of the couples, followed by chromosome 1 at 8.6%. The sex chromosomes may be under represented, given that sex-autosome translocations can have serious implications in terms of ovarian function and spermatogenesis (Gardner *et al.* 2012) or have been rejected for PGT-SR on the basis of inability to detect imbalances. Excluding the sex chromosomes, chromosome 19 was involved in the lowest number of reciprocal translocations, at only 0.6%. Given that palindromic AT rich sequences have been associated with breakpoint hot spots (Kurahasi *et al.* 2010), the high GC content on chromosome 19 may be producing the opposite effect (Piovesan *et al.* 2019) (Figure 6.5)

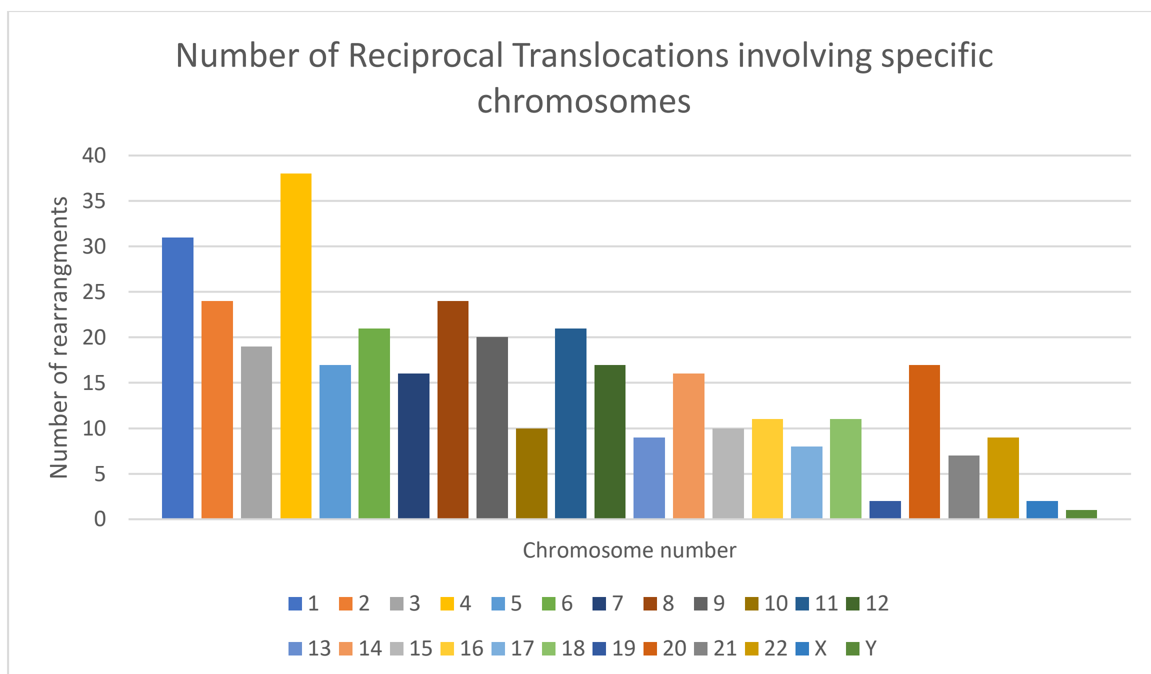

Figure 6.5. Graph illustrating the number of reciprocal translocations each chromosome was involved in.

Recombination hot spots at 11q23, 17q11, and 22q11 have been previously reported as being involved in recurrent translocations t(11;22) and t(17;22) (Gardner *et al.* 2012). However, we only observed three t(11;22) in this data group, and no t(17;22). Excluding the recurrent translocations, the 11q23 breakpoint was observed in 4 reciprocal translocations, the 17q11 breakpoint in 1 translocation, and the 22q11 breakpoint in 6 translocations. These frequencies may suggest that the reciprocal translocations which recur and are more prevalent have a lower impact on fertility.

6.4.3 Reciprocal translocations, sex and biopsy day

No significant difference was observed in outcomes between maternal and paternal reciprocal translocation carriers in terms of the proportion of euploid/balanced embryos, the proportion of unbalanced embryos, the rate of incidental aneuploidy, or the proportion of cycles with at least one embryo for transfer. This indicates that the sex of the carrier has no impact on outcomes, and while there is greater stringency of cell cycle checkpoint mechanisms in sperm, this may only apply to whole chromosome aneuploidy and not segmental changes (Table 6.2).

Cycles including samples taken on both day 5 and day 6 showed no differences in terms of the proportion of unbalanced samples. However, a significantly lower proportion of euploid/balanced embryos (p=0.0231) and a higher rate of incidental aneuploidy (p=0.0202) were seen in the day 6 samples. Given this, there was also a significantly lower proportion of cycles with at least one day 6 embryo available for transfer (p=0.0093) (Table 6.2)

	RECIPROCAL		MATERNAL		PATERNAL		DAY 5 BX		DAY 6 BX	
Average mat age	34.4		34.8		34.0		34.1			
Median mat age	35		35		35		35			
Cycle no.	323		155	48.0%	168	52.0%	124			
Sample no.	1264		577	45.6%	687	54.4%	392	60.0%	261	40%
Euploid/Balanced	278	22.0%	113	19.6%	165	24.0%	92	23.5%	42	16.1%
Unbalanced	726	57.4%	348	60.3%	378	55.0%	220	56.1%	155	59.4%
Aneuploid	986	78.0%	464	80.4%	522	76.0%	300	76.5%	219	83.9%
Aneuploid (excl unbalanced only)	643	50.1%	301	52.2%	342	49.8%	194	49.5%	154	59.0%
Cycles with embryo(s) for ET	178	55.1%	79	51.0%	98	58.3%	60	48.4%	39	31.5%

Table 6.2. Overview of NGS results in the reciprocal group broken down by the sex of the translocation carrier and by day of biopsy.

6.4.4 Reciprocal translocations and age

Comparing maternal age, cycles with increased maternal age had a significantly lower proportion of euploid/balanced embryos (<35/≥35 p=0.004, <37/≥37 p<0.0001), a higher rate of incidental aneuploidy (<35/≥35 p<0.0001, <37/≥37 p<0.0001), and a lower proportion of cycles with at least one embryo for transfer (<35/≥35 p<0.0001, <37/≥37 p=0.0002). However, maternal age had no impact on the proportion of unbalanced embryos observed (Table 6.3).

	RECIPROCAL		MAT <35		MAT <37		MAT ≥35		MAT ≥37	
Cycle no.	323		158	48.9%	214	66.3%	165	51.1%	109	33.7%
Sample no.	1264		695	55.0%	889	70.3%	569	45.0%	375	29.7%
Euploid/Balanced	278	22.0 %	179	25.8%	221	24.9%	99	17.4%	57	15.2%
Unbalanced	726	57.4 %	407	58.6%	507	57.0%	319	56.1%	219	58.4%
Aneuploid	986	78.0 %	516	74.2%	668	75.1%	470	82.6%	318	84.8%
Aneuploid (excl unbalanced only)	643	50.1 %	314	45.2%	413	46.5%	329	57.8%	230	61.3%
Cycles with embryos for ET	178	55.1 %	106	67.1%	133	62.1%	72	43.6%	44	40.4%

Table 6.3 Overview of NGS results in the reciprocal group broken down by maternal age.

6.4.5 Reciprocal translocations involving acrocentric chromosomes 13, 14, 15, 21 and 22

The acrocentric chromosomes refer to the five autosomes – 13. 14, 15, 21, and 22 – where the centromere is offset from the centre, resulting in a very short p arm. The p arm chromatin includes the nucleolar organising regions (NORs), comprising genes encoding ribosomal RNA, which may be active or inactive. The loss of NORs in the formation or Robertsonian translocations (discussed in 6.4.3) does not impact on normal cellular function (Gardner *et al.* 2012).

Unlike the analysis of all reciprocal translocations (Figure 6.5), there appears to be no pattern in terms of the association of non-acrocentric chromosomes involved in reciprocal translocations with acrocentric chromosomes (Figure 6.6). Notably, chromosomes 5, 19, and 20 were not involved in any reciprocal translocations with non-acrocentric chromosomes. Chromosome 19 also had a low involvement in all reciprocal translocations, but chromosome 5 had an expected frequency based on chromosome length, and chromosome 20 appeared to be overrepresented in the group. Conversely, the involvement of the acrocentric chromosomes appears at similar rates (Figure 6.7).

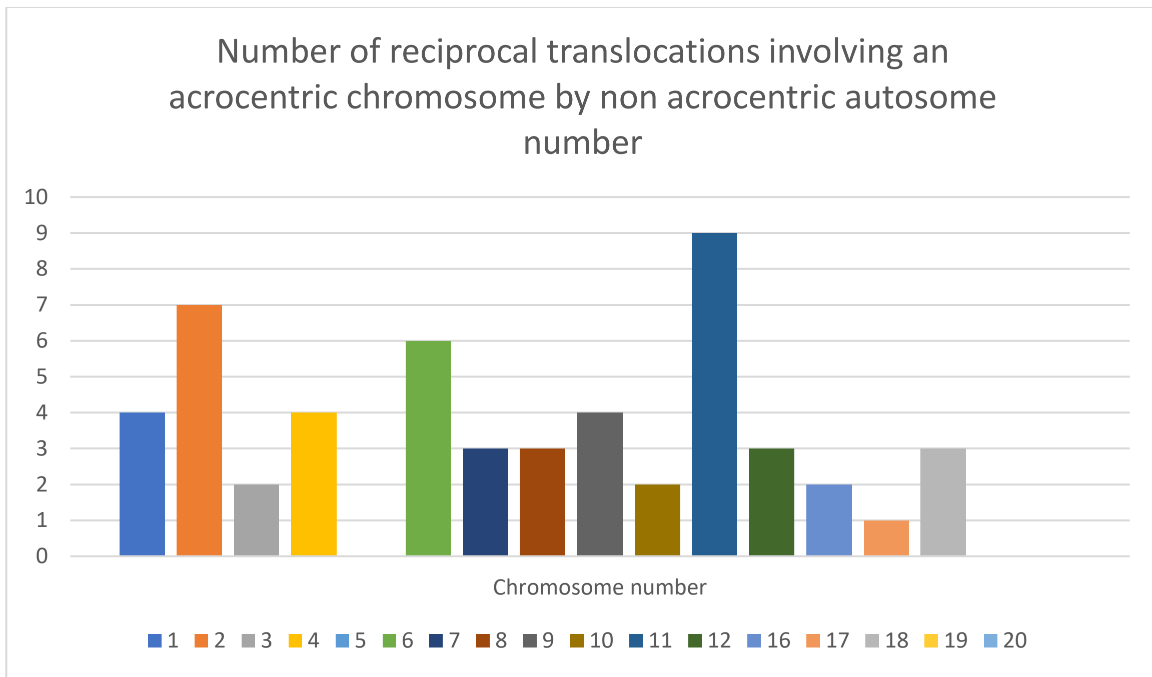

Figure 6.6 Graph illustrating the number of non acrocentric;acrocentric reciprocal translocation in which each non acrocentric chromosome was involved.

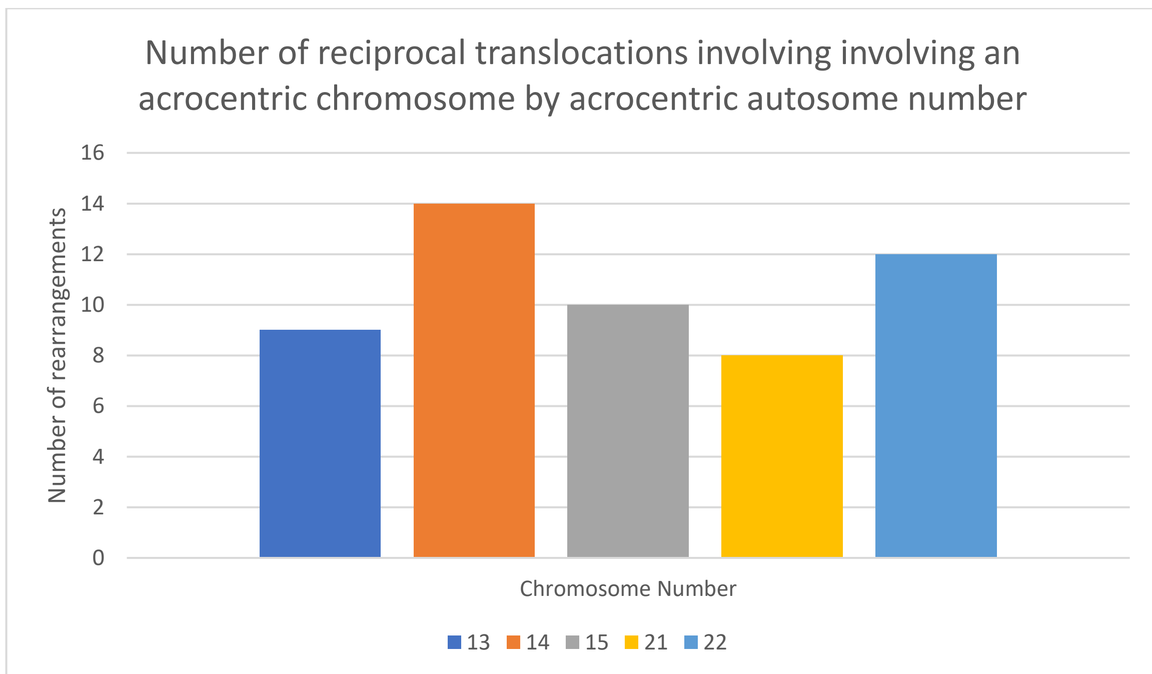

Figure 6.7. Graph illustrating the number of non acrocentric;acrocentric reciprocal translocation in which each acrocentric chromosome was involved.

	RECIPROCAL		RECIPROCAL INVOLVING AUTOSOME & ACROCENTRIC		RECIPROCAL INVOLVING NON ACROCENTRIC AUTOSOMES	
Average mat age	34.4		34.6		34.3	
Median mat age	35		34		35	
Cycle no.	323		104	32.2%	214	66.3%
Sample no.	1264		421	33.3%	825	66.5%
Euploid/Balanced	278	22.0%	90	21.4%	185	22.4%
Unbalanced	726	57.4%	259	61.5%	457	55.4%
Aneuploid	986	78.0%	331	78.6%	640	77.5%
Aneuploid (excl unbalanced only)	643	50.1%	200	47.5%	429	52.0%
Cycles with embryo(s) for ET	178	55.1%	55	52.9%	120	56.1%

Table 6.4 Overview of NGS results in the reciprocal group involving autosomes with and without the involvement of an acrocentric chromosome

The proportions of cycles:samples was comparable for the reciprocal translocations involving autosomes regardless of whether an acrocentric was involved or not, indicating no undue influence on embryo development or blastocyst formation. There were no significant differences between the reciprocal groups involving autosomes with and without the involvement of an acrocentric chromosome in terms of the proportion of euploid/balanced embryos (p=0.7182), the rate of incidental aneuploidy (p=0.1349) or the proportion of cycles with at least one embryo available for transfer (p=0.6315). The significantly higher proportion of unbalanced embryos in the group involving an acrocentric chromosome (p=0.0396) does not impact on the proportion of euploid/balanced embryos or the likelihood of reaching embryo transfer, but the more severe asymmetry of the quadrivalent impacts the segregation pattern (Zhang *et al.* 2014) (Table 6.4).

7.4.6 Robertsonian translocations

Robertsonian translocations can be heterologous (involving two different acrocentric chromosomes) or homologous (involving the fusion of two of the same chromosome). Heterologous Robertsonian translocations account for 90% of Robertsonian translocations in the general population (Therman *et al.* 1989). Homologous Robertsonian translocations can only result in either disomic or nullisomic gametes (Gardner *et al.* 2012) and so carriers are not suitable candidates for PGT-SR and not represented in this data group.

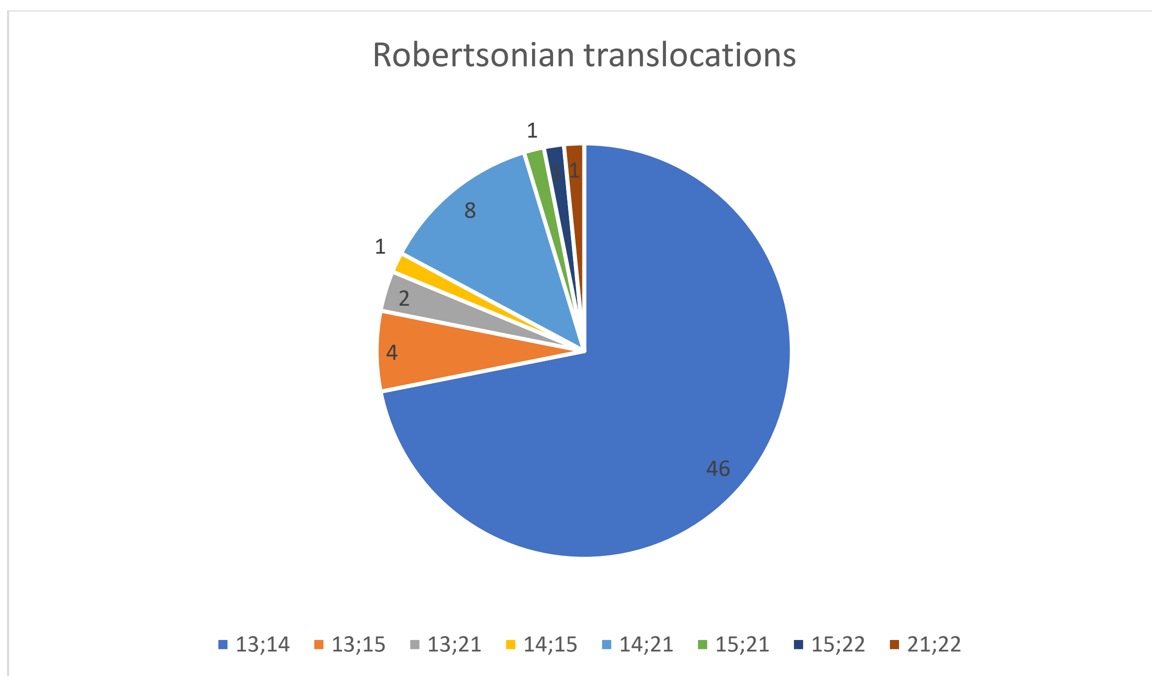

Figure 6.8 Chart showing proportion of cycles for each Robertsonian translocation

Translocation	Literature Review	Unbiased ascertainment	PGT-SR data set
rob(13;13)	3%	2%	-
rob(13;14)	33%	74%	71.9%
rob(13;15)	2%	2%	6.3%
rob(13;21)	2%	1%	3.1%
rob(13;22)	1%	2%	0.0%
rob(14;14)	0.5%	-	-
rob(14;15)	2%	5%	1.6%
rob(14;21)	30%	8%	12.5%
rob(14;22)	1%	2%	0.0%
rob(15;15)	2%	-	-
rob(15;21)	3%	0.5%	1.6%
rob(15;22)	0.5%	1%	1.6%
rob(21;21)	17%	3%	-
rob(21;22)	2%	0.5%	1.6%
rob(22;22)	1%	-	-

Table 6.5 Table comparing incidence of Robertsonian translocations in the PGT-SR group compared with relative population frequencies. Adapted from Gardner *et al.* 2012.

Literature review – Relative frequencies of Robertsonian translocations in studies with potential biased ascertainment.

Unbiased ascertainment – Relative frequencies of Robertsonian translocations in studies with unbiased ascertainment.

PGT-SR data set – Relative frequencies of Robertsonian translocation as observed in patients undergoing PGT.

While, in theory, a Robertsonian translocation can form between any two of the five pairs of acrocentric chromosomes, the rob(13;14) translocation accounts for 75% of cases in the general population, the rob(14;21) 8% of cases (Therman *et al.* 1989). In this data set rob(13;14) accounted for 71.9% of cases and rob(14;21) accounted for 12.5% cases. There was equal distribution in terms of the sex of the carrier for both these translocations (Figure 7.8, Table 7.5).

	ROBERTSONIAN		MATERNAL		PATERNAL		DAY 5 BX		DAY 6 BX	
Average mat age	34.4		35.7		33.6		34.2			
Median mat age	35		36		35		36			
Cycle no.	120		47	39.2%	73	60.8%	43			
Sample no.	423		159	37.6%	264	62.4%	120	62.2%	73	37.8%
Euploid/Balanced	139	32.9%	47	29.6%	92	34.8%	38	31.7%	30	41.1%
Unbalanced	127	30.0%	67	42.1%	60	22.7%	36	30.0%	19	26.0%
Aneuploid	284	67.1%	112	70.4%	172	65.2%	82	68.3%	43	58.9%
Aneuploid (excl unbalanced only)	238	56.3%	89	56.0%	149	56.4%	67	55.8%	37	50.7%
Cycles with embryos for ET	81	67.5%	29	61.7%	50	68.5%	25	58.1%	22	51.2%

Table 6.6. Overview of NGS results in the Robertsonian group broken down by the sex of the translocation carrier and by day of biopsy.

There was a significantly higher proportion of unbalanced samples resulting from maternal carriers ($p<0.0001$). However, despite this, there was no significant difference in the proportion of cycles with at least one embryo to transfer when comparing male and female carriers. There was also no difference in terms of the proportion of euploid/balanced embryos, or the rate of incidental aneuploidy (Table 6.6)

Cycles including samples taken on both day 5 and day 6 showed no differences in terms of the proportion of euploid/balanced embryos, the proportion of unbalanced embryos or the rate of incidental aneuploidy. Additionally, there was an equal proportion of cycles with a day 5 embryo suitable for transfer and a day 6 embryo suitable for transfer (Table 6.6).

	ROBERTSONIAN		MAT <35		MAT <37		MAT ≥35		MAT ≥37	
Cycle no.	120		49	40.8%	77	64.2%	71	59.2%	43	35.8%
Sample no.	423		169	40.0%	274	64.8%	250	59.1%	145	34.3%
Euploid/Balanced	139	32.9%	71	42.0%	104	38.0%	67	26.8%	34	23.4%
Unbalanced	127	30.0%	39	23.1%	70	25.5%	87	34.8%	56	38.6%
Aneuploid	284	67.1%	98	58.0%	170	62.0%	183	73.2%	111	76.6%
Aneuploid (excl unbalanced only)	238	56.3%	81	47.9%	142	51.8%	155	62.0%	94	64.8%
Cycles with embryos for ET	81	67.5%	35	71.4%	55	71.4%	43	60.6%	24	55.8%

Table 6.7. Overview of NGS results in the reciprocal group broken down by maternal age

Comparing maternal age, cycles with increased maternal age had a significantly lower proportion of euploid/balanced embryos (<35/≥35 p=0.0015, <37/≥37 p<0.0031), and a higher rate of incidental aneuploidy (<35/≥35 p=0.0050, <37/≥37 p<0.0.0129). While a trend is seen towards a lower proportion of cycles with at least one embryo available for transfer with increasing maternal age, this was not statistically significant. Unexpectedly, increasing maternal age was also associated with an increasing proportion of unbalanced samples (<35/≥35 p=0.0124, <37/≥37 p<0.0071) (Table 6.7). Further analysis is this is a real increase in unbalanced results, or if it is a result of incidental age-related aneuploidy.

6.4.7 Inversions

Chromosome inversions may be paracentric (involving only one arm of the chromosome) or pericentric (including the centromere, with a breakpoint on each arm of the chromosome. Unbalanced paracentric inversions produce gametes that have either no centromere (acentric) or two centromeres (dicentric) and are thus not viable. Several inversions are notes as variants of no clinical significance, including those with breakpoints in the heterochromatic regions of chromosomes 1, 9, 16 and Y (Gardner

RJM *et al.* 2012). Other clinically non-significant variants have been identified as inv(2)(p11.2q13), inv(3)(p11q11), inv(3)(p11q12), inv(3)(p13q12), inv(5)(p13q13) and inv(10)(p11.2q21.2) (Gardner *et al.* 2012). None of these variants were present in the inversion group. This could be due to non-referral for PGT-SR on the basis of being classed as clinically non-significant or could be a true indicator that they have no impact on fertility.

The frequency of both paracentric and pericentric inversions is less than 1% in the general population (Gardner *et al.* 2012) and so are over-represented in this data set, accounting for 5.6% of PGT-SR cases.

	INVERSION		MATERNAL		PATERNAL		PARACENTRIC		PERICENTRIC	
Average mat age	37.3		37.8		36.8		38.3		35.7	
Median mat age	39		39		39		39		35	
Cycle no.	27		15	55.6%	12	44.4%	19	70.4%	8	29.6%
Sample no.	95		60	63.2%	35	36.8%	51	53.7%	44	46.3%
Euploid/Balanced	31	32.6%	18	30.0%	13	37.1%	17	33.3%	14	31.8%
Unbalanced	6	6.3%	3	5.0%	3	8.6%	1	2.0%	5	11.4%
Aneuploid	64	67.4%	42	70.0%	22	62.9%	34	66.7%	30	68.2%
Aneuploid (excl unbalanced only)	63	66.3%	41	68.3%	22	62.9%	34	66.7%	29	65.9%
Cycles with embryo(s) for ET	16	59.3%	10	66.7%	6	50.0%	9	47.4%	7	87.5%

Table 6.8. Overview of NGS results in the inversion group broken down by the sex of the carrier and involvement of the centromere.

There were no significant differences between male and female inversion carriers in terms of the proportion of euploid/balanced embryos, the proportion of unbalanced embryos, or the proportion of cycles with at least one embryo for transfer (Table 6.8). There was a significantly higher rate of incidental aneuploidy in samples from female

carriers (p=0.0182) but rather than being associated with the inversion, this may be due to the higher maternal age in the inversion group as compared to other indications.

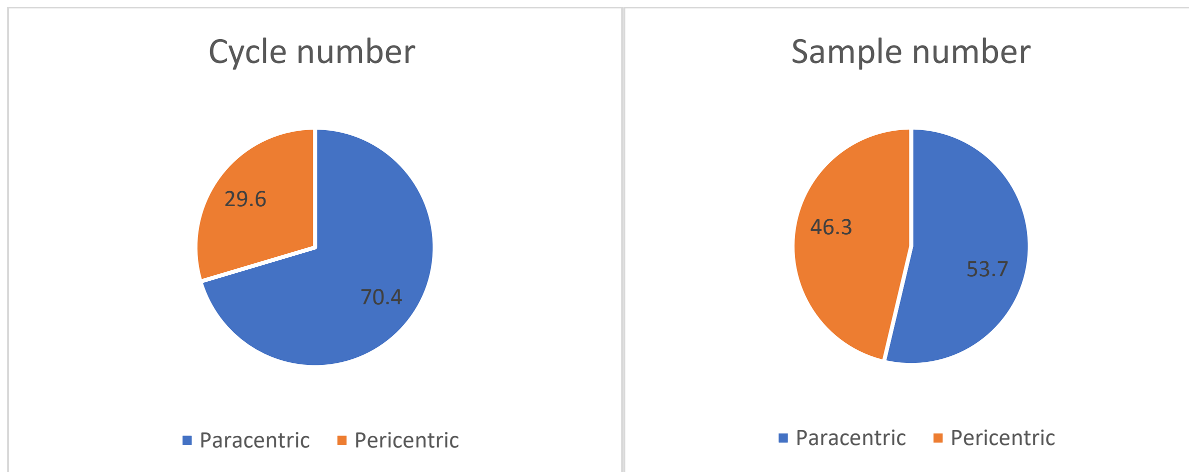

Figure 6.9. Charts showing the proportion of inversion type by cycle number and sample number.

Paracentric inversions were over-represented, accounting for 70.4% of all inversion cases, but a similar proportion of samples was seen from each, equating to fewer embryos per cycle for the paracentric inversion carriers (Figure 6.9). A higher maternal age was observed in the paracentric inversion group and so this association may be a result of this as opposed to the type of inversion.

At meiosis, the pericentric inversion forms a loop and any crossing over in the inverted segment leads to the production of recombinant chromosomes and aneuploid gametes. If crossing over occurs in the inversion loop in paracentric inversions, the recombinant chromosomes would either be acentric or dicentric, neither of which could result in viable pregnancy or livebirth, and the frequency of unbalanced gametes in carriers is low, with possible suppression of recombination in the inverted segment

(Gardner *et al.* 2012). The resulting higher risk of unbalanced livebirths in the pericentric inversion group may mean that these are identified earlier, reflected in the lower maternal age. The higher maternal age in the paracentric inversion group may reflect that these are later stage findings in fertility investigations.

There were no significant differences observed between paracentric and pericentric inversions in terms of the proportion of euploid/balanced embryos, proportion of unbalanced embryos, incidental aneuploidy, or the proportion of cycle with at least one embryo available for transfer. However, there was a trend towards a lower percentage of unbalanced embryos in the paracentric inversion group, in line with population data, which may prove significant were the sample and cycle numbers greater. There was also a trend towards a higher proportion of cycles with a least one embryo for transfer in the pericentric inversion group despite the increased frequency of unbalanced samples, which probably results from the greater number of samples per cycle in this group.

6.4.8 Insertions

Insertions may be interchromosomal (where a segment from one chromosome is transposed to another chromosome), or intrachromosomal (where a segment is transposed to another part of the same chromosome). The general population incidence is estimated to be 1 in 80000 (Van Hemmel & Eussen 2000), so although these only represent 0.6% of the PGT-SR cycles this could be considered an over representation.

While being rare, insertions are among the structural chromosome rearrangements with the highest risk of having an unbalanced livebirth (Van Hemmel & Eussen 2000). Statistical analysis was not performed due to low sample and cycle numbers, but the insertion group displayed the highest proportion of unbalanced samples.

6.4.9 Complex rearrangements

Complex chromosome rearrangements are structural rearrangements involving more than two breakpoints, and possibly more than 2 chromosomes. With fewer than 300 reported cases (Morin SJ *et al.* 2017), the frequency in this data set of 1.0% of cases could be considered higher than expected.

In the complex rearrangement group there were:

- 1 couple present with a complex structural abnormality involving 3 chromosomes with reciprocal translocations, where one of the chromosomes involved also had an interchromosomal insertion, and 2 derivative chromosomes resulting from reciprocal translocations.
- 1 couple present with a three-way reciprocal translocation
- 1 couple present with a three-way reciprocal translocation and an intrachromosomal insertion in another chromosome.

Risk figures are difficult to ascertain in this group due to their rarity and heterogeneity, but carriers are considered high risk for pregnancy loss and unbalanced livebirth (Gardner *et al.* 2012). Case studies reporting PGT-SR for such couples report healthy livebirths, but with 50 embryos generated per baby born (Escudero *et al.* 2008, Lim *et al.* 2008). Whilst statistical analysis was not performed due to low cycle and sample numbers, the data reflects this, with only 6.3% of samples being euploid/balanced.

However, an increased rate of incidental aneuploidy – 75.0% - may play a part in this, as the proportion of unbalanced samples was similar to that seen in standard reciprocal translocation cases (56.3% in complex rearrangements v 57.4% in reciprocal translocations).

6.4.10 Mosaic chromosome rearrangements

The data set included 3 cases where the carrier was mosaic for the structural rearrangement:

- A dicentric fusion of non-acrocentric chromosome and an acrocentric chromosome (resulting in the loss of the sub-telomeric region of the p arm in the non-acrocentric chromosome) in 60% of cells and an apparently normal female karyotype in 40% of cells.
- A balanced reciprocal translocation in 12% of cells and an apparently normal male karyotype in 88% of cells
- A balanced intrachromosomal inversion in 58% of cells and an apparently normal male karyotype in 42% of cells.

Mosaicism for a structural rearrangement is incredibly rare. In all cycles, balanced and unbalanced samples were identified, indicating that in each case the chromosome rearrangement was present in somatic and gonadal cells. Presumably, there is a bias here, given that those with mosaic rearrangements in their somatic cells only would be at no increased risk on aneuploid gametes and so would not present for PGT-SR.

6.4.11 Uniparental disomy

Uniparental disomy (UPD) is the inheritance of both homologues of a chromosome from a single parent with no contribution for that chromosome from the other, occurring as a result of trisomy rescue. An attempted self-correction to exclude a chromosome and restore disomy can result in both retained chromosomes originating from the same parent (Morin *et al.* 2017).

UPD is only of clinical significance when the chromosome in question contains imprinted genes, resulting in recognised syndromes depending on the parent of origin. In terms of Robertsonian translocations there is a theoretical risk of maternal or paternal UPD 14 (resulting in Temple syndrome and Wang syndrome respectively) and maternal or paternal UPD 15 (resulting in Prader-Willi and Angelman syndrome respectively). However, the estimated risk of UPD is low and given as 0.8% (Shaffer *et al.* 2006).

NGS will only detect relative losses or gains of genetic material, and so samples with UPD would be scored as euploid/balanced. At patient request, Karyomapping in 20 Robertsonian translocation cycles was performed, analysing 37 euploid/balanced samples to exclude UPD 14 and 15. UPD was not detected in any of the samples tested, but one sample was diagnosed as female triploid.

UPD testing was also performed on 3 euploid/balanced samples for reciprocal translocations at risk of paternal UPD 15 (Angelman syndrome) and paternal UPD 16. Normal biparental inheritance was confirmed in all samples tested and UPD excluded.

A previous study using PCR based CCS via STRs generated a UPD rate of 3.8% in embryos from Robertsonian translocation carriers (Fiorentino *et al.* 2010), in excess of the expected incidence of 0.8%.

6.4.12 Interchromosomal effect and mosaicism

Concern has existed that, in theory, carriers of structural chromosome rearrangements may also be at increased risk of producing gametes or embryos aneuploid for chromosomes not involved in the rearrangement, due to perturbations in meiosis or mitosis.

	RECIPROCAL NO MOSAICISM		ROBERTSONIAN NO MOSAICISM		RECIPROCAL MOSAIC REPORTING		ROBERTSONIAN MOSAIC REPORTING	
Average mat age	34.4		34.4		33.9		34.0	
Median mat age	35		35		34		35	
Cycle no.	323		120		182		58	
Sample no.	1264		423		714		224	
Euploid/Balanced	278	22.0%	139	32.9%	161	22.5%	75	33.5%
Euploid or Mosaic/Balanced					188	26.3%	81	36.2%
Unbalanced	726	57.4%	127	30.0%	415	58.1%	57	25.4%
Aneuploid	986	78.0%	284	67.1%	493	69.0%	130	58.0%
Aneuploid (excl unbalanced only)	643	50.1%	238	56.3%	298	41.7%	104	46.4%
Cycles with embryos for ET	178	55.1%	81	67.5%	110	60.4%	48	82.8%
Cycles with only mosaic embryos					9	4.9%	7	12.1%

Table 6.9 Overview of the incidence and impact of mosaicism in the reciprocal and Robertsonian groups.

In terms of impact of interchromosomal effect (ICE) at meiosis, one would expect to see a high rate of samples aneuploid for chromosomes not involved in the chromosome rearrangement. In this data set, there was no significant difference in the

rate of incidental aneuploidy between the reciprocal and Robertsonian group or between the Robertsonian group and the inversion group. A difference in incidental aneuploidy between the reciprocal group and the inversion group was significant (p=0.0039), with the inversion group having a higher proportion of aneuploid embryos, excluding unbalanced chromosome derivatives. However, I believe this is due to the higher average and median maternal in the inversion group as opposed to evidence of inter chromosomal effect. In 2019 33070 samples were analysed via NGS for PGT-A, with 54.2% of samples scored as aneuploid when excluding mosaicism (unpublished), similar to the rates of incidental aneuploidy seen in the reciprocal and Robertsonian groups (50.1% and 56.3% respectively), although the average maternal age in the rearrangement groups is 2.6yrs younger than the PGT-A group

No evidence of ICE is seen during meiosis in this data set, excluding possibly in the complex rearrangement group, where the rate of incidental aneuploidy is 75.0%. The absence of evidence for ICE is supported by sperm studies (Vozdova *et al.* 2013) and is the prevailing scientific opinion (McKinley Gardner at al 2012). Recent studies providing incidental aneuploidy as evidence of ICE (Mateu-Brull *et al.* 2019) fail to take into account the baseline levels of gamete and embryo aneuploidy in human reproduction.

ICE at mitosis would presumably lead to mosaicism within the embryo. I observed no significant difference in the proportion of mosaic embryos in reciprocal and Robertsonian carriers (8.4% and 11.2% respectively, p=0.2297) and the incidence of reported mosaic samples in the PGT-A cohort is higher (15.2%, unpublished). Mosaic

reporting meant that 16 additional cycles had embryos available for transfer (Table 6.9).

6.5 Discussion

Patients undergoing PGT-SR understandably wish to know how many of their embryos will be chromosomally unbalanced, if any will be appropriate for transfer, and their chances of pregnancy and livebirth or miscarriage. By analysing NGS results from 1814 trophectoderm samples submitted over 479 PGT-SR cycles, the purpose of this study was to identify factors impacting patient's chances of reaching embryo transfer, including the type of rearrangement, the sex of the carrier, maternal age and day of biopsy.

Statistical comparisons were made between reciprocal translocations, Robertsonian translocations, and inversions. Other indications include insertions, complex rearrangements, and dicentric fusions, but sample and cycle numbers were low (3, 3, and 1 respectively). As expected based on population frequencies, translocations accounted for 92% of cycles, , the combined population incidence of reciprocal and Robertsonian translocations being estimated at 3 in 1000 (Gardner *et al.* 2012). The proportion of cycles per indication matched the proportion of samples analysed, indicating that none of the indications examined exerted an undue impact on the number of blastocysts available for biopsy and testing.

Leaving aside insertions, complex rearrangements, and dicentric fusions because of low numbers, couples with a reciprocal translocation had the lowest chance of having embryos for transfer (55.1%) in their treatment cycle. This is significantly lower than

the Robertsonian group and inversions and reflected in the fact that the proportion of embryos that were euploid/balanced (21% for reciprocal, but 32% for both Robertsonian and inversions). This is also lower than a previously published PGT-SR NGS study (Wang *et al.* 2019) where 32.3% of embryos for reciprocal translocation carriers were balanced/euploid. However, the mean maternal age in this study was 28.8 years and so the patient population was much younger and can be expected to have a lower rate of incidental aneuploidy. Based on these data then a genetic counsellor might advise that, overall, for reciprocal translocation carriers, the chances of any of one their embryos being considered for transfer is around 1 in 5, rising to 1 in 3 for Robertsonian and inversions. Moreover, there is an overall better than even chance that at least 1 embryo will be available for transfer for reciprocal translocations, this is closer to 2 in 3 for Robertsonian and somewhere between the two for inversions.

6.5.1 Sex of the carrier

The sex of the carrier had no impact on any of the parameters analysed in the reciprocal group. In contrast, the Robertsonian group displayed a higher proportion of unbalanced samples from maternal carriers, which is a well-documented phenomenon (Gardner *et al.* 2012). However, this did not ultimately impact on the proportion of embryos available for transfer. In the inversion group, the only parameter impacted by the sex of the carrier was incidental aneuploidy, with an increased incidence in cases with female carriers. However, this was attributed to the higher maternal age in this group rather than an inherent mechanism of female inversion carriers. As with the Robertsonian group this did not ultimately impact the proportion of euploid/balanced embryos or the proportion of cycles reaching transfer.

6.5.2 Maternal age

In reciprocal translocation cases, increasing maternal age significantly increased the rate of incidental aneuploidy and reduced the proportion of cycles with an embryo for transfer. This is to be expected given the impact of maternal age on embryo aneuploidy and is unrelated to any translocation related behaviour.

This was also observed in the Robertsonian group. Unexpectedly, in the Robertsonian group we also observed a higher proportion of unbalanced embryos with increasing maternal age. This maternal age effect was previously shown by Tulay *et al.* (2016) but no other references to this could be found. Increasing maternal age was also associated with a non-significant trend towards a lower chance of having embryos available for transfer.

It is possible that in the Robertsonian group some embryos are wrongly scored as unbalanced and that they display a monosomy or trisomy for one of the chromosomes involved in the translocation, but not as a result of the translocation. However, it is also possible that the mechanisms that see increasing aneuploidy with increasing maternal age may also be responsible for the increasing proportion of unbalanced embryos in the Robertsonian group. It would also indicate different cell cycle mechanisms for dealing with segmental changes as opposed to full chromosome gain or loss, since this effect is not present in the reciprocal group. This is further evidenced by the fact that cell cycle checkpoint mechanisms in males result in a lower proportion of unbalanced samples compared to female carriers in the Robertsonian group, but not the reciprocal group. Related to this, recent studies have demonstrated that segmental

aneuploidies are predominantly paternally derived, while whole chromosome aneuploidies are more frequently maternally derived (Kubicek *et al.* 2019).

6.5.3 Day of biopsy

Day of biopsy – day 5 or day 6 – was analysed in the reciprocal and Robertsonian translocation groups. Day 7 biopsies were excluded as the number submitted was very low. To try and exclude bias introduced by a laboratory's workflow practices, only cycles with biopsies performed on day 5 and day 6 were included in the analysis. The day of biopsy had no impact on the proportion of unbalanced embryos in either group. While the day of biopsy had no impact on any of the studied parameters in the Robertsonian groups, in the reciprocal group day 6 embryos had a significantly lower chance of being euploid/balanced, a higher rate of incidental aneuploidy, and therefore a significantly lower proportion of cycles had at least one day 6 embryo available for transfer. We can think of no reason why this difference should exist between the groups other than introduction of bias through embryology practice, There is similar disagreement in PGT-A studies on the impact of day of biopsy in terms of rates of aneuploidy, but there is agreement that euploid embryos can be identified via day 6 biopsy with good clinical outcomes (Capalbo *et al.* 2014, Taylor *et al.* 2014b).

6.5.4 Inter chromosomal effect (ICE)

No evidence of ICE was observed in the reciprocal, Robertsonian or inversion groups. Although incidental aneuploidy is present, it is similar to the aneuploidy rate in the groups PGT-A patients. This could further be examined extracting age specific control groups from the PGT-A data. Mosaicism was also not present at a higher level than expected. The presence of a parental structural rearrangement, therefore, may lead

to unbalanced gametes, but does not in itself lead to the generation of aneuploidy in other chromosomes during meiosis or mitosis.

Beyond the type of structural rearrangement present, ultimately, maternal age has the largest impact on the availability of embryos for transfer. While other factors may impact the proportions of different cytogenetic constitutions, they do not ultimately impact on the proportion of cycles with embryos available for transfer. The proportion of cycles with embryos available for transfer can also be increased with the inclusion of mosaic reporting – in cycles with mosaicism reported, an extra 16 cycles had at least one embryo available for transfer.

6.5.5 Conclusions

The study is the largest appraisal of PGT-SR by CCS to date and, of course therefore, the largest by NGS. While significantly different incidences have been associated according to the type of structural rearrangement present, other factors appear to be of less consequence. That is, no evidence was found that time of biopsy, nor sex of the carrier had any effect. While maternal age also has no effect on the incidence of unbalanced embryos caused by the abnormality in the parent, the maternal age effect inevitably has an impact on the availability of embryos for transfer due to the increase in incidental aneuploidies. Moreover, no significant levels of UPD were found, suggesting no added value to the use of SNP chips over NGS. Finally, the proportion of cycles with embryos available for transfer can also be increased with the inclusion of mosaic reporting – in cycles with mosaicism reported, an extra 16 cycles had at least one embryo available for transfer, indicating the value of NGS as a diagnostic tool going forward.

7. Novel insight into the mechanism of twinning as a result of PGT

This study is an expanded version of the following abstract

Lynch C., D Maruthini, M Ragunath, L Jenner, S Fishel. 2015. First genetically confirmed monozygotic dichorionic diamniotic twin livebirth from a day 5 single blastocyst transfer: Presented at Fertility 2015, Birmingham, UK, January 2015 (Awarded best scientific presentation by a post registrant), and ESHRE 2015, Lisbon, Portugal, June 2015

7.1 Chapter summary

Monozygotic dichorionic diamniotic (MZ-DCDA) twinning has historically been believed to occur with splitting of the embryo within 72hrs of fertilisation. Here evidence is presented of it occurring later in development – post day 5 development, but prior to implantation - thereby shedding light into a possible novel mechanism. That is, I hypothesise that this mechanism may involve splitting of the embryo as the inner cell mass hatches, with potential involvement of altered zonal lysis and apoptosis.

7.2 Introduction

Multiple pregnancy is a well characterised complication of ART procedures, associated with higher risk of maternal and foetal morbidity and mortality compared with singleton gestations (Land & Evers 2003).

Twin pregnancies after IVF normally result from the transfer of multiple embryos and are therefore dizygotic. However, IVF also confers an increased incidence of

monozygotic twins – as high as 13.2% - compared to the population incidence of 0.4% (Hviid *et al.* 2018). As compared with dizygotic twins, monozygotic twins have a higher risk of complications and poor outcomes.

The phenomenon of twinning has held a long fascination; from the Greek and Roman mythologies of Castor and Pollux and Romulus and Remus, through the Kray twins of 1960s London, to current day, where the Olsen twins appear on fashion and gossip pages and the Dolan twins rack up YouTube subscribers. In mythology twins are cast as having special powers and deep bonds and may be viewed as ominous or auspicious. Castor and Pollux are a fictional presentation of the phenomenon of heteropaternal superfecundation – rare in humans but common in some animals – where two ova released in the same ovulation event are fertilised by the sperm of different fathers. Closer to home, a case of monopaternal superfecundation was recorded in an IVF patient who gave birth to quintuplets following a double embryo transfer, with genetic testing supporting that twinning was not a result of the embryos splitting, but that all babies shared the same father (McNamara *et al.* 2016).

Many studies have tried to investigate the incidence of monozygotic twinning following IVF, the factors that may impact this incidence, and the co-morbidities associated with its occurrence (Vitthala *et al.* 2008, Kanter *et al.* 2015, Hviid *et al.* 2018). However, few examine the type of twinning – beyond zygosity – and very little clinical data exists on chorionicity and amnionicity of monozygotic twins, or the mechanisms by which they may arise, the understanding of which may help in reducing the incidence in IVF.

While twins are usually described as either dizygotic (from 2 separate embryos) or monozygotic (from the same embryo), the situation is not that simple (Figure 8.1):

- Dichorionic-Diamniotic twins form when splitting takes place by the third day after fertilization.
- Dichorionic-Monoamniotic twins form when splitting takes place four to eight days after fertilization.
- Monochorionic-Monoamniotic twins form when splitting takes place from the ninth day after fertilization.

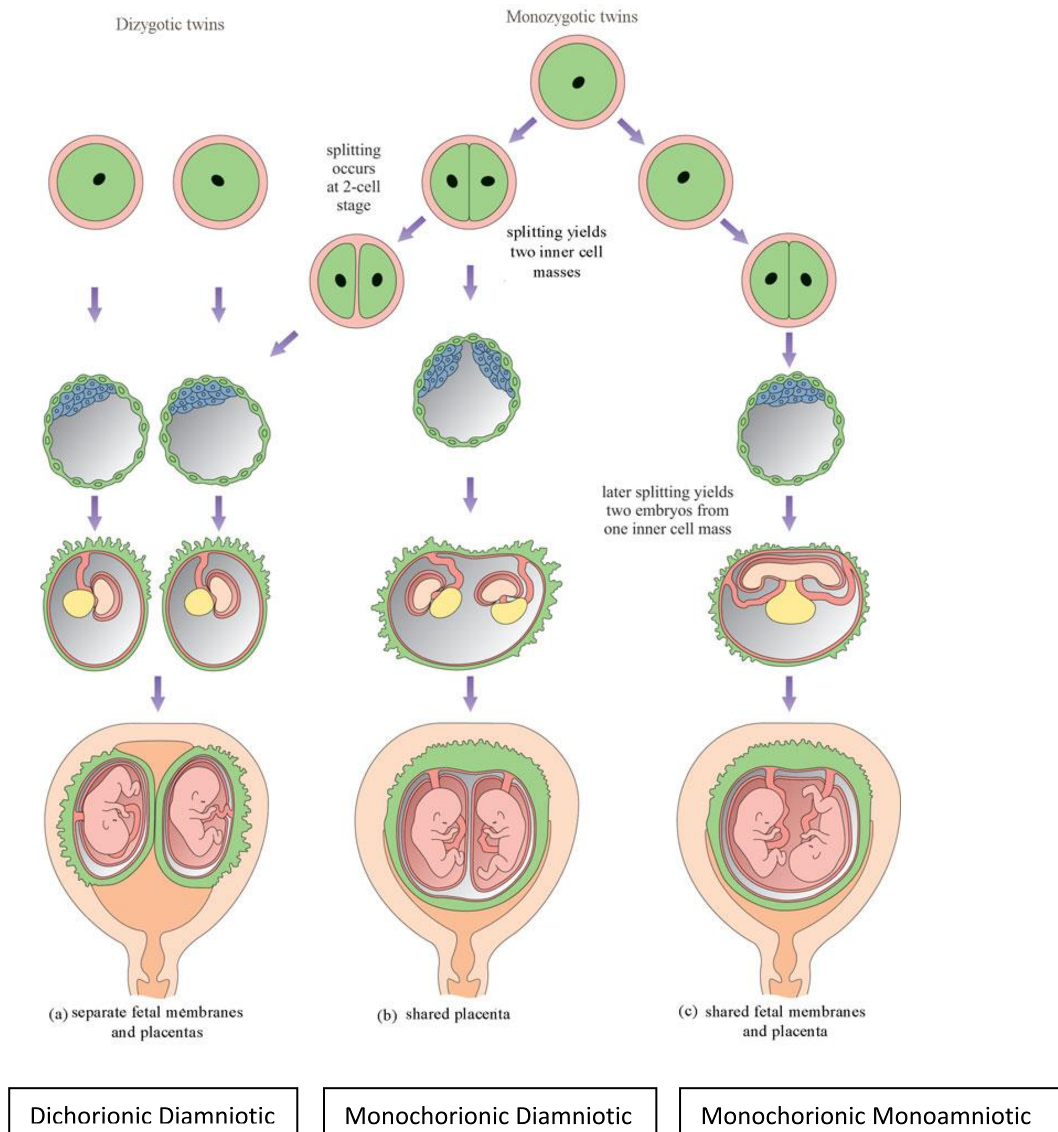

Figure 7.1. Illustration of the classical model of the types of monozygotic twinning and how and when it occurs.(Adapted from http://www.open.edu/openlearnworks/pluginfile.php/4824/mod_oucontent/oucontent/204/none/none/ldc_session8_fig8.jpg)

While dichorionic diamniotic (DCDA) monozygotic twins are characterised as splitting in the first days of embryo development a literature review of twinning studies in IVF reveals occurrences of apparently monozygotic dichorionic twins post single embryo transfer at the blastocyst stage, with incidences ranging from <1% to 14% (Kawachiya *et al.* 2011, Osianlis *et al.* 2014, Konno *et al.* 2020). The purpose of this study was to challenge previously held dogmas about the mechanisms of twinning using PGT data.

7.3 Materials and Methods

A couple underwent PGT-M treatment with embryo biopsy on day 3 and transfer of a single hatching early blastocyst on day 5 (Figure 7.2). A pregnancy scan at 7+6 weeks confirmed DCDA twins. Placental studies confirmed a DCDA fused twin placenta. The couple opted for prenatal diagnosis via chorionic villus sampling, despite warnings from their medical team in pregnancy that only one twin could be tested due to positioning in utero. The couple were confident that the pregnancy was monozygotic and that there was no risk of a natural conception and their IVF clinic, CARE Nottingham, were confident that a single embryo transfer had been performed. Despite this, the patient was told throughout her antenatal care that the twins were non identical and could not be monozygotic.

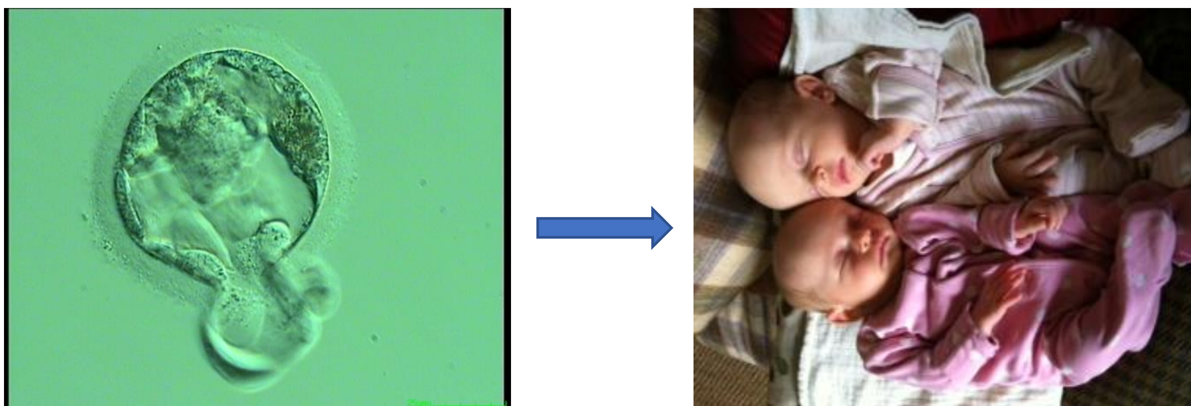

Figure 7.2. An image of the single blastocyst transferred on Day 5 and the resultant twins.

Clinic data was checked for previous occurrences of DCDA twins resulting from single blastocyst transfer. Additionally, a scientific literature search was performed to try and find papers that not only classed twin pregnancies as dizygotic or monozygotic, but examined chorionicity and amnionicity.

7.4 Results and Discussion

Testing of cord blood samples from both twins at birth by their regional genetics service using combined DNA index system (CODIS) markers confirmed both twins were healthy, and analysis of 16 STR markers excluded maternal contamination of cord samples and revealed identical profiles, confirming monozygosity.

At the time of its occurrence, we believed this to be the first case where monozygosity of a DCDA pregnancy was been confirmed by genetic testing rather than presumed by virtue of single embryo transfer (SET). However, we have subsequently discovered a similar case report occurring at a similar time (Kyono *et al.* 2013).

A retrospective analysis of monozygotic (MZ) pregnancies from 4780 single embryo transfers 2004-2014 was subsequently performed at the IVF clinic. The purpose was to review all single embryo transfers, generate an incidence of identical twinning, and identify if there were any other cases of MZ-DCDA twin pregnancies. This analysis revealed 26 sets of apparent MZ twins, 4 of which were MZ-DCDA and gender concordant as in the PGT-M case presented. Zygosity was not genetically confirmed and concurrent natural conception - superfecundation - cannot be completely excluded. However, were we to assign these as MZ-DCDA, this would equate to an incidence of 3.8% of MZ twins and 0.08% of all pregnancies following single blastocyst transfer. Two studies were found examining chorionicity and amnionisity in MZ with incidence of 0.47% and 0.8% of clinical pregnancies following single embryo transfer (Kawachiya *et al.* 2011, Osianlis *et al.* 2014). However, again, monozygosity was not confirmed with genetic testing. The numbers therefore, appear too low to identify patient or embryo specific risk factors, but it is important that patients understand a

risk of twinning still exists in single embryo transfer. A greater understanding of twinning in general might help moves to understanding why MZ twinning is higher in IVF and how we reduce it, including possibly through embryo selection.

A more recent paper (Konno *et al.* 2020) has performed the most thorough analysis of this phenomenon to date. Konno *et al.* examined 655 twin pregnancies over an eight-year period and found 43 cases of monozygotic twinning following single blastocyst transfer. Of these cases, 6 had been confirmed as DCDA using first trimester ultrasonography and postnatal placental pathology. Cord blood testing was performed on these 6 cases and genotyping was performed using 16 microsatellite markers, resulting in confirmation of monozygosity in just 3 of the cases, the other 3 being dizygotic and presumed to result from concurrent natural conception.

Our case report and the data from these studies supports a late mechanism for MZ-DCDA twinning through subdivision of the blastocyst that may be more common than we thought. Discovering the true incidence of MZ-DC twinning is complicated by the number and stage of embryos transferred and the need for genetic confirmation of monozygosity. It stands to reason that a proportion of presumed DZ twins are, in fact, MZ-DCDA twins with failure of the second embryo to implant.

Nonetheless, this data suggests that the classical model for twinning is incorrect in supposing that MZ-DCDA twinning only occurs within 72hrs of fertilisation. Further case reports exist describing various modes of atypical hatching (Van Langedonckt *et al.* 2000, Meintjes *et al.* 2001, Behr & Milki 2003) resulting in assumed MZ-DCDA twins but fail to confirm via genetic testing. The recent study from Konno *et al.* (Konno *et al.*

2020) highlights how vital this genetic confirmation is given their testing of presumed MZ twins was only confirmed in 50% of cases. Therefore, it is very difficult to draw conclusions from the available case reports describing hatching, splitting and the presence of two trophectoderm or two ICM populations, which might lead us in the wrong direction without genetic confirmation of zygosity. With increased moves to single embryo transfer at the blastocyst stage it is incumbent upon us to develop a deeper understanding of the mechanisms behind monozygotic twinning. Twinning does necessarily occur according to the classical model. A clearer understanding of how, why and when twinning actually occurs is needed to identify high risk patients or embryos and measures that can reduce risk.

8. General Discussion

The aim of this thesis was to identify variation in PGT practices that influence clinical outcomes, and both try and identify best practice and collate solid data sets to inform new approaches going forward. It was largely successful in the fulfilment of its aims, namely:

- Liaison with diagnostic labs, embryology labs and medical affairs created a new set of guidelines for embryology labs wishing to use diagnostic services (chapter 2 and Appendix) and this is now widely adopted.
- It was established that ICSI is not necessary as a preventive measure against paternal cell contamination in PGT and thus it is not necessary for all patients undertaking PGT to pay the additional fees for this procedure (chapter 3)
- It was established that re-biopsy is indeed a viable strategy when no result is obtained (chapter 4)
- It was established that there is considerable variation in "no result rate" between referring ART clinics (chapter 5)
- A retrospective analysis of over 1,800 PGT-SR samples (479 cycles of 5 years) was performed, to date on PGT-SR outcomes using CCS, finding no demonstrable ICE. A set of practical guidelines was put forward for genetic counsellors to advise patients on their likelihood of having euploid embryos for transfer based on the type of chromosome rearrangement, sex of the carrier of the rearrangement, maternal age, and any other factors that may be implicated (chapter 6)
- Novel insight into the mechanism of twinning as a result of a PGT case was provided (chapter 7)

8.1 Variation in PGT practice and outcomes

Just as we see variations in clinical outcomes for standard cycles between centres, it stands to reason we will see the same for PGT cycles. However, this variability leads to questions on the efficacy and utility of PGT and so needs to be addressed. It may be that the increased manipulations in PGT cycles expose weaknesses in embryology practice that have not previously been evident, and therefore outcomes for some centres do not show the improvements they expect. This issue is addressed in chapters 2, 3, 4 and 5.

The main guidance, therefore, that can be given to laboratories undertaking or embarking on offering PGT cycles must be to perform thorough risk assessments and identify where there are divergences from standard practice and how this might impact on clinical outcome. Then action can be taken to mitigate this where necessary without making unvalidated changes to laboratory procedures.

Given that statistically significant differences were identified between ART clinics in terms of their no result rates (chapter 5), it is important that clinics do not work in isolation and have access to information to bench mark their performance and identify when improvements are required. Thereby, innovations such as those highlighted in chapters 3, 4, 5, and 6 can be widely disseminated.

8.2 The need for ICSI in PGT cycles

While professional guidelines continue to recommend ICSI for amplification based PGT it is understandable that many clinics continue to operate on this basis. However,

given the additional cost and lack of clear benefit in the absence of male factor infertility, it seems reasonable for clinics and genetic testing laboratories to risk assess their cases, based on the actual likelihood of sperm contamination and amplification versus the severity of the outcome were it to occur, and decide their clinical policy on which insemination method is required for individual testing indications.

8.3 Efficacy of re-biopsy

There appears to be a large percentage of patients who are either not being offered re-biopsy by their ART clinics or are choosing not to undertake it based on the information they are being provided by their clinic.

It is clear that when an embryo does not yield a result there was a known on hidden technical issue in the embryology or genetic testing laboratory, as re-biopsy and retesting gives information on the genetic status of the embryo. Given protocols within genetics labs in terms of the WGA reaction occurring in the submitted sample tube, the use of multichannel pipettes and the volume of samples processed simultaneously, the balance of probability points to an issue in the embryology lab in terms of the quality or integrity of the sample taken or in the way it is handled in cell preparation and tubing.

Re-biopsy will give patients information on the genetic status of their embryos and it will provide embryos suitable for transfer to patients who did not have any euploids from their initial round of testing. However, it is becoming apparent that these repeat procedures are impacting on the implantation potential and sustained implantation potential of the embryos. While clinical outcomes appear better than the transfer of a

no result embryo, more data is required to be able to say whether this is another outcome that will show variability between ART clinics. A lot of euploid re-biopsied embryos remain in storage, as do a lot of no results embryos that may be transferred or undergo re-biopsy in the future. It is important that data collection on the use and clinical outcomes of these embryos remains ongoing to be able to properly inform patients on the benefits and drawbacks of re-biopsy, helping them make informed choices in their treatment while maximising their chances of success. Re-biopsy is certainly an option for PGT-SR (chapter 6) when no result is obtained.

8.4 PGT-SR data

The data collated on PGT-SR is the biggest data set of its kind to date. The data was remarkably consistent, and it has given the genetic counselling team within our organisation very useful information to tailor their consultations with PGT-SR patients in terms of being able to give them a more individualised risk figure.

However, some of the most interesting data related to what we can learn about chromosome rearrangements on a wider scale. The absence of translocation hot spots and recurrent translocations in our data set points to a lesser impact on fertility, and again raises the question of the true incidence of chromosome rearrangements in the population if there are many than fail to be identified through having no adverse impact throughout a person’s life. The over and under representation of certain chromosomes in our data sets can potentially help inform on the mechanisms by which these rearrangements form or which specific rearrangements are likely to be benign incidental findings.

The data also adds to the increasing body of evidence that interchromosomal effect does not exist either at the meiotic or mitotic level.

The most surprising finding was the maternal age effect in Robertsonian translocation carriers. It could be that the mechanisms which see increasing aneuploidy with increasing maternal age may also be responsible for the increasing proportion of unbalanced embryos in the Robertsonian group. It also indicates different cell cycle mechanisms for dealing with segmental changes as opposed to full chromosome gain or loss, since this effect is not present in the reciprocal group. We will be collating PGT-SR data from our other testing sites to further improve this data set and see if these findings remain consistent.

8.5 The Prospects for non-invasive PGT

The vast majority of this thesis has dealt with PGT performed by the removal of 5–10 cells from the trophectoderm, followed by WGA and subsequent diagnosis. While this is thought not to harm the embryo if done correctly, it can be harmful if performed badly. Moreover, it is time consuming at best, requiring a highly advanced skill set and this there is considerable interest in developing methods for non-invasive PGT (niPGT).

Blastocentesis involves the suction of blastocoelic fluid, which contains embryonic using an ICSI pipette. Results from some groups suggest that 82% of blastocyst yield a result, with 97% concordance rate with trophectoderm biopsy results. Other however suggest that rates of concordance, are as low as 40% (reviewed in Victor *et al.* 2020). True niPGT-A however involved no direct manipulation of the embryo at all and

considerable research is focussed on analysis of spent medium. Cell-free DNA derived from the embryo is present in the medium through as yet poorly understood processes that may include apoptosis or other cell death pathways. Whether such DNA is of sufficiently good quality and quantity to effect a reliable PGT is currently under investigation. A myriad of recent reports using contemporary NGS platforms observed ploidy concordance rates between spent medium and blastocyst cells between 80%–95% range. An important note of consideration from these studies however is that, often some procedure that could elevate the quantity of DNA in the medium was usually performed. These included assisted hatching, freeze-thaw cycle, or performing a trophectoderm biopsy so that a comparative study could be performed. Only such study avoided such confounding factors returning a 78.7% concordance rate for ploidy and sex. It is important to note that 100% concordance may not necessarily be a realistic prospect however, in part because of the possibility that the trophectoderm is a more accurate representation of the embryonic genotype. Better results have been returned when embryos are cultured to day 6 or 7 however maternal DNA contamination (cumulus cells) can present a problem (reviewed in Victor *et al.* 2020).

In a recent optimistic white paper, Cooper Genomics have assessed the prospects for niPGT from spent medium. In a prospective study they examine metadata parameters and parental dosage, concluding that the body of evidence that niPGT is certainly possible and feasible. Evidence exists that minimally disruptive embryology protocols may be amendable to non-invasive testing, thereby expanding the potential of niPGT to more clinics and diagnostic labs. Nonetheless the majority of published reports of niPGT are limited to a single clinical centre studies and quite small cohorts of patients and embryos. They also highlight a lack of scientific rigor in the measurement of

contaminating maternal cell DNA, something that they are hoping to address in the future.

8.6 Artificial Intelligence (AI) in Reproductive Medicine

Artificial intelligence (AI) has seen an exponential growth in all areas of science and healthcare in recent years and reproductive medicine is no exception. From theory, to algorithm development, to implementation uptake has been expedited by the availability of huge datasets and much enhanced computing power. Machine learning (ML), a division of AI, permits computers to identify patterns from such datasets and thereby make predictions for treatment strategies. Numerous machine learning techniques have been used to augment providing much needed direction. Examples of how it is being applied to reproductive medicine are given in figure 9.1

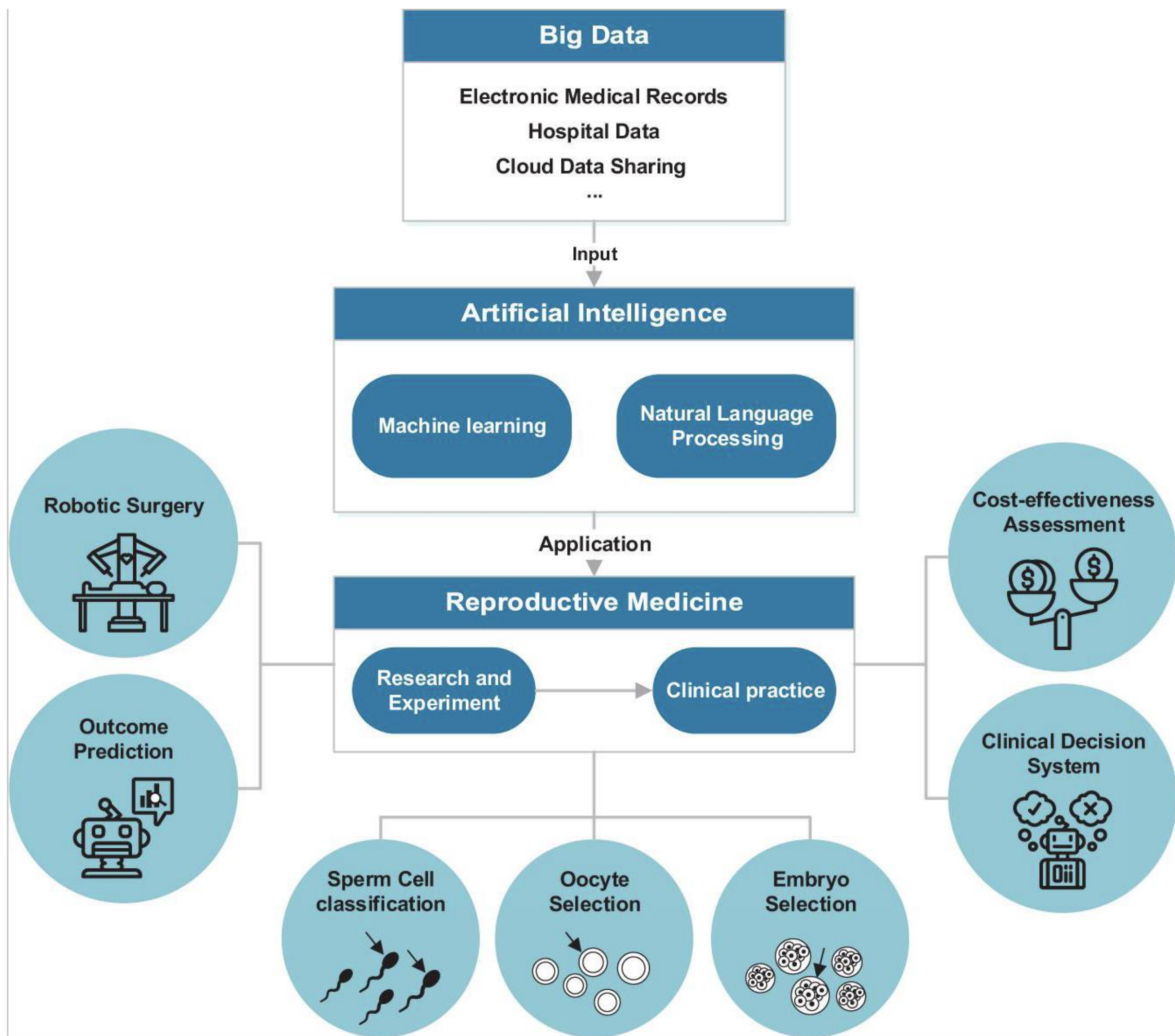

Figure 8.1. The role of artificial intelligence in Reproductive Medicine. Big data include electronic medical records and other data. AI such as machine learning and natural language processing can be used in the many aspects of reproduction, from research to medical practice. This diagram gives an overview the seven main applications of AI in reproductive medicine (Wang *et al.* 2019).

Figure 8.2 gives an example of how a workflow of AI might be employed in Reproductive Medicine.

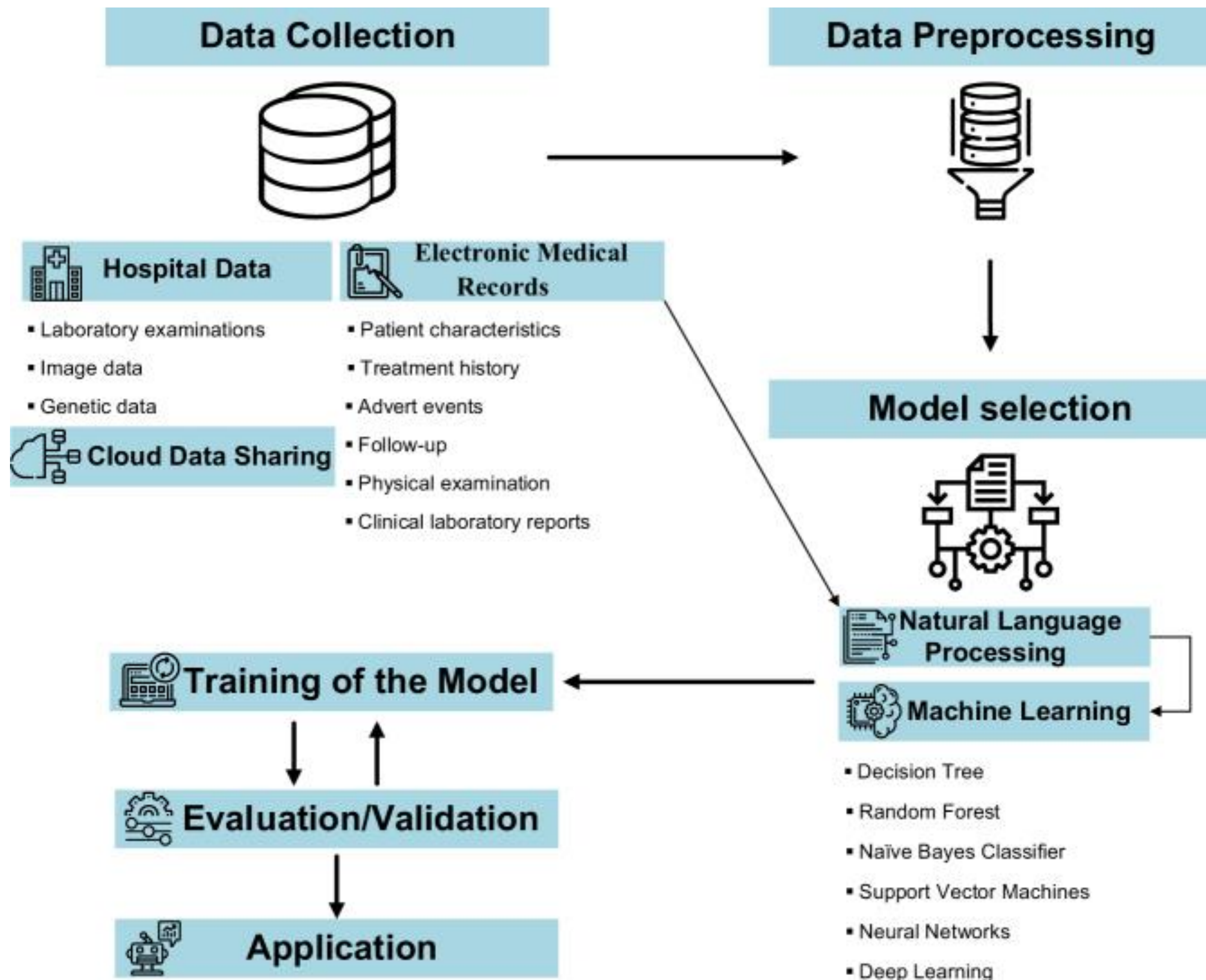

Figure 8.2 Flowchart providing a brief overview of a typical AI workflow. A) data collection including electronic medical records (EMRs), hospital data and cloud data sharing. B) data pre-processing. C) selection of an appropriate model. D) data analysis using AI. E) the training dataset is used to train the model. F) evaluation and validation of the model (Wang *et al.* 2019).

To this end, a new CooperGenomics PGTai Solution claims to be the first software to deliver true statistical analysis of PGT-A data based on 1,000 pregnancy outcomes used to generate

a baseline. Over 10,000 samples were run to generate robust statistical analysis, thereby producing user-Independent automated diagnoses through optimized and validated algorithms. This is highlighted as a "leap forward" in PGT-A accuracy. A second PGTai 2.0 platform combines copy number variation analysis with global SNP (single nucleotide polymorphism) analysis, powered by AI. This includes detection of female triploidy (69XXX) and haploidy which were (see chapter 4) hitherto a problem. Also incorporating the detection of meiotic aneuploidy, it provides IVF centres with a greater level of confidence for diagnosis. Figure 9.3 indicates how AI incorporated into the diagnostic pipeline.

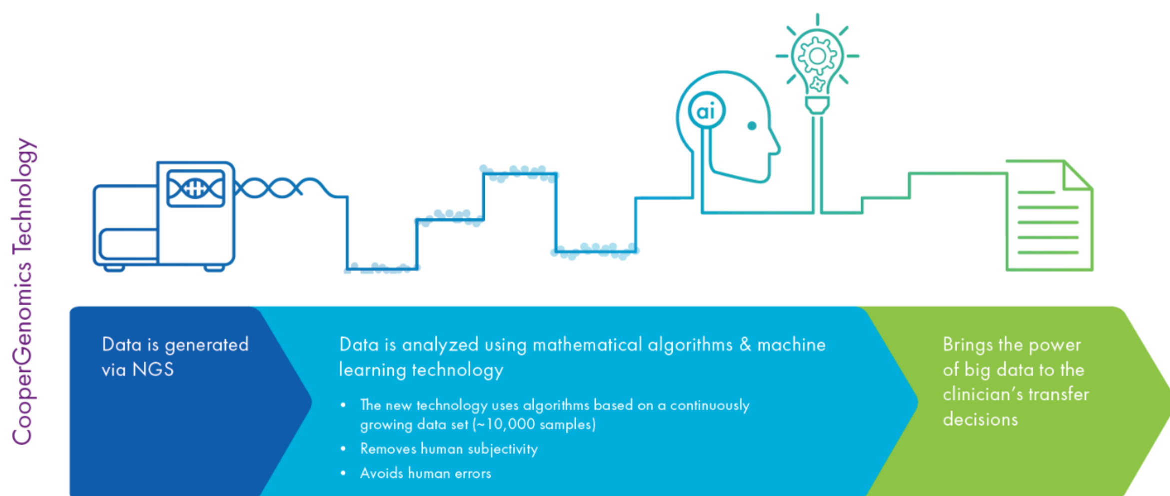

Figure 8.3. CooperGenomics AI solution for future PGT-A

8.7 Final perspectives

From the outset, I set on a journey to see if I could make the world of PGT a little better. While it would be wrong of me to say that "everything went to plan," the overall outcomes I hope point to this thesis making a small but significant contribution in this direction. Finding out a little bit about specific biological mechanisms such as those involved in chromosome segregation (chapter 6) and twinning (chapter 7) were an added bonus.

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Appendix

Fertility and Genomic Solutions

TROPHECTODERM BIOPSY

Advised standard operating procedure V11.2019

1. MATERIALS AND EQUIPMENT

Equipment
Embryo Biopsy Kit CooperGenomics REF 096101016 v1
Tube rack for PCR tubes
Biopsy buffer
Sample tubes
Barcodes stickers
Biopsy worksheet
Electronic pipette controller
Stripper pipette holder
Pipetman micropipette
Laminar flow hood
Stereomicroscope 16x zoom advised
RI Ambiplate for manipulations on room temperature on heated stage
RI Integra 3
Inverted microscope with heated stage
Micromanipulator with microinjectors
Active laser
Air injectors
Mini centrifuge for PCR tubes
Tri-gas incubator for culture of embryos
Non-gassed incubator for equilibration of dishes
Consumables
Biopsy pipettes
ID ID 18-22µm XS or ID 23-27 µm XXS
Holding pipettes
Medium holding OD 95–120 µm and ID 15–20 µm
Handling/denudation pipettes
Embryo handling: 275 µm
Sample handling: 75–150 µm
Light or mineral oil for embryo culture
Origio Handling medium – dual MOPS/HEPES buffered medium
ICSI dish (embryo biopsy dish 9mm height)
Serological pipette 5 ml
LifeGlobal 4 well GPS dish
Sterile disposable Gilson tips Eppendorf – DNA RNAse free 2–200 µl
Cleaning solution and cloths for DNA/RNA removal of working surfaces
Markers

RI Witness labels
Tubes cfr PGTai pack
Cold block – in freezer
Sterile cloth
Gloves – powder free, preferably sterile
Styrofoam cooler and shipping box

2. PROCEDURES

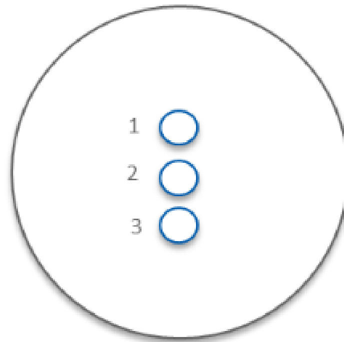
2.1 Preparations

Preparation prior to day of biopsy

- Ensure all oocytes/zygotes are carefully denuded and no cumulus cells remain
- Perform assisted hatching on day 3, 4 or 5 (optional)
- Prepare post-biopsy single embryo culture dishes on day 4
- Check stock material and consumables (see list above)
- Check stock and shelf life Embryo Biopsy Kit
- Check cool pack is in freezer

Preparation on day of biopsy

- Embryos may be hatched on the morning of day 5 if not performed previously, or hatched at the time of biopsy
- Prepare the embryo biopsy dishes – 9mm height dish – using HEPES or MOPS buffered media supplemented with HAS like Origio Handling medium. Label with patient ID. (Do not use lid of culture dish for biopsy dish.) It is advised to prepare 1 biopsy dish per embryo
- Make 3 x 10–20 µl drops for biopsy washing and equilibrating tools and overlay with pre warmed oil



- Incubate the dishes for a minimum of 30 minutes prior to biopsy in a non-gassed incubator at 37°C

2.2 Biopsy Procedure

ALL EMBRYO MOVEMENTS REQUIRE AN APPROPRIATE WITNESS AND WITNESS EVENTS MUST BE RECORDED

1. Check laser alignment and function
2. Check temperature on heated stage
3. Install the holding and biopsy pipettes
4. Label the biopsy dish with embryo ID
5. Using a large diameter denudation pipette under a stereomicroscope, transfer one embryo from the culture dish to the first drop of the biopsy dish. Rinse in the first drop to remove excess culture medium and place in second biopsy drop
6. At the inverted microscope prime the holding and biopsy pipettes in the equilibration (first) drop

7. Position the embryo on the holding pipette with the inner cell mass between 6 o'clock and 12 o'clock
    - Where the embryo is hatching, the herniating area will ideally be 90–180° away from the inner cell mass
    - Where the ICM is hatching, a biopsy sample may be taken adjacent to the ICM or a second zona breach can be made 90–180° away from the ICM
    - Where the embryo is not hatching, a zona breach can be made 90–180° away from the ICM. Gently aspirate out from the biopsy pipette at the site of the breach to initiate collapsing
8. Perform biopsy using either the laser and flick (see 2.2.1 below) or laser and stretch (see 2.2.2) method

2.2.1 Laser and Flick

- Trophectoderm cells are aspirated into biopsy pipette and pressure neutralized
- A maximum of 3 laser pulses (6-10 µm hole size) fired at cell junctions along the outside of the opening of the biopsy pipette
- Embryo released from the holding pipette
- Biopsy pipette is positioned above the holding.
- Biopsy pipette is moved to the edge of the holding pipette. They are pushed against each other, causing the biopsy pipette to flick across the opening of the holding pipette.

2.2.2 Laser and Stretch

- Trophectoderm cells are aspirated into biopsy pipette as the embryo is held securely on the holding pipette
- A maximum of 3 laser pulses (10-12µm hole size) fired at cell junctions between the embryo and the biopsy pipette aiming to perforate while stretching the tissue
- Continue to laser and pull until the trophectoderm sample separates from the embryo
- Procedure should be repeated one pulse at a time until the trophectoderm sample separates from the embryo

9. Move the biopsy dish back to the stereomicroscope
10. Label the post-biopsy culture dish with the patient ID, and each drop with the embryo number
11. Take a large denudation pipette to wash the blastocyst in fresh culture medium and transfer it to its corresponding drop of the post-biopsy dish in the presence of a witness
12. Bring the post-biopsy dish to the incubator until vitrification.
13. Vitrification can be performed within 30 min from the biopsy procedure, prior to re-expansion, if laboratory practice is to collapse prior to vitrification. Otherwise, embryos can be allowed to re-expand prior to vitrification
14. It is recommended to perform cell tubing prior to vitrification

2.3 Tubing Procedure

2.3.1 General guidelines

- All cell sample movements require an appropriate witness and witness events must be recorded
- The cell preparation stage is the stage of the process most vulnerable to contamination with foreign DNA. Sample preparation and collection should be performed at room temperature in an DNA free environment in an unheated class II laminar flow hood
- The working space should be cleaned in order to remove all sources of contamination (cells, DNA, RNA, etc.)
- Gloves (powder free, preferably sterile), mask, a surgical mask and sleeves should be worn throughout the tube loading procedure and changed immediately if there is contact with any potential source of contamination
- The collection tube rack should be placed during the tubing procedure on a cool block that is covered with a sterile cloth. An RI Ambiplate can be used under the stereomicroscope over a heated surface
- The sample rack should be sealed and stored in the freezer until shipment and shipped with frozen ice packs to the genetic testing laboratory

2.3.2. Barcode and labelling

- Ensure that only the materials and forms for one patient are in the working space
- Place one of the provided barcode stickers on the biopsy worksheet
- The remaining barcode stickers will be used to label each sample tube
    - Place the sticker around the tube as close to the top of the tube as possible
    - The stickers should overlap at the ends, this is necessary to ensure the sticker stays in place during temperature changes
    - Doublecheck to ensure that the barcodes on the tube match the barcode placed on the biopsy worksheet
    - Discard any extra bar code stickers
- Label the top of each tube with a unique identifier (e.g. 1, 6 vs 9 should be clarifies with an underline)

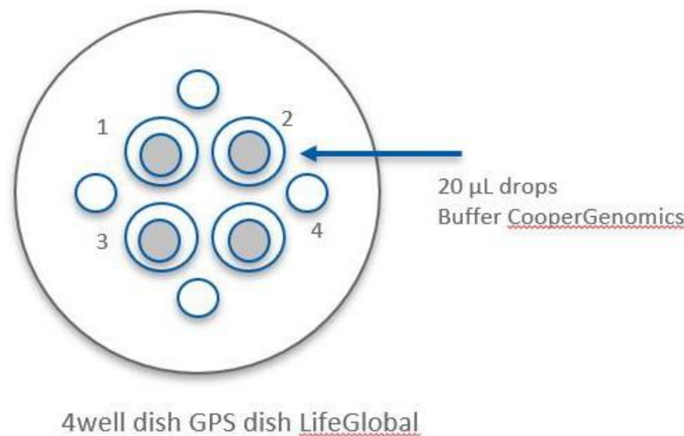
Typically, one barcode will be used per patient. Multiple barcodes should be used for one patient only when:

- The number of samples exceeds the number of barcodes/tubes per kit
- Results will be reported on separate report OR
- Any time the samples will be sent as a separate submission
- In these cases, please ensure all barcodes used are on the biopsy worksheet and designate the date each barcode was used

2.3.3 Cell preparation and tubing

Buffer drops

- Prepare a washing dish with a minimum of 3 drops of 20µl of the buffer supplied by CooperGenomics, in a sterile, DNA/RNA-free petri dish, taken from a fresh sleeve and clearly label with the corresponding unique identifier – patient's name and embryo number
- Once the drops are made, they should be covered with the lid until use. Buffer drops should never be reused



4well dish GPS dish LifeGlobal

- Prepare a maximum of 5 labeled sample tubes by adding 2µl buffer to each and close the lid. Place in the rack on the cool block

Cell sample washing and tubing

- The cell sample washing procedure should be performed at room temperature
- Take a fresh small handling pipette and pre-rinse the pipette by filling and expelling the washing buffer three or four times outside the well.
- The buffer-rinsed pipette will be used to transfer the sample from the embryo biopsy dish to the first buffer drop
- Take the biopsy dish and visualize the cell sample
- Expel a few microliters of buffer over the cell sample
- Then pick up the sample in a small volume of buffer and transfer to the washing droplets one by one
- Each time the sample is moved, the pipettes should be rinsed twice and preloaded with buffer
- Minimize the amount of buffer transferred from one drop to the next
- The cell samples should be washed through 4 clean buffer drops before they are transferred to a PCR collection tube
- After the final drop, transfer the sample with a minimal volume of buffer (maximal 0.5µl buffer) to a PCR collection tube

- Balance the tube in the side of the dish under the microscope and watch as the sample isexpelled into the tube
- Visualize the cell sample in the tube
- Avoid blowing bubbles when expelling the sample
- Wash the sample handling pipette in the last buffers drop to doublecheck that the pipette isempty
- Note the condition of the sample on the biopsy worksheet. It is very important to note if lysis is suspected in the sample, this will enable CooperGenomics to determine why certainresults may present
- Ensure the sample number is written on the lid of the tube, and doublecheck the numberson all dishes before loading the sample into the tube
- It is advised to quick centrifuge the tubes before storage, in order to spin the content downin the tube
- To keep the sample stable, so DNA will, not degenerate, the tube rack is sealed as soon aspossible and placed in the freezer (see sample packing)

CooperSurgical®
Fertility and Genomic Solutions

Guide to successful embryo biopsy and tubing

Tips and tricks

Insemination – IVF or ICSI

Current professional guidelines recommend ICSI for all cases where a PCR-based test will be employed on the biopsied cell material. If IVF is used, there is the small possibility that a biopsy practitioner will contaminate the biopsied cell sample with extraneous bound sperm. It is also possible that non-decondensed sperm are present in blastomeres and hence could contaminate thesample.

Tip: ICSI is the preferred insemination technique.

Embryo culture

Single embryo culture is advised for embryos undergoing biopsy for PGT. This can have an impact onthe number of culture dishes needed for one patient, as well as the space needed in the incubators for culture. Moreover, the process of biopsy increases the number of incubator door openings, with each embryo being moved individually from culture dish to biopsy dish and back again and may further increase if embryos are checked at multiple times to see if they are suitable for biopsy. It should be considered how many embryos should be in each culture dish, and what type of incubation is best suited to the requirement for additional morphology checks and multiple door openings.

Tip: The use of culture dishes with preformed single embryo culture wells isadvised. A dedicated incubator for the biopsy procedure is advised.

Biopsy dish preparation

The medium used for blastocyst biopsy should remain pH stable during manipulation, so should contain a GMOPS and/or HEPES-buffer supplemented with an albumin source.

Unlike biopsy at the cleavage stage, calcium–magnesium-free media should NOT be used. A standard ICSI dish is used and should contain at least three small drops of medium under oil: one drop for tool equilibration, flushing and re-equilibration of the biopsy pipette; one drop for washing the embryo before biopsy;and one drop for biopsy of the embryo. It is not recommended to place the dish back in the incubator when it contains biopsy samples. Dishes need to be equilibrated at 37°C for at least an hour before use.

Tip: A standard ICSI dish with three small drops of HEPES and/or GMOPS bufferedmedium under an overlay of oil is used during the biopsy procedure.

Equipment and consumables

A microscope equipped with a heated stage, an ICSI rig and a laser is preferred. Lasers can be fixed –the embryo/dish must be moved to the laser target – or directional – the laser target can be moved to a specific area on the embryo. A directional laser aids precise targeting during the biopsy procedure.

A large holding pipette and a narrow flat and polished biopsy pipette should be used. The biopsy and holding pipette should be flushed and re-equilibrated before each use and also between embryos.

The aspiration pipette should be changed if there is visible cellular material stuck to the pipette, if there had been lysis and fragmentation of the biopsy sample in the pipette, or if cells or cell fragments have moved upward into the pipette.

When the biopsy sample sticks to the biopsy pipette, firmly tapping on the tool holder with a solid object like a pen or EZ Squeeze™ handle may release it.

A large diameter pipette is required for moving blastocysts, while a narrow diameter pipette is required for moving and tubing biopsy samples.

Tip: Using a new holding and aspiration pipette avoids the risk of contaminating the biopsied sample.

Assisted hatching

Embryos can be assisted in their hatching by drilling a <u>small</u> opening in the zona pellucida on day 3 or 4. The laser should be directed at a spot in the ZP where the perivitelline space is the largest to avoidany damage by heat noise to the cells.

Tip: A large opening in the zona pellucida can lead to hatching of a very large part of the TE. This is to be avoided.

Which embryos to biopsy?

Blastocyst biopsy is performed at a specific stage in development rather than a standard time point. Two approaches to this exist: multiple timepoint biopsy for the same patient; or biopsy of all embryos at the same time point.

Multiple timepoint biopsy involves monitoring the embryos until they have reach an expanded blastulation stage, with differentiation of the inner cell mass and trophectoderm. This this can lead to biopsying at multiple timepoints from day 4 to day 7 for the same patient.

An alternative option is to leave all embryos in culture until day 6 and biopsy them at this timepoint.

When there are limits to the number of embryos to be biopsied, grade A and B embryos should be prioritized.

Slower developing embryos, i.e. those with morulae on day 5 or 6, can be biopsied after exposure toCa-Mg free media for 20 minutes.

Tip: Time lapse is an excellent tool as the development of the embryos can be remotely checked.

Biopsy technique

The alignment of the laser should be checked before use. Where it is confirmed that the pilot lightand target align, no further steps need to be taken. However, if the pilot light and target are not aligned, a laser alignment must be performed. Embryos can either be hatched on day 3 or day 4 to encourage herniation for biopsy, be hatched at the time of biopsy, or left to hatch naturally.

Ideally the embryo should be held with the inner cell mass at the 8 to 10 o'clock position and the hatching area at the 2 to 4 o'clock position. Trophectoderm cells should be gently aspirated into the biopsy pipette (the embryo may collapse) and stretched slightly to help visualize the cell junctions. Current recommendations are that at least five cells should be taken when reporting on mosaicism. The laser should be used minimally at a low pulse length; 2–3 pulses at cell junctions is sufficient to create a weakness in the trophectoderm.

It is important to watch for movement within the biopsy pipette. There should be no positive or negative pressure and if cells are seen moving up the biopsy pipette this should be corrected by blowing out slightly. This is to ensure the weak point in the trophectoderm remains at the end of thebiopsy pipette and that post-biopsy the sample remains at the opening of the biopsy pipette. The embryo should be released from the holding pipette with the trophectoderm cells still inside the biopsy pipette. The biopsy pipette is positioned above the holding pipette and the pipettes can be touched against each other to confirm they are in the same plane of focus. The biopsy pipette can then be moved to the edge of the holding pipette, and as the pipettes are pushed against each otherthe biopsy pipette will flick across the opening of the holding pipette, separating the biopsy sample. The holding pipette should be removed from the dish before the biopsy sample is released away from the embryo, but not too close to the edge of the drop to aid later identification under the stereomicroscope.

Tip: With each laser shot, there is a risk of DNA damage by heat (noise), not only in the embryo but also to the biopsied cells. Fewer laser shots is better!

Tubing – preparation of environment

This is the stage of the process most vulnerable to contamination. It is important to understand clean practice in the context of the genetic testing laboratory. The concern is contamination via DNA

rather than the embryology concerns of fungus or bacteria. Therefore, it is important that any cleaning or sterilization procedures effectively degrades DNA.

Clean practice in the IVF lab involves preventing contamination from bacteria and fungus

Clean practice in the genetics lab requires prevention of contamination from human DNA and RNA

Genetics laboratories use:

- Chemical reagents, i.e. wiping with hypochlorite-based solutions or DNA/RNA-free water to remove all traces of amplifiable DNA
- UV irradiation, 83.4% effective at 8cm, 70.7% effective at 48cm
- NOTE: Ethanol does NOT remove DNA and should not be used

IVF laboratories can employ:

- Amended cleaning practices
- Dedicated working areas
- Dedicated equipment/consumables
- Protective personal equipment (PPE)

Where a dedicated area is not available it is acceptable to use a workstation in the embryology laboratory taking into consideration:

- Physical layout (i.e. minimizing movement of personnel and items)
- Primary use of workstation and cleaning level required

In areas where hypochlorite-based solutions cannot be used, a combination of UV irradiation and 'mechanical' cleaning with DNA/RNA-free water is acceptable, as long as other recommendations are followed.

Kampmann M.L., Borsting C., Morling N. (2017) Decrease DNA contamination in the laboratories. Forensic Sci Int: Genet. 6 e577-e578

Preuße-Prange et al. (2009) The problem of DNA contamination in forensic case work—How to get rid of unwanted DNA? Forensic Sci Int: Genet. 2(1):185-186

Tubing of cell samples

Each biopsy sample should be washed through at least three drops of buffer with a narrow diameter pipette before being placed into the corresponding 0.2ml PCR tube. If there is concern that the sample is fragile and may be lost or degenerate it is acceptable not to wash it. The pipette should be changed between samples. The PCR tubes should only be opened directly before placing the sample inside and should be closed again immediately afterwards. On washing, the sample can be aspirated from the final drop in 2μl of buffer and placed on the inner side of the PCR tube as it is held horizontally under a phase contrast microscope. Alternatively, the PCR tubes can be loaded with 2μl of buffer and the sample can be pipetted in minimal volume into the buffer at the bottom of the

tube, again while visualized under phase contrast. It is important to note that when the sample is

not visualized in the PCR tube, this does not mean it has not successfully been tubed and taking a second biopsy may not be necessary.

The tubes need to be spun in a microcentrifuge to ensure the sample is at the bottom of the tubeand stored in the freezer immediately.

Tip 1: Avoid touching the tubes with warm fingers/hands. The heat can degrade the DNA in the sample.

Tip 2: The tube can be placed on the ridge of the cover of a petri dish during the tubing. This immobilizes the tube and frees the hands for tubing and focusing the stereomicroscope

Tip 3: A good quality stereomicroscope is mandatory for tubing. The operator can change the contrast to visualize the cells more easily.

Training and competency

A written procedure should be in place to assess initial training and ongoing competency for both biopsy and tubing. Effective training requires the availability of training material, equipment and time. Training courses will quickly teach the basics of the techniques but do not replace the in-house training program. The training program should outline the minimum number of embryos to be biopsied/tubed successfully and the parameters to be examined as a measure of a successful procedure. A minimum of 20 successful embryo biopsies and tubing sessions should demonstrate competency for at least the following parameters: survival of the embryo post-biopsy, 24 hours development post biopsy and positive signal.

Similarly, competency should look at key performance indicators (KPIs) in clinical practice but also confirm adherence to laboratory standard operating procedures (SOPs). The Vienna consensus on the development of ART laboratory performance indicators suggests successful biopsy rate as a KPI for the reference group. The successful biopsy rate is: (number of biopsies with DNA detected/the number of biopsies performed) x 100. A competency value of at least 90% is suggested with a benchmark of at least 95%.

Witnessing

A robust labeling and witnessing system is essential to retain the chain of custody between embryo, biopsy sample and genetics report. Every movement of the embryo or biopsy sample must be recorded along with an electronic witness record or physical signature to reflect a manual witness. This includes the vitrification and warming procedures and receipt and interpretation of the results. Vitrifying embryos in carriers of different colors can be an additional visual identifier at warming.

Tip: Ideally two operators are involved in the biopsying and tubing procedure: one to do the biopsy, the other the tubing and both can do the witnessing checks.

Vitrification and warming

Embryos can be vitrified after biopsy when still collapsed or at a time convenient in terms of laboratory workflow, when they may have re-expanded. There is currently no strong evidence to suggest a preferred time frame for vitrification, but it is important that the biopsy samples are tubedbefore the vitrification and that timings used in vitrification are appropriate to the stage and expansion of the blastocyst. Embryos should be warmed as per standard lab protocol for frozen embryo transfer. It is becoming more common to hatch PGT blastocysts on warming but before re- expansion to increase implantation rates.

Tip: Do not vitrify the embryos before the tubing process is finished. In case of a tubing failure a re-biopsy is mandatory.

Troubleshooting

Non-functioning laser

First confirm the laser objective is in use and that the laser is aligned. Try switching the laser off and on again and check that all leads are firmly connected. Finally, contact the laser provider for support.A contingency plan should be in place for the eventuality that the laser is not working when a biopsy is scheduled. This would preferably involve mechanical hatching and biopsy, but it is acceptable to vitrify the embryos to warm and biopsy at a later date, or transfer in the right conditions to another laboratory for biopsy.

Inner cell mass hatching

While it is good practice to biopsy away from the inner cell mass, this does not need to a full 180° separation. Where the inner cell mass is hatching, the zona breach can be enlarged allowing more of the embryo to herniate and a biopsy taken from the trophectoderm adjacent to the inner cell mass. Alternatively, a second zona breach can be made at the preferred biopsy site, but this must be recorded, and hatching considered on warming. A third option is to remove the embryo from the zona and biopsy as a fully hatched embryo.

Fully hatched embryos

Fully hatched embryos can be biopsied by the previously described procedure. It is important that only very gentle suction is applied on the holding pipette.

Biopsy sample lost at tubing

The embryo should be re-biopsied prior to vitrification. Ideally the second sample should be taken from the original biopsy site. It may be possible to maneuver the biopsy pipette into the zona to aspirate the trophectoderm without having the zona breach in the exact plane of focus. It is also acceptable to re-biopsy by making a second zona breach or removing the embryo from the zona.

No results on PGT report

In case of failure of amplification/no result a re-biopsy may be considered.
The embryos can be warmed, re-biopsied, and vitrified again. The re-biopsy is performed aspreviously described.

However, the patient should be informed that the embryo may not survive the re-biopsy, approximatively a 90% amplification success/successful reporting rate after re-biopsy is noted, and iftransferred, the implantation potential may be reduced.

It is not advised to re-biopsy aneuploid or mosaic embryos.

Depending on the indication for testing, it may be acceptable to the clinic and patient to transfer theembryo without a result.

References

ESHRE Special Interest Group of Embryology, Alpha Scientists in Reproductive Medicine, 2017. TheVienna consensus: report of an expert meeting on the development of art laboratory performance indicators. Human Reproduction Open 2017, hox011. https://doi.org/10.1093/hropen/hox011