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Novel Insights into Preimplantation Genetic Testing for Aneuploidy and Non-invasive Prenatal Testing

A thesis submitted to the University of Kent for the degree of

DOCTOR OF PHILOSOPHY

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Kathryn Denise Sanders

School of Biosciences

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

Kathryn Sanders

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List of Abbreviations

aCGH	Array comparative genomic hybridisation
ADO	Allele dropout
AFP	Alpha-fetoprotein
AMA	Advanced maternal age
AMH	Anti-mullerian hormone
ART	Assisted reproductive technology
BMI	Basal metabolic index
cfDNA	Cell-free deoxyribose nucleic acid
cffDNA	Cell-free fetal deoxyribose nucleic acid
CPM	Confined placental mosaicism
CNV	Copy number variation
CVS	Chorionic villus sampling
dCTP	Direct cytidine triphosphate
DF	Degree of freedom
DNA	Deoxyribose nucleic acid
EB	Elution buffer
EEM	Extra-embryonic mesoderm
ET	Embryo transfer
FET	Frozen embryo transfer
FISH	Fluorescence in situ hybridisation
FSH	Follicle-stimulating hormone
hCG	Human chorionic gonadotropin
HT1	Hybridisation buffer
HLA	Human leukocyte antigen
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IUGR	Intrauterine growth restriction
IVF	In vitro fertilisation

LASER	Light amplification by stimulated emission of radiation
LNA1	Library normalization additives 1
LNB1	Library normalization beads 1
LNS1	Library normalization storage buffer 1
LNW1	Library normalizing wash 1
MPS	Massively paralleled sequencing
MPSS	Massively parallel shotgun sequencing
NaOH	Sodium hydroxide
NIPT	Non-invasive prenatal testing
NIPS	Non-invasive prenatal screening
NGS	Next generation sequencing
NT	Nuchal translucency
PAPP-A	Pregnancy associated plasma protein-A
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGT	Preimplantation genetic testing
PGT-A	Preimplantation genetic testing for aneuploidy
PGT-M	Preimplantation genetic testing for monogenic diseases
PPV	Positive predictive values
RAT	Rare autosomal trisomy
RCT	Randomised controlled trial
RPM	Revolutions per minute
RSB	Resuspension buffer
SD	Standard deviation
SNP	Single nucleotide polymorphisms
SSC	Saline sodium citrate
TE	Trophectoderm
uE3	Unconjugated estriol
UPD	Uniparental disomy

VTA	Veriseq tagment amplicon
WGA	Whole genome amplification
ZP	Zona pellucida

Abstract

Aneuploidy (chromosome copy number imbalance) is the leading cause of implantation failure, miscarriage, and live birth aneuploidy. Preimplantation genetic testing for aneuploidy (PGT-A) aims to select chromosomally normal (euploid) embryos produced during in vitro fertilisation (IVF) treatment for transfer. Ensuring that the resulting pregnancy is unaffected by aneuploidy aiming to avoid adverse outcomes and improve IVF success rates. Non-invasive prenatal testing (NIPT), is applied at a later stage of fetal development than PGT-A, after 10 weeks of gestation, to detect predominantly the chromosome abnormalities associated with live births. DNA for both PGT-A and NIPT genetic analysis is most commonly from the same region of the developing fetus, the trophoblast, part of the placenta. For PGT-A the trophectoderm (trophoblast) is currently the preferred site for biopsy, which, after developing into the placenta, the subsequent apoptosis of the trophoblast cells provides cell free fetal DNA for NIPT analysis. PGT-A and NIPT are both considered screening tests for the genetic status of the developing fetus, but genetic variation (or mosaicism) between the placenta and fetus in some instances can lead to false positives and negatives for both PGT-A and NIPT. As such, PGT-A and NIPT have limitations in their degree of accuracy, this has generated criticism regarding their application. With this in mind, the aims of this thesis were to investigate the application of PGT-A and NIPT on success over conventional methods and explore the causes and frequency of false positives and mosaicism.

Specifically:

1. Following PGT-A, to assess how often are there no euploid embryos but only a mosaic embryo available for transfer? And if using the CoGEN position statement to rank mosaic embryos, what proportion of these mosaic embryos would be considered for transfer?
2. To investigate if the transfer of embryos after PGT-A results in improved live birth rates over conventional IVF without genetic analysis, and to what extent this varies by centre.
3. To investigate the rates of aneuploidy detected following NIPT, based on referral indication.
4. To test the hypothesis that NIPT cases previously reported with a suspected aneuploidy and found to be discordant suspected common aneuploidies, is caused by the presences of rare autosomal trisomies (RATs) on the untested chromosomes.

Results found that 10-11% of PGT-A cases only had a mosaic embryo for transfer, and that around 4% of cases would have a mosaic that would be considered for transfer. PGT-A offers improved live birth rates per embryo transferred for patients over 35 years old, and that there is a large degree of variation between referring centres. This work also found that patients referred following an abnormal ultrasound scan were the most likely to have aneuploidy detected with NIPT. This work also demonstrated that in this instance the false positives following the reporting of a suspected common aneuploidy was not due to RATs on the untested chromosomes.

The results presented in this thesis demonstrate that both PGT-A and NIPT offer improved accuracy and in turn better outcomes with continued technological improvements. It also demonstrates that by extending the availability of both genetic screening methods to more patients, improvements could be seen in terms of increased live birth rates for PGT-A and increased detection of live birth aneuploidies for both high and low risk patients.

1 Introduction

1.1 Preimplantation Genetic Testing

1.1.1 Perspective

Preimplantation Genetic Testing (PGT) is essentially a medical intervention designed to minimise the chances of transfer of genetically abnormal embryos in an ART setting. Its primary utility is to help families at risk of transmitting genetic disorders conceive a normal child and/or to improve IVF success rates by the selective implantation of chromosomally normal embryos. Typically, the process involves referral and genetic counseling for the nature of the specific problem, standard IVF, embryo biopsy, genetic diagnosis of the biopsied cells then selective transfer of embryo(s) thought to be genetically normal.

1.1.2 The first PGT-M cases

The first recorded PGT-M case in model species was a chromosomal one, performed to control sex ratio in rabbits (Gardner & Edwards, 1968). Gardner and Edwards successfully assessed trophoblast fragments for inactive sex chromatin (Barr body) in females and thereby accurately determined the sex of blastocysts. Application of this technology to humans clearly had the potential to screen for X-linked recessive diseases before implantation of an embryo, avoiding invasive prenatal assessments and the possibility of a

difficult decision deciding whether to terminate. Following the development of in vitro fertilisation (IVF) in 1978 (Steptoe & Edwards, 1978), clinical progress in this area became possible and thus, in 1990, the first human embryos underwent blastomere biopsy and the sex was established by PCR amplifying a Y-specific repeat sequence. The unaffected female embryos having two copies of the X chromosome and thus no amplified signal lacking the Y were transferred resulting in successful pregnancy and healthy live birth free from the X-linked condition (Handyside *et al.,* 1990). This led the way to PGT-M in other monogenic conditions such as cystic fibrosis, which was successfully achieved in 1992 (Handyside *et al.*, 1992). Early PGT-M used polymerase chain reaction (PCR) to amplify short fragments of the known affected region of DNA using nested primers; providing confirmation if the cell and thus embryo possessed the sequence which coded for the condition in question. Thereafter, for most of the history of PGT-M therefore diagnoses were either monogenic, usually involving increasingly sophisticated variants of PCR, or chromosomal, initially involving fluorescent in-situ hybridisation (FISH), but later involving whole karyotype screening approaches.

FISH was first introduced clinically in 1992 to sex embryos using probes specific to X and Y chromosomes (Griffin *et al*., 1993; Delhanty *et al*., 1993). It is thus more than 25 years since the first chromosomal PGT-M cases were peformed. Later in 1993, the first aneuploidy screening cases using FISH were carried out, assessing chromosome copy numbers of the most common trisomies associated with live birth defects X, Y, 13, 18 and 21 (Schrurs *et al*., 1993; Munné *et al*., 1993). The number of chromosomes that could be

screened simultaneously was limited by the colours of the probes: red, yellow, green, aqua and blue. PGT-A most commonly was used for patients undergoing IVF with the following indications: advanced maternal age (AMA), recurrent miscarriage, recurrent implantation failure and those with severe male factor infertility. By screening embryos to identify and transfer chromosomally normal embryos, IVF success rates and pregnancy outcomes should be improved.

1.1.3 The trouble with PGT-A

Initial, retrospective, studies of PGT-A indicated that there was an increase in implantation rates and decrease in pregnancy loss following PGT-A with FISH (Munné *et al*., 1993; Gianaroli *et al*., 2005). Several, randomised controlled trials (RCTs) challenged this however, showing either no significant improvement or a detrimental effect on successful outcomes of IVF (Schoolcraft *et al*., 2009; Mastenbrook *et al*., 2007). There are differing opinions regarding why these studies had varying outcomes. Firstly, there is concern that the process of embryo biopsy at the cleavage stage could have an adverse effect on the embryo (Mastenbrook *et al., 2007)*, at this stage in embryo development there are normally 8 cells, removing one of these could reduce the success of the future development of that embryo. Related to this, the other remaining cells (and hence the developmental potential of the embryo) could be damaged in the biopsy process and this could be operator dependent (Cimadomo *et al*., 2016). Therefore, the studies that saw a decrease in implantation rate when compared to standard IVF without PGT-A, have been

criticised for inadvertently causing damage during the biopsy process in those embryos which were later transferred and failed to implant. Secondly, it is known that embryos can present some degree of chromosomal variation between cells or mosaicism (Taylor *et al*., 2019). For some cases of PGT-A it is possible that the cell that is screened will present as chromosomally normal, where in fact the other cells are abnormal, creating a false negative result (Griffin & Ogur, 2018). Thirdly, another possible contributor is that with most PGT-A studies using FISH, not all chromosomes are analysed. The chromosomes that are screened may have appeared euploid, but those chromosomes that have not been screened could be aneuploid, resulting in the transfer of an abnormal embryo (Griffin & Ogur, 2018). The practice of PGT-A in the clinical setting ultimately declined following the publication of these RCTs. FISH in most clinical IVF cases is now not the method of choice, in part due to lack of confidence in the technique but also from the emergence of advanced technology which was subsequently applied to PGT-A.

1.1.4 Trophectoderm biopsy and improved methods for PGT-A and PGT-M

Improved culture conditions leading to a greater number of embryos reaching the blastocyst stage in regular IVF presented an opportunity for an improved approach to PGT-A protocols. Trophectoderm biopsy on day 5 of embryo development was now an attractive option over the conventional blastomere biopsy on day 3. The advantage of trophectoderm biopsy is clear, by day 5 of embryo development there are many cells that make up both the inner cell mass (ICM) and the trophectoderm (Harton *et al.,* 2013). Removing a few cells from the trophectoderm while leaving the ICM undisturbed in theory

has the potential for less adverse effects on the developing embryo and the advantage of providing more cells for analysis, than blastomere biopsy. A study by Kokkali *et al*. 2007 demonstrated an improved implantation rate with blastocyst biopsy over cleavage biopsy and subsequent studies support these findings (Harton *et al*., 2013). More recently, it has been shown that trophectoderm biopsy can be more consistent and reproducible across different practitioners and clinics compared to blastomere biopsy (Capalbo *et al*., 2015).

Perhaps the major technical advance in our ability to screen biopsied cells for chromosome abnormalities was the development of whole genome amplification (WGA) (Handyside *et al*., 2004; Wells *et al*., 1999). This approach increases the amount of available DNA where only small amounts are initially available from single cells. WGA enables multiple tests to be carried out, for example, PGT-A and PGT-M simultaneously, while benefiting from an increase in accuracy and sensitivity. Another benefit is that WGA products can be stored for later subsequent analysis in the instance of test failure or to confirm findings (Handyside, 2010). WGA enabled array comparative genomic hybridisation (aCGH) for the analysis of all chromosome copy number. aCGH essentially compares the amplified DNA labeled in one fluorescent colour with known, normal DNA labeled in another colour simultaneously hybridised to a genome-wide microarray (Gabriel, 2011). Chromosome-by-chromosome ratio analysis gives an indication of cytogenetic gain or loss e.g. trisomy or monosomy. Randomised clinical trials suggest benefits for screening by aCGH in terms of the usual outcomes used to measure IVF success (Rubino *et al*., 2013).

Another application of WGA was that multiplex PCR was successfully adapted for PGT-M, this allowed for the simultaneous analysis of linked markers to screen for monogenic conditions as well as aneuploidy for selected chromosomes. This permitted screening of multiple conditions, with greater accuracy as allele drop out (ADO – where a heterozygous individual was erroneously called as homozygous due to allele specific amplification) presented less of an issue with this technique than that seen with earlier applications of PGT-M due to the fact that multiple loci could be screened (Handyside, 2010). Human leukocyte antigen (HLA) typing, also known as saviour siblings, could also be combined with aneuploidy and monogenic PGT and was first successfully performed using PGT-M in 2000 (Verlinsky *et al*., 2001). This process establishes a pregnancy and live birth that is a HLA-match to an existing sibling, by selecting an embryo which is a HLA- match for transfer who can then be a stem cell transplant donor for their older brother or sister. As most couples requiring this form of PGT-M are of AMA there is therefore a potential benefit to be able to combine this with PGT-A (Rechitsky *et al*., 2006).

1.1.5 Is there still a problem with PGT-A?

Despite improvements in technology there is still an ongoing debate regarding the effectiveness of PGT-A for improved implantation and ongoing pregnancy rates. There have been several studies that have shown an improvement when PGT-A is used. A systematic review by Lee *et al*., 2015, where they combined the findings of 19 articles,

which were comprised of 3 RCTs and 16 observational studies, showed that PGT-A overall had improved success rates when compared to morphology based embryo selection. While, RCTs are considered the best design for research, the nature of ART in the clinical setting makes it difficult to create studies that meet all the criteria of a RCT, for example patients will always want to be in the group with the best outcome, they may wish to switch groups during the study to get what they perceive to be the best outcome, this can skew results, but would be unethical not to let patients have a choice (Lee *et al.*, 2015). There are also many more unknowns associated with this area of medicine, such as the complex interaction of the physiologies of two people (in order to produce a third). The comparison of different retrospective studies carried out in different clinics with varying approaches to ART and differing levels of biopsy practitioner skill can still play a big part in the variation of results presented (Gleicher, 2014). Ideally, all clinics would be uniform in their techniques to draw conclusive comparisons however this is not always practicable. It should also be kept in mind, in those cases where PGT-A does improve outcome, whether the cost implications associated with PGT-A match the increase in success rates (Neal *et al*., 2018). PGT-A techniques remain relatively high cost when added to a conventional IVF cycle. The effect on the patient, however, is difficult to quantify. Couples undergoing IVF cycles with PGT-A may potentially avoid the transfer of an embryo which has a high chance of miscarriage, meaning that they will be able to progress to the next possibly successful cycle much quicker than if an aneuploid embryo is transferred, implanted and miscarried (Neal *et al*., 2018).

1.1.6 Karyomapping and Next Generation Sequencing (NGS)

Karyomapping, first developed in 2010, is a method that uses the principles of linkage analysis and the inheritance of chromosomal haploblocks, in which the mother, father and a reference affected family member or grandparents are compared to map the origin of each chromosome inherited (and any crossovers between grandparental chromosomes) (Handyside *et al*., 2010). Karyomaps allow the tracking of affected genes that reside on these haploblocks, which can then subsequently be used for PGT-M to identify unaffected embryos before transfer. When applied to screening embryos this can also give valuable additional information to detect monosomy, uniparental disomy and trisomies. The karyomaps that are produced offer easy visualisation of the chromosomes and present clearly where there is crossover of genetic material. The SNP data for the karyomaps can also be applied to B Allele Frequency Graphs and Log R Ratio can be calculated from this to determine to what degree the embryo sample differs from normalised data, once these are plotted this information can allow inferences to be made of chromosome copy number (Estkei *et al,* 2015). Karyomapping has the advantage of allowing for diagnosis of genetic conditions, while simultaneously screening for chromosomal imbalances however its potential for the use in aneuploidy screening has yet to be realised (Natesan *et al*., 2014). This method has been made possible through whole genome sequencing; it is more commonly used with single nucleotide polymorphisms (SNPs) chip technology but can also be used alongside next generation sequencing (NGS). To date however NGS has primarily been used for aneuploidy screening.

NGS is a high-resolution whole genome sequencing technology that allows for the processing of samples at high throughput with a high level of accuracy. Recent studies show NGS to have 100% specificity and sensitivity making it superior to aCGH for PGT-A (Kung *et al*., 2015). NGS provides the ability to run samples simultaneously which gives the potential to make this technology lower cost and quicker than that seen with aCGH. It also has the potential to identify small copy number variations (CNVs), which can affect embryo development and result in severe birth defects (Fan *et al*., 2015).

1.1.7 The impact of cryopreservation and "freeze all" strategies on PGT-A

ART has seen improvement in embryo cryopreservation techniques. In PGT-A, there has been a shift towards the cryopreservation of embryos and transfer later, when the status of embryos has been confirmed (Harton *et al.,* 2013). Increased pregnancy success rates when screening for aneuploidy, have been attributed to the advancement of PGT-A technologies, such as aCGH and NGS. However, studies have shown there to be an improvement associated with frozen embryo transfer (FET) compared to fresh embryo transfer (Wang *et al.,* 2018). This improvement is thought to be due to ovarian stimulation having a detrimental effect on the endometrium, which lowers implantation rate. This stimulation is not encountered during a frozen embryo transfer and could therefore lead to higher implantation rates (Roque *et al*., 2013). Further research is required to determine if PGT-A is offering increased pregnancy success rates in addition to those seen with FET.

1.1.8 What we have learnt from research into PGT-A?

A greater understanding of meiosis, crossing over and molecular biology has led to improvements in PGT-M and PGT-A (Thornhil *et al.,* 2015). Similarly, however by studying the chromosomal aspects of PGT-A we can understand the basic biology of early human development better. One example is the incidence of mosaicism, previously it was believed that mosaicism was very rare and that in the case of trisomies it would be throughout all the cells in an embryo (Delhanty *et al.,* 1993). Indeed, some studies have suggested that most human embryos are aneuploid and mosaic (McCoy, 2017). In a recent study by Maxwell *et al*., WGA products that had initially been assessed using aCGH were retested using NGS (Maxwell *et al*., 2016). Embryos that had originally been determined to be euploid by aCGH were found to be mosaic by NGS, some of these mosaic embryos that had been transferred resulted in miscarriage, this provided an explanation as to why these pregnancies subsequently failed. However, other embryos that had been found to be mosaic through retesting using NGS were found to have resulted in a healthy live birth (Maxwell *et al,* 2016). Two percent of all normal pregnancies are post zygotically mosaic; therefore, caution is required when considering mosaic embryos at the time of transfer (Kalousek & Vekemans, 1996). Further research is required to ascertain the prevalence of mosaic cells in embryos and the implications on pregnancy outcome.

We have learnt that aneuploidy in embryos is commonplace, but we are learning more about the effect of abnormalities on embryo development. It has for instance been found that aneuploidy rates are lower at day 5 than day 3, raising the possibility that some aneuploidies are corrected or selected against between day 3 and day 5 (Harton *et al*., 2013). It has also been suggested that meiotic abnormality, for example in patients who are Robertsonian translocation carriers; can affect the segregation of other structurally normal chromosomes, this may results in an interchromosomal effect on the subsequent mitotic divisions and thus a higher abnormality rate in these patients than in other unaffected patients (Alfarawati *et al*., 2012). All the biological implications of PGT-A findings are not going to be listed in detail here, however the fact remains that it is a unique, fundamental system to study and much further research is still needed to address basic biological questions. For instance, we are still not entirely sure of the precise incidence, cell by cell, of aneuploidy in blastocyst embryos and, indeed, if a small amount of abnormality is commonplace in most embryos.

1.1.9 The future and conclusions

PGT-M and PGT-A have both come a long way since their first use in the early 1990s. We now believe that most embryos are aneuploid and that by transferring embryos that are aneuploid it is likely to result in failure to implant, miscarriage or the birth of an affected child. Pregnancy rate increases are consistently being reported more often when PGT-A is used however we still do not know if or when it is safe to transfer embryos with some

level of post-zygotic aneuploidy. Aneuploidy screening is of course only one of several selection strategies for assessing and determining embryos for transfer. All will require further review, ensuring the highest possible chances of IVF. It is undeniable that further research in the future is required to optimise PGT-A for clinical use. For instance, we need to understand mosaicism better; where a non-mosaic euploid embryo is available this will always be the first choice for transfer, but where mosaic embryos are all that is available we need to better understand under what circumstances these are safe to transfer. We need to understand the origin of trisomies; embryos with trisomies that are meiotic in origin should not be transferred, however we need to be better informed when detecting if a trisomy is post zygotic and the clinical outcome this will lead to, if transferred.

A final question therefore is: if PGT-A can be demonstrated to increase IVF significantly, should it be offered to all IVF patients? While aneuploidy is more prevalent among patients of AMA, there are still many embryos that are aneuploid in younger patients. Moreover, as previously mentioned, CNVs can affect any age group of patients, PGT-A optimised for CNV screening can be used to benefit all patients of any age. Such a suggestion is likely to be contentious, particularly among the opponents of PGT-A. In any event, the debate for and against PGT-A is likely to rage for some time yet. A consideration rarely aired however is the issue of whether it is unethical not to offer PGT-A, given its potential benefits.

1.2 Non-invasive prenatal testing

1.2.1 Perspective

Non-invasive prenatal testing (NIPT) (sometimes more appropriately referred to as non-invasive prenatal screening (NIPS)) has revolutionised prenatal screening for fetal live birth trisomies (e.g. trisomy 21, Down syndrome, trisomy 18, Edwards' syndrome and trisomy 13, Patau syndrome), since it was first introduced clinically in 2011 in the USA (Go *et al.,* 2011). The number of copies of each chromosome in the fetal DNA are counted by sequencing the fetal DNA that is present in the maternal blood plasma. This technology produces an accurate, reliable, sensitive, and specific determination of fetal trisomies and, as such, has been rapidly taken up as the preferred method of prenatal screening for live birth trisomies (Futch, 2013).

Live birth of children with an extra copy of chromosome 21 (Down syndrome), or chromosome 18 (Edwards' syndrome), or chromosome 13 (Patau syndrome) has long been correlated with AMA. Among these syndromes, Down syndrome has the highest prevalence of live birth, rising from 0.84 per 1000 births for women between 25-29 years old, to 15.22 for women between 40-44 years old (Wu & Morris, 2013).

Conventional methods of prenatal screening for such fetal trisomies, prior to the introduction of NIPT, involve the screening of biochemical markers in the blood of pregnant women, in conjunction with ultrasound scanning. If a high risk of fetal trisomy

pregnancy is detected, then an invasive prenatal test is advised, either chorionic villus sampling (CVS) from 11-14 weeks' gestation, or amniocentesis following 15 weeks' gestation (Bianchi, 2004). Biochemical screening has a significant false positive rate (4.2%) and there are elevated risks of complications and/or possible pregnancy loss associated with such invasive prenatal testing. In contrast, NIPT has a comparatively low false positive rate (0.5%) and has seen rapid uptake in order to reduce the unnecessary use of invasive diagnostic testing (Bianchi *et al*., 2014).

It has been known since the 1940s that fetal cells are present in the blood of pregnant women as these cells' 'leak' from the placenta into the maternal blood stream. Walknowska *et al*. (1969) first suggested that these cells could be collected for prenatal diagnosis. Subsequently, it became the goal of scientists working in the field of prenatal testing, to isolate these rare fetal cells and produce a non-invasive screening test (Lo *et al.*, 1996). However, fetal cells are present in less than 1 in 1 million of the cells in the blood of pregnant women (Lo *et al., 1996)*. Furthermore, fetal cells from previous pregnancies can also persist in the maternal blood stream for years (Lo *et al.,* 1997). Therefore, producing an effective screening test for these rare cells has been a huge challenge.

It was not until Lo *et al*. in 1997 first identified that fetal DNA was present in the blood plasma of pregnant women that an alternative method of non-invasive prenatal screening became a possibility. Non-invasive prenatal screening became truly feasible by the early 2010s due to the exponential increase of capacity of DNA sequencers. These sequencers

allowed the accurate quantification of fetal trisomies in cell-free DNA (cfDNA) from maternal plasma (Fan *et al.,* 2010). The first commercial test became available in 2011 and since that time more than 1 million cfDNA NIPT samples have been processed, leading to a whole new area of prenatal medicine (Bianchi, 2017).

1.2.2 Standard prenatal screening methods and invasive diagnostic tests

Prenatal screening for fetal trisomy is routinely offered to most pregnant women as a combination of ultrasound scanning to measure nuchal translucency (NT) thickness and several biochemical markers (Shajpal & Siddiqui, 2020). Various combinations of first and second trimester biochemical markers can be combined with ultrasound scanning to produce the various integrated / combined / quadruple tests (Table 1.1) (Shajpal & Siddiqui, 2020).

The integrated test is considered to have the best performance characteristics of the ultrasound and biochemical test. Although, even with the best combination of ultrasound and biochemical markers the sensitivity is lower and the false positive rates are significantly higher, than that seen with NIPT, leading to much lower positive predictive values (PPV) for these tests (PPV being the likelihood of being given a positive screen result and that actually being confirmed as a positive test) (Ferreira *et al.,* 2016). The integrated and quadruple tests, whilst not being as effective as NIPT for screening for live birth trisomies, do benefit from having reasonable detection rates (85% for integrated test) and false positive rates (1% for integrated test) for open neural tube defects (Shajpal

& Siddiqui, 2020). The results of the integrated and quadruple tests are generally expressed as a risk factor e.g. 1 in 100 risk of Down syndrome live birth, whereas many of the different NIPT tests offered provide detected/not detected results. Integrated and quadruple tests are intended for screening and are not fully diagnostic tests since they determine the potential for the presences of live birth trisomies. This is in contrast to CVS and amniocentesis, which are considered diagnostic tests since they can accurately confirm the absence/presence of live birth trisomies (Alfirevic *et al.,* 2017).

Test	Types of prenatal screening for fetal trisomy
Combined test	• Blood taken at 10-13 weeks pregnancy • Ultrasound scan for nuchal translucency • Blood screen for pregnancy associated plasma protein-A (PAPP-A) • Blood screen for β-human chorionic gonadotropin (free β-hCG) • Sensitivity (detection rate) of 82%-87% and a false-positive rate around 5%
Integrated test	• First stage at 11 weeks pregnancy of ultrasound scan for nuchal translucency/gestational age and blood screen for PAPP-A • Second stage ideally at 15-16 weeks' pregnancy. Blood screen for alpha-fetoprotein (AFP), total hCG, unconjugated estriol (uE3) and inhibin-A (inhibin) • Integration of results from first and second stage to single screening result • Sensitivity (detection rate) of 88-95% with a 2-5% false-positive rate for Down syndrome
Quadruple test	• Blood sample taken at 15-20 weeks pregnancy • Blood screen for AFP, total hCG, uE3 and inhibin-A (inhibin) • Sensitivity (detection rate) of 80% with a 5% false-positive rate for Down syndrome

Table 1.1: Various combinations of first and second trimester biochemical markers can be combined with ultrasound screening to produce the various combined, integrated, and quadruple tests

1.2.2.1 Chorionic villus sampling

CVS is performed from the 11th to the 14th weeks of pregnancy. A small sample of placental tissue containing chorionic villus cells is removed by ultrasound-guided aspiration. CVS can be carried out either by a transcervical or transabdominal method (with the latter most commonly performed) (Alfirevic *et al.,* 2017). The tissue could be prepared directly for chromosome analysis or cultured over a 1-2-week period and a result obtained following chromosomal harvest. Discrepant results between the two types of

chromosome abnormality heralded the widespread study of confined placental moscaicism (CPM) in humans (Griffin *et al.*, 1997). A direct CVS preparation derives its chromosome preparations from the rapidly dividing syncytiotrophoblast layer, whereas a cultured preparation assays cells from the extra-embryonic mesoderm (EEM – mesenchymal core) (Griffin *et al.*, 1997). As such there are advantages of both types of preparations. The former achieves a diagnosis quicker, but the chromosomes are of lower quality and less likely to represent the fetus. The latter take longer to return a result, but the chromosomes are of better quality and more likely to represent the fetus (Griffin *et al.*, 1997). Both methods detect the presence of a fetal trisomy, as well as other cytogenetic abnormalities (usually as indicated from an abnormal ultrasound scan). However, as CVS is an indirect test, testing the placenta rather than the actual fetus, confined placental mosaicism (CPM) can occur, where the cytogenetics of the placenta differ compared to the fetus.

1.2.2.2 Amniocentesis

Amniocentesis is performed between the 15th and 20th weeks of pregnancy. The process involves the use of a needle to remove a sample of the amniotic fluid from the amniotic sac (Alfirevic *et al.,* 2017). As with CVS, the amniotic fluid is either cultured to provide a cytogenetic karyotype, or DNA is extracted from the amniotic fluid and a microarray test performed. Amniocentesis is the most diagnostic prenatal test for live birth trisomies that can be performed since the process tests cells that are essentially direct from the fetus,

therefore this test largely superseded CVS despite the later stage of testing (Alfirevic *et al.,* 2017).

1.2.2.3 The timing of testing

Early testing is preferred as this is when termination is safest and can reduce the amount of distress encountered. There are similar risks of miscarriage associated with both CVS and amniocentesis of 0.5%-1%, although the exact risk can also depend on the skill of the practitioner (Alfirevic *et al.,* 2017). CVS is the method of choice for the first trimester, as amniocentesis prior to 15 weeks is not generally offered. This is because amniocentesis can lead to a higher risk of club foot (talipes equinovarus) and miscarriage in the first trimester (Alfirevic, *et al*., 2003). The associated risk of miscarriage from both invasive procedures is a major consideration, especially if it has been conducted due to a false positive screen. The minimisation of screening false positives, and therefore minimisation of invasive procedures, has been a major advantage of NIPT and one of the main reasons for its rapid uptake (Pettit *et al.* 2014). NIPT can also be carried out from 10 weeks of pregnancy, offering a means of screening pregnancies at a more advantageous early stage.

1.2.3 Non-invasive screening

1.2.3.1 Biology

Cell-free fetal DNA (cffDNA) is formed due to a natural process of cell death (apoptosis) in the placenta (Bischoff *et al.,* 2005). This is part of the constant turnover of the trophoblast within the placenta to maintain its healthy function. Apoptosis leads to the release of small fragments of DNA, around 150 base pairs into the maternal blood stream (Li *et al.,* 2004). CffDNA appears in greater amounts as the pregnancy progresses, with male sequences of cfDNA being detectable after 6-7 weeks and averaging at around 11-13% of total cell free DNA (Chan *et al.,* 2004).

NIPT is not offered until 10 weeks of gestation since this is when the fraction of fetal DNA is greater than 2-4% of total cfDNA and averages around 11-13% of the total cfDNA (Tagleur *et al,* 2013). The fetal fraction is important in NIPT, as higher fetal fraction can lead to more accurate results. When a maternal blood sample is taken for NIPT, it is centrifuged to separate the plasma from the blood cells. The cffDNA remains in the plasma, together with a larger proportion of maternal cfDNA. It has been demonstrated that cffDNA is most commonly present in smaller fragments than that of maternal cfDNA, averaging <0.3kb and >1kb, respectively (Li *et al.*, 2004). Therefore, by enrichment of the smaller fragments (<0.3kb) of cfDNA, it is possible to direct the testing towards the DNA that is representative of the fetus, as opposed to the mother (Li *et al*., 2004).

There are two further areas of cffDNA biology that need to be considered in relation to screening tests. Firstly, the biological source of cffDNA must be considered. It is widely accepted that the majority of cffDNA originates from the trophoblast of the placenta (Masuzaki *et al*., 2004). As a result, NIPT is not a fully diagnostic test, but a screening test. This is because NIPT is not directly testing the fetal genome, but the placenta as a proxy for the fetus, much like that carried out in CVS testing. Due to the similarity of the source of DNA for NIPT and CVS testing, a positive NIPT test result should not be confirmed by CVS testing but by amniocentesis (Zelig *et al,* 2016).

Secondly, cffDNA is present in an excess of maternal cfDNA, and despite enhancement of smaller DNA fragments, many tests (although not all) cannot differentiate and quantify between fetal and maternal DNA. This is a problem if there is an underlying maternal chromosomal abnormality, as this may lead to a false positive NIPT result (Beulen *et al,* 2017). In some rare cases, the pregnant women may be mosaic for trisomy 21 (Down syndrome) and since the maternal genome is present in often 10-20 times greater concentration in the cfDNA than the fetal DNA, even a low level maternal mosaic trisomy 21 can lead to a false-positive NIPT (Song *et al.*, 2013). The most frequently seen maternal chromosome abnormality, when sex chromosomes are included in screening, is low level mosaic Turner Syndrome (where a single chromosome X is missing, alternatively known as 45,X) (Pescia *et al.*, 2017). Again, for some NIPT cases where the cffDNA cannot be distinguished from maternal DNA, this can lead to a false positive screen for Turner syndrome.

Factors such as maternal basal metabolic index (BMI) and whether the conception was a result of IVF can cause fetal fraction to be too low to produce a result (Bergh *et al,* 2013). With high BMI, there appears to be a 'dilution effect', where there is more maternal cfDNA circulating, causing the fetal fraction to reduce, possibly due to an increased turnover of adipocytes. For the latter, it has been suggested that there may be problems with impairment of placentation in IVF pregnancies, which can result in a lower amount of cffDNA in the maternal blood plasma (Bevilacqua *et al*., 2015).

NIPT false positives associated with CPM can be a signal for placental dysfunction (Snyder *et al,* 2016). These false positive results can be an indicator of other problems that can occur later during pregnancy. Follow-up testing with amniocentesis, which rules out chromosomal abnormalities in the fetus, could be an indication that the chromosomal abnormality is in fact present but only in the placenta (Masuzaki *et al.,* 2004). This could potentially interfere with the normal functioning of the placenta. Placental dysfunction can lead to a higher rate of miscarriage, intrauterine growth restriction (IUGR) and hypertension (Grati *et al.,* 2019). It has also been shown that high levels of fetal fraction can be linked to pre-eclampsia (Bischoff *et al,* 2005). Increased levels of apoptosis, and thus increased levels of cfDNA in the maternal circulation, can be due to oxidative stress on the placenta. It has been suggested that those patients that have false-positive results and high levels of fetal fraction should be closely monitored during their pregnancy (Taglauer *et al*., 2014).

1.2.3.2 Technology

The first successful application of NIPT was possible due to the introduction of massively parallel genomic sequencers that enabled millions of sequence reads to be made in parallel from each cfDNA sample (Song *et al*, 2013). This has made it possible to sequence multiple samples at the same time and reduce the turn-around time. This has further reduced the cost of testing and made this form of prenatal screening commercially viable. There are currently three different methods to carry out NIPT. These are, quantitative massively parallel shotgun sequencing (MPSS), quantitative targeted massively paralleled sequencing (MPS) and qualitative single nucleotide polymorphism (SNP) based targeted sequencing. Here, we briefly discuss the differences in these methods and how this can affect the performance of the tests.

MPSS, reads from all chromosomes rather than from specific chromosomes (i.e. 13, 18, 21, X and Y). Maternal and fetal DNA are sequenced at the same time, but it is not possible to distinguish between the maternal and fetal DNA. An algorithm is used to count the number of chromosomes, and a ratio is produced to see if one chromosome is over- or under-represented (Buysse *et al,* 2013). The accuracy of this test is related to fetal fraction, in that the higher the fetal fraction, the easier it is to distinguish when one chromosome has a higher representation than the others. The algorithm uses a reference database of previously analysed population to determine thresholds for trisomy. When the number of reads per sample is lowered, this allows for more samples to be analysed in the same sequencing run, although a lower number of reads can result in an increase in no

results returned (Buysse *et al,* 2013). There can be variation in the efficiency of amplification in individual chromosomes; this can alter the ratio of reads, which can result in false positives. Sex chromosome detection is not as sensitive in this method as with other chromosomes. When testing for chromosomes 13, 18, 21, X and Y, this accounts for only about 14% of the reads from sequencing, therefore, the rest of the genome which has been sequenced would be wasted. However, this means that there is potential to expand screening to all other chromosomes (Kotsopoulou *et al*., 2015).

Targeted MPS, is similar to MPSS but only amplifies those chromosomes of interest i.e. 13, 18, 21, X and Y. The chromosomes are then counted in the same manner. A benefit to targeted MPS is that more samples can be sequenced simultaneously and at higher reads (Go *et al,* 2011).

Qualitative SNP-based targeted sequencing using a genotype analysis as opposed to a counting analysis. This method targets SNPs on specific chromosomes. This method gives information regarding chromosome copy number but can also give further information and allow for more complex analysis such as, inheritance patterns (Dar *et al.,* 2014). The maternal and fetal DNA contributions are identified. Some forms of this testing are not suitable for patients that have received oocyte donation, as the maternal information is not available. One test that is commercially available, simultaneously, tests for DNA which is exclusively from the mother, this information is used to remove her DNA from the

cfDNA that is detected in the plasma (Kotsopoulou *et al*., 2015). SNP based targeted sequencing offers very low false positives and the ability to detect triploidy.

1.2.3.3 Clinical applications

NIPT is most commonly available to women who are at a higher risk of having a pregnancy with a live birth trisomy. High risk pregnancies are those associated with AMA (35 years or older at delivery), positive screening test results (as previously mentioned), history of pregnancies with a trisomy, previous miscarriage, multiple gestation and parental balanced Robertsonian translocation (Oepkes *et al*, 2016). It has been suggested that NIPT should be available for all pregnancies; however, the benefits of this are debated. For example, women who are considered low risk can still have pregnancies which result in a live birth trisomy. In the United States, it is estimated that 1 in 1300 pregnancies will be affected by trisomy 21 for a woman of 25 years old (Newberger, 2000), and this increases to 1 in 400 at 35 years of age. However, '80% of all children born with Down syndrome are born to mothers under 35 years' (Bunt & Bunt, 2014). Yet, there is concern that the number of true positive results from NIPT will be outweighed by an increased number of false positive results leading to unnecessary invasive procedures and possible miscarriages as a result (Bianchi, 2017).

It is highly recommended that women seeking NIPT are adequately counselled before and after testing. There is concern that with the rapid uptake of NIPT, clinics are not able to keep up with providing appropriate counselling. It should be emphasised that NIPT is a

screening test and not diagnostic. Despite this, it is still a highly sensitive and specific screening test, by comparison to conventional screening methods mentioned previously. A study in 2016 by Mackie *et al*. reviewed the results from 117 studies to determine the accuracy of NIPT. The results for fetal trisomies were, for sensitivity and specificity, respectively:

- Trisomy 21: 99.4% and 99.9%
- Trisomy 18: 97.7% and 99.9%
- Trisomy 13: 90.6% and 100%

Factors that can lead to a false positive result include those mentioned previously and CPM, where the aneuploidy is confined to the placenta and the fetus is unaffected.
It is recommended that counselling should be offered for other problems that can arise due to a false positive and aneuploidy confined to the placenta e.g. placental dysfunction, suggesting that further monitoring later in the pregnancy could be beneficial (Bianch *et al*, 2012). Maternal chromosomal abnormalities could be identified from screening and can lead to a false positive result for the fetus. This should be explained to women before NIPT to determine if they would like these findings disclosed. It has also been shown that false positives for multiple aneuploidies can come about when there is undetected cancer in the mother (Snyder *et al*, 2016). Another factor which can cause false positive results is if there has been co-twin demise (Snyder *et al,* 2016). If it is known that one twin has 'vanished', it is important to counsel patients that a positive result could be due to a chromosomal abnormality in the demised twin. Although the twin is no longer detectable through ultrasound, the placenta could still be intact and able to contribute to the cffDNA

in the maternal circulation (Niles *et al*., 2018). In these instances, it can be recommended to wait a couple of weeks and repeat the NIPT to see if the result is still positive prior to carrying out an invasive procedure.

False negative results are much rarer than false positives. It is estimated that potentially 0.2% of cases in high risk patients could be missed and 0.02% in the low risk patients (Van Opstal *et al*., 2016), whilst this is rarer than false positives. The two main factors which can lead to a false negative result are, CPM and low-level trisomy mosaicism. As with CPM seen in false positives, the trophoblasts from the placenta could be chromosomally normal, presenting a normal karyotype with NIPT. Whilst, in fact, the fetus has a trisomy, therefore this would be missed by NIPT. Low-level trisomy mosaicism is where not all cells in the fetus and the placenta possess the trisomy. Where the trisomy is very low, for example <30% of the cells are affected, the fetal fraction which represents the trisomy will be further reduced (Wang *et al.,* 2013). Therefore, the normal cells will have a greater representation, indicating a negative NIPT result and the low-level trisomy would be missed. However, low-level trisomy mosaicism is known to present a problem for prenatal clinicians, as it is not clear what percentage level will result in an affected live birth. For example, Tang *et al*. 2017, have reported on cases of low-level trisomy 3 mosaicism, where investigation through amniocentesis has presented mosaicism between 8 and 10% but resulted in a normal live birth. Normal outcomes have also been seen at 16 and 22% mosaic, but congenital anomalies at 36% (Tang *et al.,* 2017). It is important that patients are counselled that there is a chance for false negative results. It is also recommended

that follow up screening for structural abnormalities should be offered to all patients, to aid in detection of those low- level mosaic trisomies that may be missed by NIPT.

It is advantageous to be able to apply NIPT to twin pregnancies as the risks of aneuploidies in these pregnancies is higher than with singletons (Bevilacqua *et al*., 2015). The risk of miscarriage associated with invasive techniques for testing are also higher in twin pregnancies. Twin pregnancies present challenges when it comes to NIPT. It is important to understand that, in the case of twins, the amount of cffDNA does not double (Bevilacqua *et al*., 2015). It appears that cffDNA increases by approximately a third when comparing twins to a singleton and the contribution of cffDNA from each twin can vary. With dizygotic twins (those from two different oocytes) there is a greater challenge than with monozygotic (those from the same oocyte), as it is not possible to identify which fetus is affected when a trisomy is detected (Tagleur *et al.,* 2013). If one twin has a trisomy, that twin could be under-represented in terms of cffDNA and a false negative result could occur. This is particularly true of trisomy 18 and trisomy 13 as these are often related to a low fetal fraction. Despite this, testing is possible with NIPT, accepting that the accuracy is not as high as that seen in singleton pregnancies and there is a higher testing failure rate seen (Bevilacqua *et al*., 2015). It is not possible to be conclusive with the sex of the twins, however in the case of Y chromosome being detected it indicates that at least one of the fetus is likely to be male.

The most common NIPT offered is for trisomy 21, trisomy 18, trisomy 13 and sex chromosome aneuploidy. However, testing for microdeletions, cri-du-chat and Prader-Willi/Angelman syndrome is beginning to emerge. These tests may not be as sensitive as that seen in conventional NIPT, due to the biological nature of these micro-deletions and the early stage of testing (Schwartz *et al.,* 2018). Most importantly, NIPT does not screen for all birth defects and it is important that women have additional screening for all other chromosomal abnormalities and neural tubal defects. One downside to the increased uptake of NIPT is that there has been an increase in the number of infants born with the conditions which are not screened for using cffDNA. This is thought to be linked to the decrease in the amount of invasive procedures carried out (Beaudet, 2016).

Overall, there are less pregnancies being exposed to invasive procedures because of the uptake of NIPT. For women who receive a positive NIPT, it is vital to ensure that they are fully informed regarding the importance of follow up diagnostics for confirmation. While NIPT can be offered early in the pregnancy, the wait for a subsequent invasive diagnostic test may seem unacceptable to some women. For example, one study found that 6.2% of cases had terminated directly after a positive NIPT result (Dar *et al*., 2014). It has been suggested that offering early amniocentesis might be worth considering for patients who are unwilling to wait and would otherwise opt for termination of pregnancy following a positive NIPT result (Zelig *et al*., 2016).

1.2.4 The future of non-invasive prenatal screening

NIPT is a relatively recent advance to the world of prenatal screening, and as such we would expect to see new developments and improvements in the future. With improvements in technology, expanding the scope and reducing the cost of NIPT seems increasingly possible. It would be ideal to expand the limits of testing for NIPT, so that it would be possible to screen for a wide range of fetal abnormalities in one test. The first area of expansion of NIPT, is to screen for all chromosomal trisomies, or rare autosomal trisomies (RATs). It has been suggested that trisomies on other chromosomes can influence the algorithm, resulting in false positive results for those chromosomes that are reported and test failures (Bianchi *et al*., 2017). Therefore, while it might be beneficial to offer as much information regarding all chromosomes, avoidance of false positive results needs to be considered.

An area of much recent focus is that of microdeletion screening. NIPT for microdeletions involves more time and cost to detect subchromosomal abnormalities. For example, 71.8% of these abnormalities can be detected at 3.5M reads, yet this increases to 94.5% with 10M reads (Liu *et al*., 2016). It is hoped that in the future, with improved technolog and cost-effectiveness, microdeletion screening will become a more viable option.

Another future possibility is for NIPT to screen for single gene disorders, such as cystic fibrosis, sickle cell anaemia and Huntingdon's disease. One suggested method involves the analysis of both parents to identify the affected alleles which may be inherited from each

parent. However, this can present a similar challenge to that seen when screening for trisomies i.e. the fetal DNA exists within an abundance of maternal DNA. To identify whether the fetus has inherited the affected alleles, the cfDNA will need to be analysed for relative mutation balance or imbalance. For example, a mother who is a carrier of a single gene disorder, possessing one affected allele and one unaffected allele will have 50:50 (affected, unaffected, respectively) represented in her cfDNA. Where there is a fetal fraction of 10% the mutant allele load will increase (55:45) when affected, decrease (45:55) when unaffected or remain the same (50:50) when a carrier, depending on fetal inheritance of the affected alleles (Liu *et al*, 2016). Another method is analysis of haploblocks, to build a picture of the direction of allele inheritance. As these methods involve the sequencing of, at least the parents and the fetus there is increased cost involved. It is therefore, thought that targeted sequencing for the genes affected may be more cost effective; however, methods to avoid ADO would be necessary to increase accuracy (Liu *et al,* 2016).

There are still some unanswered biological questions relating to fetal cfDNA. For example, whilst it is widely accepted that the majority of cffDNA originates from trophoblast cells from the placenta, the amount contributed from this source is not known, or, if it is the only source. Details of the mechanism of cffDNA production and the nature of cfDNA clearance, both maternal and fetal, from maternal plasma during pregnancy, is not fully understood. Further knowledge in these areas would greatly benefit NIPT to aid in future developments and increased accuracy.

Advances in technology could see a return to analysis of whole fetal cells. If WGA and analysis were successfully applied to isolated whole fetal cells, it would make it possible to screen for all abnormalities, including copy number variation, microdeletions, and point mutations. WGA and analysis of whole fetal cells, would then make prenatal testing truly diagnostic whilst being non-invasive.

1.3 Rationale for this thesis

PGT-A and NIPT are subject to criticism relating to their levels of accuracy due the origin of the DNA for assessment, the trophectoderm for PGT-A and later the trophoblast of the placenta for NIPT. Both methods are screening assays for aneuploidy in the preimplantation embryo and developing fetus and thus indicative rather than necessarility diagnostic of the karyotype of the subsequent fetus. For PGT-A, increasing knowledge of mosaicism within the embryo, the frequency of mosaicism, whether these embryos if transferred will result in healthy live births are all important information to know for the future application of PGT-A. The controversy regarding PGT-A and if it does result in increased live birth rates following IVF continue and will remain a key topic for future research. For NIPT, more information is required to reduce the frequency of false positives and identifying which patients will benefit most from its application. Retrospective analysis of data pertaining to NIPT and PGT-A could offer large amounts of information available for research areas which as of yet have not been addressed. Some areas that have been identified to be missing in the literature are outlined below:

As mentioned under section 1.1.8, NGS is capable of detecting mosaic embryos that may have formerly been identified as euploid following aCGH. It is currently not clear that when mosaic embryos are reported following PGT-A, how often clinicians are likely to be presented with the dilemma of having to decide if a mosaic embryo should be considered for transfer, when faced with no euploid embryos available for transfer. An investigation

regarding how often a patient is likely to only have a mosaic embryo for transfer, and what type of chromosomal abberations may be considered would be valuable information for clinicians and patient counselling.

As described in section 1.1.5, variation between different clinics included in retrospective studies and RCTs could be playing a big part in the results that are presented. Factors such as approaches to ART, differing methods of biopsy, and biopsy practitioner skill levels could have a significant impact on the outcomes following PGT-A. To date no studies have separated out clinics included within their analysis. The degree to which PGT-A success rates vary between clinics would be invaluable, especially if insight could be learned regarding which variables are having the most impact.

NIPT is most commonly offered to high risk patients as mentioned under section 1.2.3.3, there are a variety of referral indications that lead to patients being referred for NIPT. The more common referral reasons are abnormal ultrasound and positive biochemical screen but can also include (but are not limited to): previous affected pregnancies, previous miscarriages, and multiple gestation. It has not been demonstrated in the literature which of the referral reasons are most likely to result in an aneuploidy being detected following NIPT. By reviewing NIPT cases in terms of their likelihood to have an aneuploidy detected based on their referral reason, can assist with future guidance regarding the most appropriate patients to refer for NIPT.

There are a number of reasons why false positives can occur with NIPT. One such reason that has been proposed is aneuploidy being present on a non test chromosome, which affects the reliability of the algorithm for detecting aneuploidy on the intended test chromosomes, as discussed under section 1.2.4. Currently, there are no details in the literature of false positive NIPT results being interrogated to assess if there was, in fact, aneuploidy present on another chromosome which was not the subject of initial testing. By doing so, this could indicate whether this is a common cause for false positives and if by extending NIPT to test for all chromosomes, if these false positives could be avoided in the future.

1.4 Specific aims

With reference to the above rationale, the specific aims of this thesis were:

Specific Aim 1: To establish how often only a mosaic embryo is available for transfer in a PGT-A cycle? To consider what proportion of these would be considered for transfer when reviewed using the position statement established by CoGEN (the "Controversies in Genetics" forum)?

Specific Aim 2: To determine, by retrospective analysis of the largest cohort to date, if there is an improvement in live birth following PGT-A compared to conventional IVF. To test the hypothesis that there is inter-clinic variation between these figures.

Specific Aim 3: To determine which of the many referral indications are more likely to return a result of "aneuploid" following NIPT.

Specific Aim 4: To test the hypothesis that previous cases identified as "suspected aneuploidy" for chromosomes 13, 18, 21, XY, but subeseqently identified as normal at follow up, were identified because of the presence of a rare autosomal trisomy, or a technical fault.

2 Materials and methods

The projects carried out within this thesis were based on retrospective data analysis. The samples had been processed by CooperGenomics UK. I did, however, observe all the processes involved and was involved in some of the processing of NIPT samples. Here, I outline the methods involved to obtain the genetic results for both PGT-A samples and NIPT samples.

CooperGenomics UK validated their aCGH pipeline by testing blinded cell lines, once completed the results from aCGH were compared to the cell lines for concordance. NGS was subsequently validated by the processing of samples through both the aCGH pipeline and the NGS pipeline to see if the same result was achieved. The second phase of validation for NGS involved running samples in tandem with the already validated US groups NGS pipeline. Additional mosaic chromosome variants were detected by NGS when compared to aCGH for a small number of embryo samples, this was expected given the increased sensitivity of the NGS process. All NGS results were concordant with the US groups results.

It has been estimated that the false positive rate for PGT-A is around 10%, following a non-selection study carried out by Scott et al, 2012, here 4 live births were reported following the transfer of 99 embryos deemed to be aneuploidy following PGT-A, based on the live birth rate for the euploid group of 41.4%, it is assumed that a roughly similar amount that were viable in the aneuploid group would have implanted, leading to a false positive rate of 10% being inferred (Rosenwak et al, 2018). Although there has only been

one non-selection study carried out to date, and this was carried out using SNP based analysis. There is no current false positive rate known for NGS.

2.1 Preimplantation Genetic Testing for Aneuploidy Studies

2.1.1 Embryo biopsy samples

Blastomere and trophectoderm embryo biopsy samples used in retrospective clinical data analysis studies were from patients undergoing ART cycles with PGT-A at multiple fertility clinics, where genetic analysis of the biopsy samples were processed at CooperGenomics UK & CooperGenomics USA.

Embryos were biopsied in accordance with each fertility clinics standard operating procedures, prior to being shipped to CooperGenomics where embryo biopsy samples were processed in accordance with CooperGenomics standard operating procedures between January 2011 and December 2017.

2.1.2 Embryo biopsy practice

Embryo biopsy practice has varied over the years, dependent on emerging evidence for best practice for embryo biopsy. However, each clinic will assess their own biopsy technique and success rates to determine the method of embryo biopsy used. It is therefore not possible to include the method used by each individual clinic for embryo

biopsy samples which were included in the following research studies. Below I outline the most common embryo biopsy methods used.

There are three stages of oocyte and embryo biopsy that have been conventionally performed, these are, 1st and 2nd polar body biopsy, cleavage stage biopsy on day 3 of embryo development and trophectoderm biopsy on day 5 or 6 of embryo development. At all stages the oocyte or embryo is observed and handled using a micromanipulator, this allows for the oocyte or embryo to be viewed under a high powered microscope and finely manipulated, whilst being kept at a constant temperature on a heated stage. This reduces negative impact on the embryo during the procedure. The oocyte or embryo is positioned in the optimum position for biopsy using biopsy micromanipulation pipettes, whilst held in place using the holding pipette, a perforation is made in the zona pellucida using a LASER at the biopsy site. The biopsy site can also be perforated mechanically, using a pipette to pierce through the zona, or a chemical called Tyode's solution can be used to dissolve part of the zona. However, LASER is the most common method currently in use clinically. The biopsy aspiration pipette is then used to remove the required cells from the oocyte or embryo biopsy site. The biopsied cells are subsequently washed in phosphate-buffered saline (PBS) using a manual pipette and 'tubed', placed into a PCR tube containing 2.5ul of 1x PBS. A 'blank' PCR tube is also filled with PBS to ensure there is no genetic contamination at this stage and as a negative control.

Polar body biopsy is most frequently carried out after insemination of the oocyte on day 0

to retrieve the first polar body and after fertilisation check on day 1 to retrieve the second polar body. Although both the first and second polar body can be retrieved together on day 1.

Cleavage stage biopsy is usually carried on day 3 embryos that have reached at least 5 cells, 1 or 2 cells can be removed for analysis, although most commonly 1 cell is removed to reduce detrimental impact on the developing embryo.

Blastocyst or trophectoderm biopsy is most frequently carried out on day 5 of embryo development but can also be carried out on day 6 of embryo development, for those embryos which take longer to reach the blastocyst stage. 4 to 10 cells from the trophectoderm are retrieved for analysis. Biopsy is normally carried out at the furthest point from the inner cell mass (ICM) to avoid any inadvertent damage to the ICM.

In the past cleavage stage biopsy was the most popular stage for biopsy, however, trophectoderm biopsy in recent years has become more often the preferred stage for biopsy. Trophectoderm samples make up the majority of samples analysed and recorded within my retrospective data analysis.

Occasionally, whole embryos will be sent for analysis. These are sent in the following two cases: firstly, embryo arrest, once the embryo has arrested it is no longer viable for transfer and a clinic may send the sample in to gain further information as to whether the

embryo has arrested due to chromosomal abnormality. The second is for follow up information, if an original sample has been reported as aneuploid and unsuitable for transfer, the clinic may send the embryo for analysis to see if the whole embryo is the same as the original biopsy result.

2.1.3 Whole genome amplification

Whole genome amplification (WGA) was carried out on all biopsied samples received by CooperGenomics as soon as possible, to extract the biopsied cells' DNA and to amplify the DNA to make 2-4ug of DNA available for sequencing. The SurePlex DNA amplification system (Illumina, Inc, San Diego, CA, USA) was used to extract and to amplify the samples.

Each WGA reaction contained a positive control, which is a diluted DNA sample and negative control, containing only mastermix from each step, these were prepared in 0.2ul PCR tubes. The samples were first put through a cell lysis DNA extraction process as follows: Cell extraction buffer and extraction enzyme dilution buffer were vortexed for 15-20 seconds. All samples and reagents plus cell extraction enzyme were spun in a mini centrifuge for 15-20 seconds to ensure the biopsy samples and reagents are at the bottom of their tubes. Biopsy samples were then kept in a cooling block at 4°C. The reagents were mixed together in a 0.6ml eppendorf tube in accordance to how many samples were being extracted up to 98 in one extraction at a ratio of 3:4.8:0.2 (Cell extraction buffer: Extraction Enzyme Dilution Buffer: Cell Extraction Enzyme). Once the "extraction cocktail"

had been vortexed, 8µl was added to each biopsy sample. The samples were centrifuged to ensure that the "extraction cocktail" mixed with the sample. All tubes were placed into a thermal cycler with the following programme: 1 cycle: 75°C for 10 minutes; 1 cycle: 95°C for 4 minutes; 1 cycle: hold at room temperature (23°C). The samples were then transferred back to the cooling block at 4°C.

Following cell lysis, the samples went through a pre-amplification stage. A "Pre-Amp cocktail" was prepared in a 0.6ml eppendorf tube using pre-amplification buffer and pre-amplification enzyme, in accordance to how many samples were being amplified at a ratio of 4.8:0.2. The "Pre-Amp cocktail" was vortexed and 5µl was added to each biopsy sample. The samples were centrifuged to ensure that the "Pre-Amp cocktail" mixed with the biopsy sample. All tubes were placed into a thermal cycler with the following programme: 1 cycle: 95°C for 2 minutes; 12 cycles: 95°C for 15 seconds, 15°C for 50 seconds, 25°C for 40 seconds, 35°C for 30 seconds, 65°C for 40 seconds, 75°C for 40 seconds; 1 cycle: 4°C hold. The samples were again transferred back to the cooling block at 4°C.

The samples at this stage were ready to go through the amplification stage of the process. The "amplification cocktail" was prepared in a 1.7ml eppendorf tube using nuclease free water, amplification buffer and amplification enzyme, in accordance to how many samples were being amplified at a ratio of 34.2:25:0.8. The "amplification cocktail" was vortexed and 60µl was added to each sample. The samples were centrifuged to ensure that the "amplification cocktail" was mixed with the samples. All tubes were placed into a thermal

cycler with the following programme: 1 cycle: 95°C for 2 minutes; 14 cycles: 95°C for 15 seconds, 65°C for 1 minute, 75°C for 1 minute.

Following the WGA step, the samples were checked using gel electrophoresis to confirm that DNA was present from the biopsied samples. An additional positive control (previously amplified samples, which were subsequently aneuploid and not transferred) and negative control (millipore water) were added to the gel electrophoresis. 5µl of each WGA sample was mixed with 1.1µl of gel loading dye into a strip of PCR tubes, they were vortexed and centrifuged prior to loading 5µl into each well of the gel. The gel was run for 5 minutes. The gel was then visualised in the dark on a UV light box, the samples were confirmed for amplification where banding is present along with control positives. All blanks were also checked for signs of DNA contamination, as well as control negatives. Any samples that had not amplified did not proceed to sequencing and the clinics were informed of amplification failures.

2.1.4 Copy number analysis

Two forms of copy number analysis have been used on PGT-A samples between January 2011 and December 2017. Initially aCGH was the copy number analysis method of choice, however with developments in technology, this progressed to NGS. Samples have been processed using NGS from August 2015 where there was a slight overlap in the technologies between August 2015 and October 2015.

2.1.4.1 aCGH preparation, imaging, and analysis

All samples which were successfully amplified were prepared for hybridisation and digital fluroscent imaging. During preparation of the samples, it is advised that the lights be turned off. Two reference DNA samples were required, a SureRef reference male DNA and SureRef reference female DNA.

The samples were first labelled with a corresponding dye. Master mix cocktails were prepared, cyanine dyes were used: Cy3 labelling mastermix in a green 1.7ml tube and Cy5 labelling mastermix in a red 1.7ml tube. The mixes were prepared by adding 73.5µl of dCPT labelling mix, 73.5µl of reaction buffer and 14.7µl of Klemow enzyme to both tubes, then 14.7µl either Cy3 or Cy5 dye to their corresponding tube.

5µl of primer solution was aliquotted to all red and green PCR strip tubes. 8µl of whole genome amplified product or control was added to its corresponding position in the PCR strip tubes (the position for each patient and control is set prior to beginning sample preparation). The tubes were vortexed and centrifuged for 10-15 seconds. The tubes were then placed into the thermal cycler, which was pre-set at 80°C and a 'denature' cycle was run, where the samples were heated to 94°C for 5 minutes. Once the cycle had completed the samples were immediately transferred to a cooling ice-block and put into the fridge for 5 minutes. After the samples had been cooled, 12µl of Cy3 master mix cocktail was added to all the green PCR strip tubes and 12µl of Cy5 master mix cocktail was added to the red PCR strip tubes. The PCR strip tubes were subsequently vortexed and centrifuged.

The tubes were then placed into the thermal cycler and run on either a 'labelling' programme or a 'short labelling' programme. The 'labelling' programme incubated the samples at 37°C for two to four hours and can be extended where required (e.g. overnight), the 'short labelling' programme, incubated the samples at 37°C for one hour and then was followed by 15 minutes at 65°C. If the 'short labelling' programme was used, the samples needed to be removed from the thermal cycler immediately after the 15 minutes at 65°C and processed immediately.

Following the labelling step, the samples were combined and evaporated down. The PCR strip tubes containing the samples were centrifuged for 10-15 seconds. The Cy3 samples (green PCR strip tubes) were combined with the Cy5 samples (red PCR strip tubes), (the positions of the corresponding samples were set prior to beginning sample preparation). 25µl of COT Human DNA was added to each sample of the PCR strip tubes. They were then vortexed and centrifuged for 10-15 seconds. The tubes were transferred with their caps open to a pre-warmed centrifuge. The centrifuge was set to heat to 80°C for 45 minutes. This allowed the samples to evaporate leaving a 2-3µl pellet in each tube.

The samples were then ready for the hybridisation step. 22µl of hybridisation buffer which had been pre-warmed to 75°C, was added to the pellets which had formed in the PCR strip tubes, the pellet was mixed using a pipette with the buffer to re-suspend. The samples were then vortexed for 10-15 seconds before placing them into the thermal cycler which was pre-warmed to 75°C, the samples were incubated for 5-10 minutes. Once the samples

had completed incubation, the samples were vortexed and pulse centrifuged and placed back into the thermal cycler, if required, for a further 5 minutes, until the pellet was dissolved. The tubes were further pulse centrifuged following the pellet being dissolved. The Hybex Microsample Incubator (SciGene, CA, USA) needed to be turned on and set to 47°C. The hybridisation chamber was prepared by first saturating an absorptive pad with millipore water, this pad was placed into the base of a metal slide holder, which was placed into the hybridisation chamber. A hybridisation template planner (placed on top which comes with 24sure V3 microarrays), was taken and cover slips were positioned in accordance with pre-determined positioning. 18.5µl of labelled DNA solution from the PCR strip tubes was added to the centre of each cover slip, the slide was then lowered into the cover slip with the barcode facing down, to ensure the position of the DNA on the slide. After the sub-arrays have attached onto the array slide, the slide was flipped over and placed onto the hybridisation template and aligned with the template.The arrays were placed into the hybridisation chamber and the lid was firmly applied. The hybridisation chamber was incubated at 47°C for 3-4 hours.

The slides with hybridised DNA were at this point ready for the wash step. The slides were removed from the Hybex Microsample Incubator, and cover slips carefully removed. Wash buffer 1, (which consists of 899.5ml of millipore water, 100ml 20x saline sodium citrate (SSC) and 500ul 20 x Tween 20) was added to a glass bowl which was placed onto a magnetic stir plate and the slides were incubated in wash buffer 1 for 10 minutes. Next wash buffer 2, (which consists of 950ml millipore water and 50ml 20x SSC) was added to a

new glass bowl which was placed onto the magnetic stir plate, the slides were incubated for a further 10 minutes in wash buffer 2. Wash buffer 3, (which consists of 995ml millipore water and 5ml of 20x SSC), was placed into the Hybex Microsample Incubator and preheated to 60°C, the slides were placed into wash buffer 3 in the incubator for 5 minutes. The slides were placed into another glass bowl containing more wash buffer 3 and incubated at room temperature for a further minute. The slides were then placed into a centrifuge for 3 to 5 minutes until dry.

The slides were then ready to be scanned. The slides were loaded into the scanner facing down and the lid closed. On the computer using GenePix® Pro 7.0 (Molecular Devices, LLC, USA), the whole array was previewed to align the scan area. Once the area had been defined the scan was started. The scanned images were then saved, and the process repeated for each array. The scanned image files were imported into BlueFuse Multi (Illumina, Inc, San Diego, CA, USA), where the fluorescence data from the microarray was processed and analysed against the reference DNA. The images could be viewed in BlueFuse, interpreted for copy number on each chromosome and finally the results were then reported to the referring clinics.

2.1.4.2 Library preparation for NGS

All samples which were successfully amplified were prepared for sequencing using the VeriSeq Library Prep Kit (Illumina, Inc, San Diego, CA, USA). The samples were all vortexed and put into the centrifuge, to ensure that the extracted DNA was mixed evenly within the

sample and spun to the bottom of the tube. A positive control was added at this stage. A 'dilution PCR plate' was prepared by diluting 4µl of each sample with 36µl of water; one well per sample. Once the diluted samples had been mixed using a pipette, the plate was sealed, vortexed and placed into the centrifuge for 1 minute at 1300rpm. 4µl of each sample from the dilution plate was further diluted with 96µl of water into a new PCR plate, this PCR plate was stored on a cold block ready for the tagmentation step.

Earlier NGS samples would have gone through a quantification of WGA DNA using a Qubit 2.0 fluorometer (ThermoFisher Scientific, Ltd, UK) step, this step was later deemed unnecessary and removed. The process was carried out after the first dilution of the samples under dim lights. Qubit working solution was prepared into a 15ml tube; buffer at a volume of 199µl x twice the number of samples and dye at a volume of 1µl x twice the number of samples, was added to the tube before vortexing. The working solution would then be aliquotted into 50 Qubit tubes. 10µl of standards are added to the first two tubes. 5µl of each sample from the dilution plate was added to a corresponding Qubit tube. All tubes were then mixed, centrifuged, and incubated for 2 minutes. The Qubit 2.0 fluorometer was first calibrated using each tube containing a standard and then each sample was quantified. The reading for each sample was assessed, to see if the sample required further dilution. The samples were further diluted if required, based on calculations from the Qubit and Bluefuse Workflow manager, to achieve a DNA concentration of 0.2ng for all samples.

The tagmentation step adds transposomes which cleave to the DNA of the sample, this gives the samples a universal overhang, to which a primer and known index can be added, to identify the sample during sequencing (Figure 2.1). A new PCR plate is labelled VTA, and 8µl of Tagment DNA buffer is added to the first 4 rows. 4µl of Amplicon Tagment Mix was added to these first 4 rows of the Veriseq Tagment Amplicon Plate 'VTA plate'. 4µl of each sample from the last dilution plate was added to a corresponding well on the 'VTA plate'. The plate was sealed, shaken at 1800rpm for 1 minute and centrifuged at 1300rpm for 1 minute. The 'VTA plate' was placed onto a thermal cycler and run on a VS TAG programme for 5 minutes. Once the cycle had finished and the samples had reached 10°C, 4µl of Neutraliza Tagment buffer was added to all wells, the plate was resealed, shaken, and centrifuged as before. The plate was left to incubate for 5 minutes at room temperature.

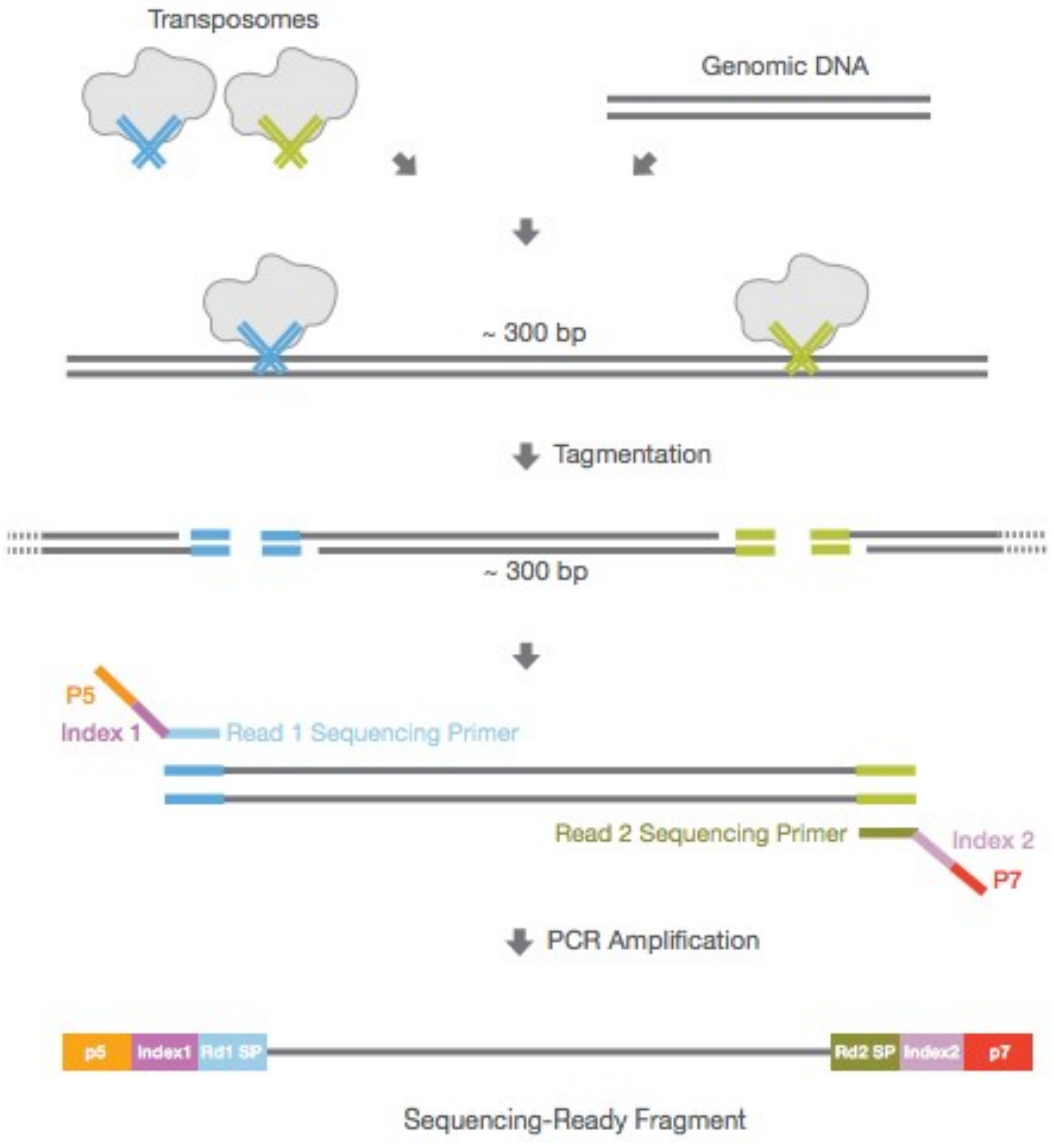


Figure 2.1: Nextera tagmentation reaction step for NGS. "Nextera XT transposome with partial adapters is combined with template DNA Call out. Tagmentation to fragment and add partial adapter. Limited cycle PCR to add sequencing primer sequences and indexes". (Image source: www.illumina.com)

Following tagmentation the indexes were added, and a PCR amplification step was performed. 4µl of the indexes were added to their corresponding wells in the 'VTA plate'. 12µl of Nextera PCR Master Mix was added to each well. The plate was sealed, shaken, and centrifuged as previously described. The plate was then put onto the thermal cycler and the VS PCR protocol was run for 30 minutes.

A PCR clean up stage was then carried out. The 'VTA plate' was first centrifuged for 1 minute at 1300rpm to collect any condensation that may have formed from the PCR step. A new deep-well plate was labelled 'clean-up' and 36µl of AMPure XP beads (PCR purification beads) (Beckman Coulter Life Sciences, Inc, IN, USA) were transferred to 4 rows of the plate. 36µl of the samples from the 'VTA plate' were transferred to the 4 rows of the 'clean-up' plate, the samples were mixed with the beads. The 'clean-up' plate was sealed and shaken for 1 minute at 1800rpm. The plate was incubated at room temperature for 5 minutes and pulse spun in the centrifuge. The 'clean-up' plate was then placed onto a magnetic stand for 2 minutes, checking that the supernatant had cleared once the time was up. The supernatant was carefully discarded whilst the plate remained on the magnetic stand. Whilst the plate was still on the stand, 200µl of 80% Ethanol was added to each well, the plate was incubated at room temperature for 30 seconds. The supernatant was again discarded whilst the plate remained on the magnetic stand, and the ethanol step was repeated, leaving to incubate for a further 30 seconds, the supernatant was again discarded, ensuring all of the ethanol was removed. The plate was left to air-dry whilst on the magnetic stand for 5-15 minutes until completely dry. The

plate at that point was removed from the magnetic plate, 40µl of resuspension buffer (RSB) was added to each well and mixed 5-10 times using a pipette. The plate was sealed and shaken for 2 minutes at 1800rpm. The plate was left to incubate at room temperature for 5 minutes then centrifuged for 1 minute at 1300rpm. The 'clean-up' plate was then placed onto the magnetic stand again for a further 2 minutes. A new plate was labelled 'dsDNA_2', 36µl of the supernatant was moved from the 'clean-up plate' into clean rows of the new 'dsDNA_2 plate'.

The clean double stranded DNA of each sample was at that point in the 'dsDNA_2' plate ready for the library normalization stage. Library Normalization Additives 1 (LNA1) and Library Normalization Beads 1 (LNB1) were both vortexed. In a new 5ml tube, 1.76ml of LNA1 was added, quickly followed by 320µl of LNB1, this was now mixed 15-20 times. A fresh deep-well plate was labelled 'LNP' for library normalization plate, and 36µl of the LNA1-LNB1 mixture was added to 4 clean rows of the plate. 16µl of the DNA from all wells of the 'dsDNA_2 plate' was added to corresponding wells of the 'LNP plate'. The 'LNP plate' was sealed and placed onto the microplate shaker at 1800rpm for 30-35 minutes. The plate was then pulse-spun at 1300rpm before being placed onto a magnetic stand for 2 minutes, ensuring that the supernatant had cleared after that time. Whilst the plate was still on the magnetic stand 52µl of the supernatant was removed, leaving the undisturbed beads in the plate. The plate at that point could be removed from the magnetic stand, 36µl of library normalizing wash (LNW1) was added to all wells which contained the DNA sample. The 'LNP plate' was then sealed and shaken for 5 minutes and then pulse spun.

The 'LNP plate' was again placed onto the magnetic stand for 2 minutes, whilst the plate remained on the stand the supernatant was removed and discarded. The 'LNP plate' was removed from the magnetic plate and the LNW1 step was repeated, adding the LNW1 reagent again, sealed, shaken, pulsed, and incubated on the magnetic stand prior to removing and discarding the supernatant. The 'LNP plate' was once again removed from the magnetic stand, this time 24µl of 0.1N NaOH was added to each well, the plate was sealed and then shaken on the microplate shaker at 1800rpm for 5 minutes. The 'LNP plate' was centrifuged for 60 seconds at 1300rpm and placed on to the magenetic stand for a further 2 minutes. A new 96 well PCR plate was labelled 'LNS' for library normalization storage, 20µl of library normalization storage buffer 1 (LNS1) was added to 4 clean rows of the 'LNS plate'. 20µl of the supernatant from the 'LNP plate' was then transferred to the corresponding wells of the 'LNS plate' and mixed 5 times to combine with the LNS1. The 'LNS plate' was sealed and spun at 1300rpm for 1 minute.

2.1.4.3 NGS

Following the library normalization stage, the samples were ready to pool ready for loading to the Miseq (Illumina, Inc, San Diego, CA, USA). 5µl of each library sample was added to a DNA low-bind PCR tube. Once all the samples had been added, the tube was lightly vortexed. 20µl of the pooled library was then added to a new low-bind PCR tube, 80µl of hybridisation buffer (HT1) was added to the tube to bring the volume up to 100µl. The remaining library pool could be stored in the freezer for up to one week. The new tube containing the pool/HT1 mixture was gently vortexed and centrifuged prior to being

placed into the thermal cycler, the NGS pool protocol was run. Once the thermal cycler reached 4°C the lid could be opened whilst waiting for the program to finish. In a fresh 1.5ml low-bind microcentrifuge tube, 593µl of HT1 and 7µl of denatured 20pM Phix control DNA was added whilst the tube was on a cold block. Once the thermal cycler was finished, the 100µl of the pooled library solution was added to the tube containing the HT1/denatured Phix control mixture, these were mixed together using a pipette, 3 to 5 times before all of the contents were aliquotted to reservoir 17 of the MiSeq cartridge (MiSeq Reagent Kit v3) (Illumina, Inc, San Diego, CA, USA). The MiSeq cartridge and a clean flow cell were loaded into the MiSeq and the sample sheet, containing specific information for each patient sample was uploaded, the Miseq at this point was ready to carry out the sequencing run.

The following is a description of the process undertaken by the MiSeq to sequence the patient samples:

Before the sequencing commences the library adapters which were added to the fragmented DNA become bound to the flow cell at complimentary locations and the fragments were amplified through bridge amplification. This process provides a template for sequencing. During the sequencing step, each single base is detected as it is added to the template DNA strand. As the DNA is fragmented, multiple fragments are sequenced simultaneously. Early NGS was carried out as single-end sequencing, that is the DNA stand fragments are sequenced in a singular direction, this later changed to paired-end sequencing from February 2017, which meant the DNA fragments would be sequenced

from both ends of the fragment, there is a degree of overlap between each end of the paired end read to align the sequencing data during data analysis, this allows paired-end sequencing to have a greater level of accuracy.

2.1.5 Data analysis, interpretation, and reporting

When the MiSeq finished its sequencing run, the data were processed using BaseSpace® (Illumina, Inc, San Diego, CA, USA). The sequencing data, indexes and patient information are matched up. The sequencing fragments are aligned alongside a reference genome. The data were imported into Bluefuse Multi v3 software for NGS to carry out the reading, interpreting, and reporting of the PGS cases. Each sample was looked at independently and results were recorded into the data reader. All cases were read independently by two trained scientists to ensure that they are read and reported correctly.

The Bluefuse software provides an image of the sequencing data points where chromosome copy number can be interpreted, the images that follow are examples of the type of results that can be detected:
In these images the chromosome number is listed along the x axis and the chromosome copy number is listed along the y axis.

A Euploid sample will posses 2 copies of all chromosomes except X and Y, which will have 2 copies of X and 0 copies of Y present for a female and 1 copy of each for a male.

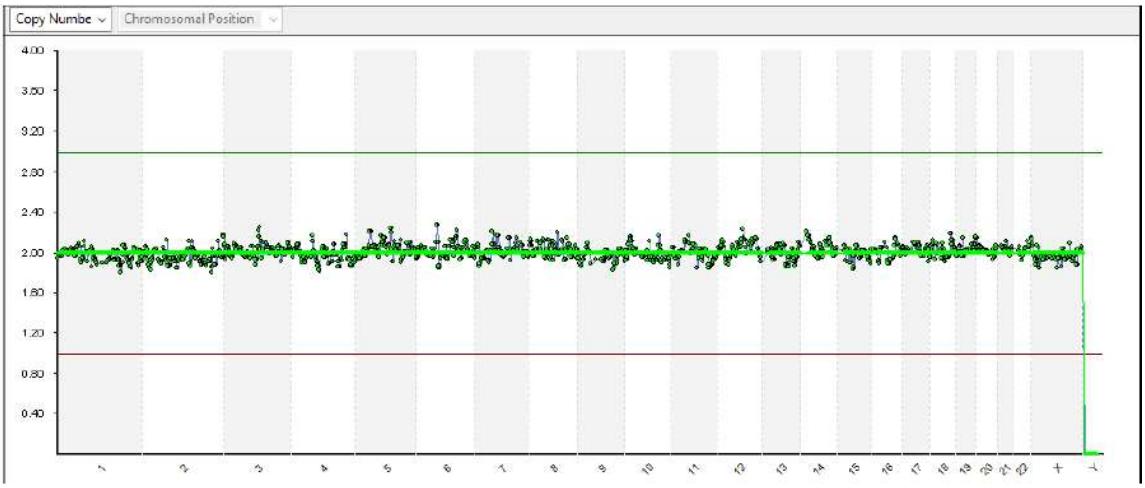


Figure 2.2: Bluefuse PGT-A image, Euploid, XX (with thanks to CoopergGenomics for sharing these images)

In Figure 2.2 we can observe a euploid sample which is XX for sex chromosomes. In the image you can see that the sequence reads two copies of each chromosomes except Y which it detects none present.

A monosomy will present as a whole chromosome dropping to one on the y axis, but the remaining chromosomes will have two copies, except X and Y, which will have two copies of X and 0 copies of Y present for a female and one copy of each for a male. However, in the case of Monosomy X, all chromosomes except X and Y will have two copies, but the X chromosome will appear as 1.0 on the Y axis with the Y chromosome will appear as 0.

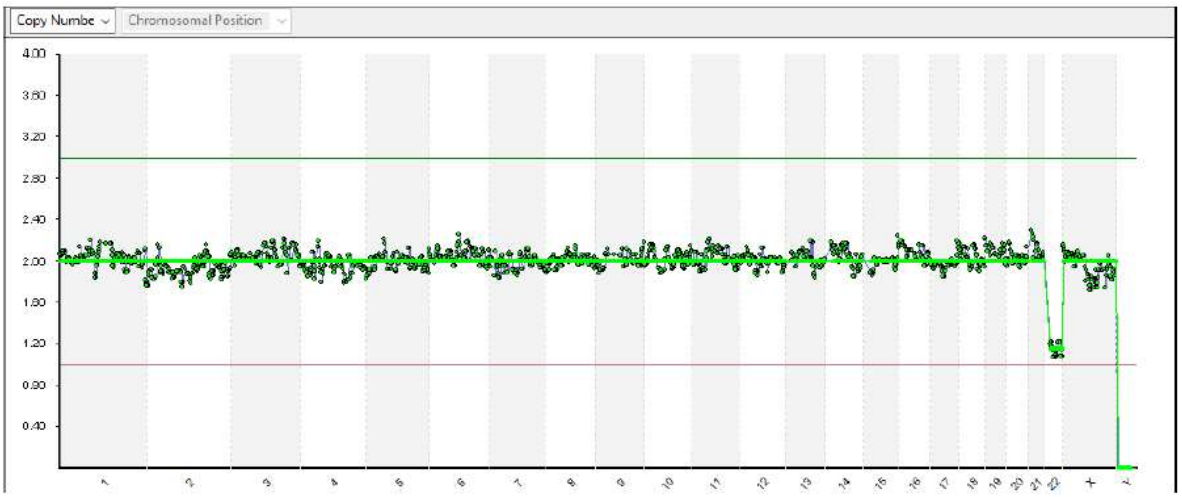


Figure 2.3: Bluefuse PGT-A image, Monosomy, XX (with thanks to CoopergGenomics for sharing these images)

In Figure 2.3 a monosomy at chromosome 22 can be observed where there is one copy of the chromosome being detected. All other chromosomes have two copies of the chromosomes present except at Y.

Trisomic chromosomes will appear at three on the y axis, for a single trisomy, a whole chromsome will increase to 3.0 along the y axis and the remaining chromosomes will have two copies, except X and Y as mentioned previously. However, in the case of sex chromosome abnormalities, you can see that there are two copies of X and one copy of Y in the case of XXY, or one copy of X and two or more copies of Y in the case of XYY.

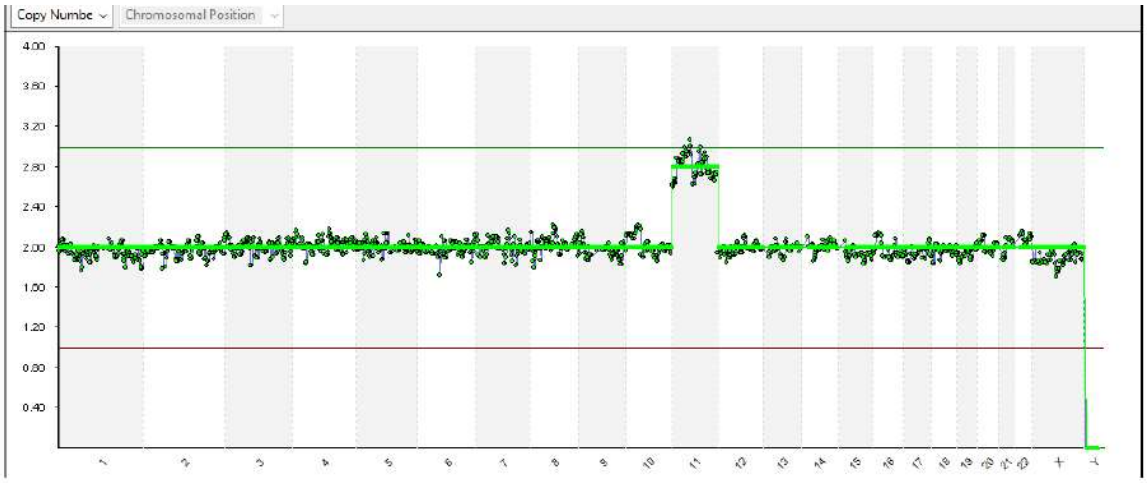


Figure 2.4: Bluefuse PGT-A image, Trisomy 11, XX (with thanks to CoopergGenomics for sharing these images)

In Figure 2.4 an extra copy of chromosome 11 is observed. You can also see that all other chromosomes except Y have two copies of the chromosomes present.

Values between one and two of the whole width of the chromosome on the y axis would represent a mosaic monosomy. A percentage of cells affected can be inferred by comparing the number of cells tested against the copy number presented. For example, if six cells were in the trophectoderm biospy sample and the copy number fell at 1.5 on the y axis, you could establish that three or 50% of those cells processed monosomy and 50% were euploid.

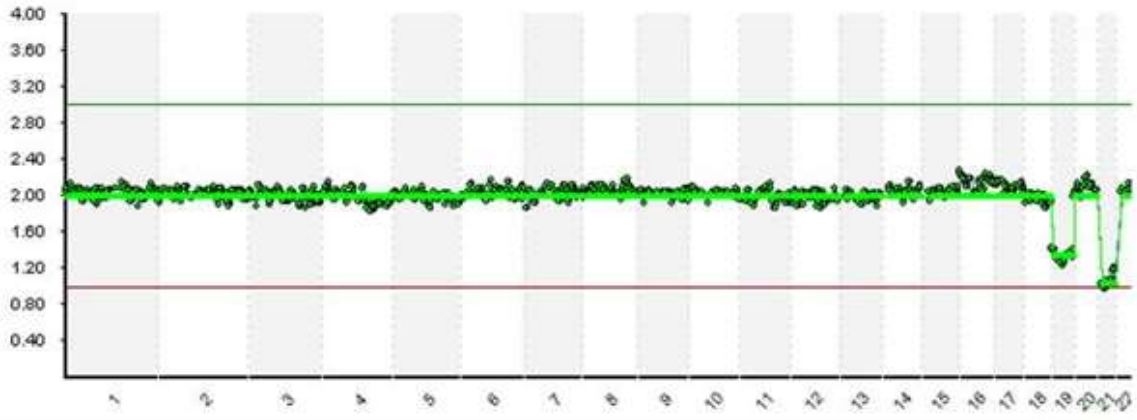


Figure 2.5: Bluefuse PGT-A image, Monosomy 21, Mosaic Monosomy 19 (with thanks to CoopergGenomics for sharing these images)

In Figure 2.5 we can see that there is a mosaic loss of chromosome 19, ranging between 60 and 70% of the cells that were tested. All the other chromosomes possess two copies except chromosome 21 which is a full monosomy.

Values between two and three of the whole width of the chromosome on the y axis would represent a mosaic trisomy. A percentage of cells affected can be inferred by comparing the number of cells tested against the copy number presented. For example, if six cells were in the trophectoderm biopsy sample and the copy number fell at 2.5 on the y axis, you could establish that 3 or 50% of those cells possessed a trisomy and 50% were euploid.

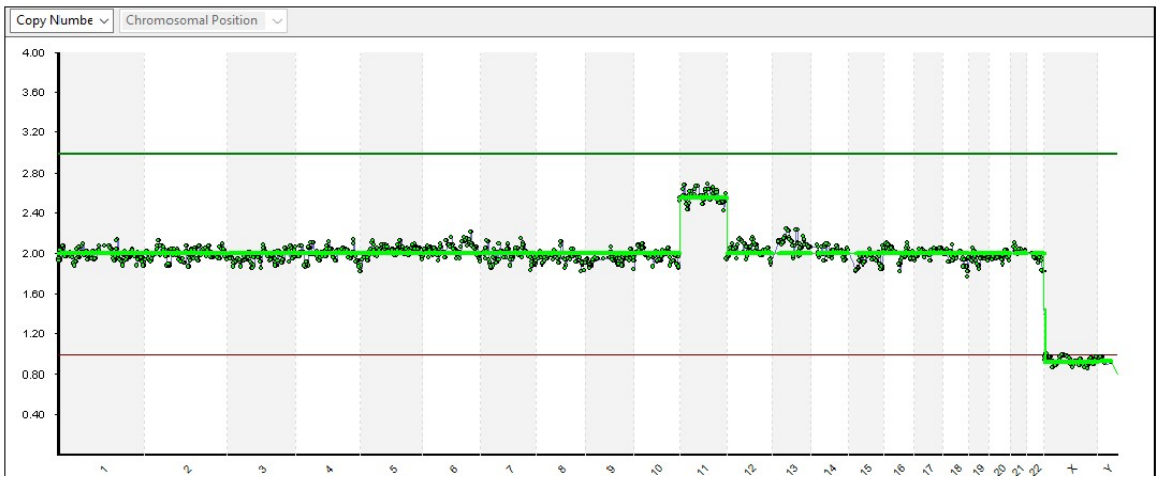


Figure 2.6: Bluefuse PGT-A image, Mosaic Trisomy 11, XY (with thanks to CoopergGenomics for sharing these images)

In Figure 2.6 you can see an example of a mosaic trisomy 11, there is a distinguishable gain on chromosome 11, although this does not reach the 3.0 copy number on the 'y' axis, and therefore we can infer that not all the cells which were included in the biopsy possessed this trisomy. This copy number appears to reach between 2.5 and 2.6 on the y axis, therefore it is likely that between 50% and 60% of the biopsied cells are affected. All other chromosomes possess two copies except for X and Y, which have one chromosome each.

Segmentals gains, losses, mosaic gain and mosaic loss are detected in the ranges mentioned previously, but only affect part of a chromosome instead of the whole chromosome. These can be detected in fragments as low as 1.5Mb. Here are some examples of these types of profiles:

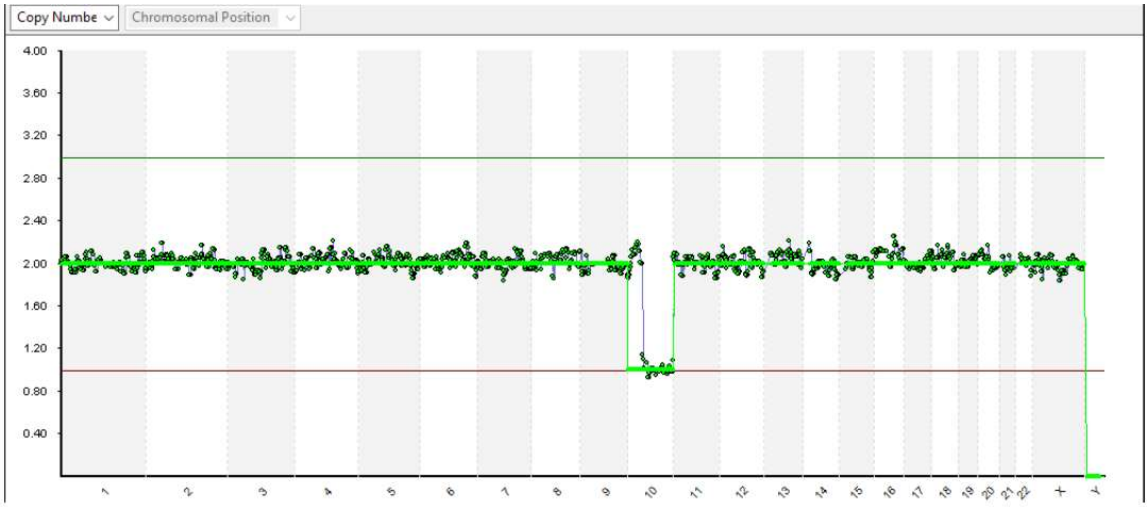


Figure 2.7: Bluefuse PGT-A image, Segmental loss 10, XX (with thanks to CoopergGenomics for sharing these images)

In Figure 2.7 we can see a segmental loss at chromosome 10. You can note that the chromosome along its entire length on the x axis has not been lost, therefore these are interpreted as segmental loss. The individual chromosome can be investigated in more detail on another screen to provide further information where these segmental's lie along the p and q arms if required. All other chromosomes possess two copies except for Y.

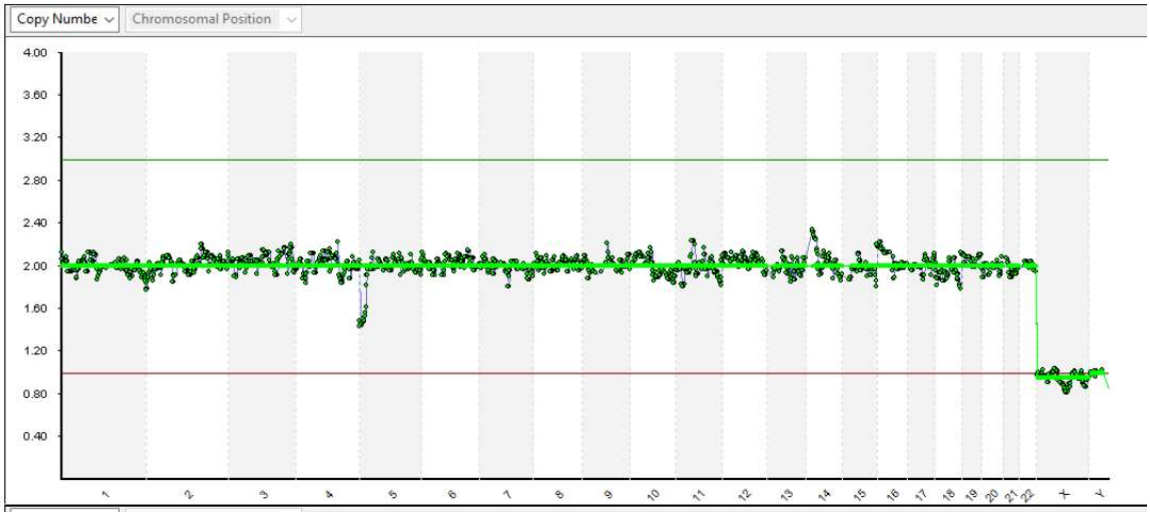


Figure 2.8: Bluefuse PGT-A image, Mosaic Segmental Loss 5, XY (with thanks to CoopergGenomics for sharing these images)

In Figure 2.8 we can see a small dip in the profile on part of chromosome 5. This is a mosaic segmental loss. Only part of chromosome 5 is affected along the x axis, and this drops to between 1.4 and 1.5 on the y axis, indicating that 60% to 50% of cells which were biopsied were affected. All other chromosomes possess two copies except for X and Y.

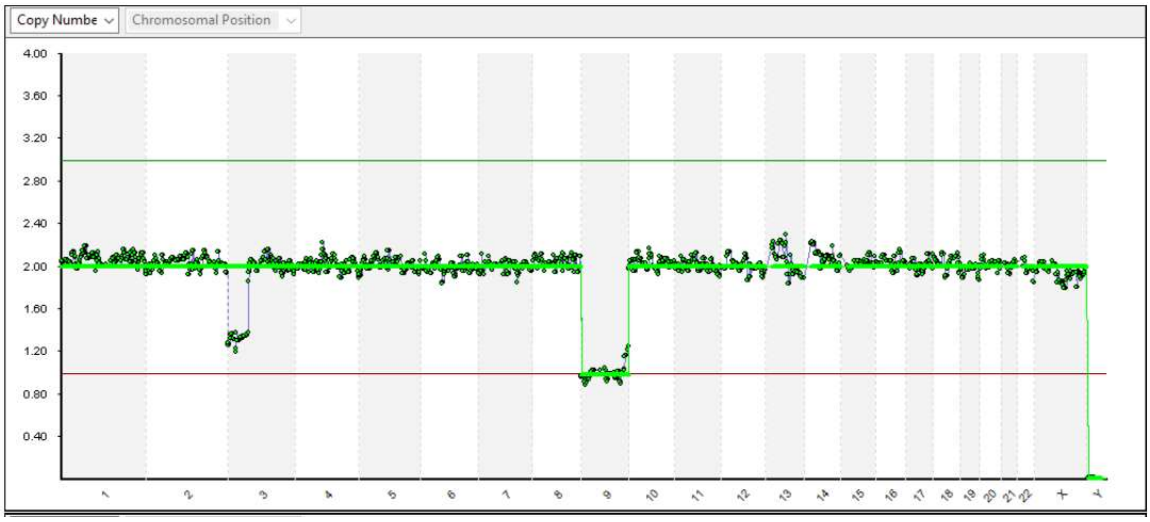


Figure 2.9: Bluefuse PGT-A image, Mosaic Segmental Loss 3, Monosomy 9, XX (with thanks to CoopergGenomics for sharing these images)

In Figure 2.9 we can see that this biopsy sample possess a full monosomy at chromosome 9, this is complete across the chromosome on the x axis and reaches to 1.0 on the y axis. However, it also has a mosaic segmental loss on chromosome 3, not only does this loss not spread across the entire width of the profile at chromosome 3 on the x axis, it doesn't reach all the way to 1.0 on the y axis, instead it lies around 1.3, indicating that around 70% of the biopsied cells are affected. All other chromosomes possess two copies except for Y.

Sometimes the profiles that occur can appear chaotic and can become difficult for operators to interpret. Where more than two chromosomes are affected it is routine to classify these samples as complex abnormal.

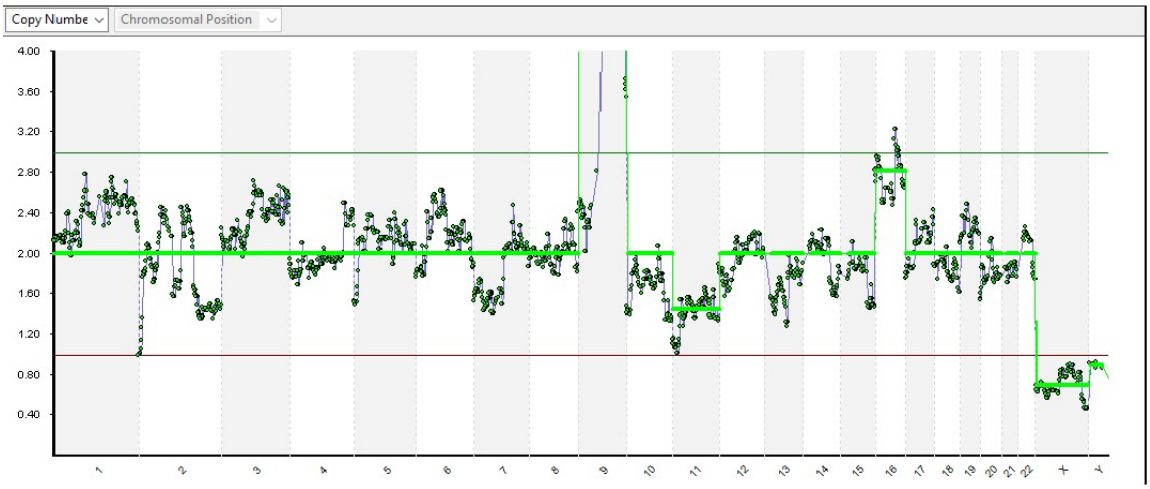


Figure 2.10: Bluefuse PGT-A image, Complex Abnormal (with thanks to CoopergGenomics for sharing these images)

Figure 2.10 is an example of a chaotic profile. It is difficult to list all the abnormalities, gains and losses, as there are more than two chromosomes affected, this profile would be recorded as complex abnormal.

Once the biopsy profiles had been interpreted. The results were written up into a report, there were three stages to producing a report, all of which are performed by a different scientist. The first scientist wrote the report, the second scientist performed a first edit, and the third scientist performed a second edit. This is to ensure that the report has been

produced accurately. Any discrepancies in reading of the results would need to be looked at by a senior scientist or lab manager to ensure that these were resolved before the report is sent to the clinic.

2.1.6 Consolidation of reported PGT-A cases, embryo fate and transfer outcome follow-up

All PGT-A cases received at CooperGenomics UK between January 2011 and December 2017 from CARE Fertility UK were collated into a database using Excel. Each PGT-A case was recorded in the database alongside a karyotype result (where applicable) for each embryo biopsy, whether euploid embryos were transferred and the outcome after transfers.

The following information was recorded in the database:
Clinic name; Patient ID; Cycle ID; Submission ID; CooperGenomics family ID; Cycle number; Infertility diagnosis; Patient karyotype (where applicable); Patient date of birth (or oocyte donor date of birth, where applicable); Maternal age at time of oocyte collection; Donor status (donor oocyte or sperm, or both); BMI; Anti-mullerian hormone (AMH) levels; Follicle-stimulating hormone (FSH) levels; Stimulation drugs used; Stimuation start date; Stimulation end date; Stimulation duration; Follicle count; Ovulation trigger type; Date of oocyte collection; Number of oocytes collected; Number of Mature oocytes; Type of fertilisation (IVF/ICSI); Number of oocytes fertilised; Number of embryos biopsied; Biopsy date; Biopsy type; Biopsy embryologist; PGT-A screening type (aCGH/NGS); Number of

biopsied samples that amplified; Number of euploids; Number of aneuploids; Number of no result; Number quality too low to report; Embryo biopsy ID; Embryo morphology grade; Karyotype; Meitotic abnormality (Yes/No); Mosaic chromosome A; Mosaic chromosome B; Was the embryo transferred?; Date of embryo transfer; Fresh or frozen embryo transfer; Biochemical pregnancy (Yes/No); Clinical pregnancy (Yes/No); Live birth (Yes/No); Miscarriage? (Yes/No); Other comments; Embryo fate (Discarded/Transferred/Frozen).

Due to patient data protection, I collected all the data for the database internally at each CARE Fertility Clinic. The raw identifiable data were kept on-site at CARE Fertility, and de-identified for analysis. Final collated numbers were taken away for the numbers presented within this thesis. I carried out all the data collection, processing, cleaning, and analysis.

The following definitions were used to record the outcome after embryo transfer:

- Biochemical transfer: After the transfer of an embryo from an IVF cycle a biochemical pregnancy test was carried out after 14-16 days, where the test was negative, this was recorded as a failed cycle and no further pregnancy follow up was performed. Where the test was positive, the pregnancy was recorded as a biochemical pregnancy until a clinical pregnancy was confirmed. If the pregnancy was not confirmed at the clinical pregnancy stage, this was recorded as a biochemical pregnancy only and recorded as an early miscarriage.

- Clinical pregnancy: After a positive biochemical pregnancy test, the patient would be invited for an ultrasound scan 4-6 weeks following embryo transfer. This detects for on going pregnancy by observing a gestational sac and a fetal heartbeat. If pregnancy was confirmed by ultrasound, this was recorded as a clinical pregnancy.
- Live birth: The term collectively used for determining the pregnancies which made it to term and result in the birth of a baby. Additional notes were recorded where applicable of any live birth defects detected or any significant anomalies. Still births and neonatal deaths were not recorded in this category and additional notes were made in these circumstances. Any pregnancies which were terminated, were not recorded in this category and additional notes were made for this, and the reasons for doing so, e.g. birth defects detected in pregnancy.
- Miscarriage: Where a biochemical test was returned positive but not confirmed by the first ultrasound scan, this was recorded as a biochemical pregnancy, but also recorded as an early miscarriage in the database. Where clinical pregnancy was confirmed by ultrasound, but was lost before 12 weeks of pregnancy, this was classed as an early miscarriage and recorded as a miscarriage in the database. If the timing of the miscarriage was not recorded, this was noted in the database with a comment. A miscarriage between 13 and 24 weeks was classified as a late miscarriage and was recorded in the database as a miscarriage. If the timing of the miscarriage was given, this was recorded with a comment. A pregnancy loss after 24 weeks, was classed as a still birth and recorded in the database.

2.2 Non-invasive Prenatal Testing Studies

2.2.1 Blood samples

Maternal blood samples from patients from 10 weeks of gestation were received into CooperGenomics UK from multiple referring centres for analysis using Serenity Basic and Serenity 24. These maternal blood samples were processed at CooperGenomics UK, to determine if aneuploidy is detected using Non-Invasive Prenatal Testing (NIPT).

Maternal blood samples were processed in accordance with CooperGenomics UK standard operating procedures between September 2015 and July 2018. Serenity Basic offered testing for chromosomes 21, 18, 13 and sex chromosome aneuploidies. Serenity 24 was offered from January 2018 this expanded testing to all other chromosomes.

Between September 2015 and July 2017, samples were processed manually through the laboratory, from July 2017 samples were processed using the Hamilton Microlab STAR to automate the process. Details of both methods will be outlined in the following sections.

2.2.2 Plasma isolation

2.2.2.1 Manual plasma isolation

Sixteen blood samples were processed simultaneously in the manual process, fourteen patient samples as well as one positive and one negative control. The handling of the uncovered plasma samples was carried out within a class II safety cabinet. One 5.0ml tube and three 2ml micro tubes were labelled with patient name and CooperGenomics ID number, for each patient sample. The details on the tubes were witnessed by another operator to ensure that these were correct. The streck blood collection tubes containing the patient plasma samples were loaded into the centrifuge adapters, the adapters were weighed to ensure that there was no more than 0.50g difference between them to ensure that the centrifuge was balanced. This was done by using an additional blank streck tube in each adapter loaded with water, adding, and removing water as needed. Once the weight was balanced the adapters were loaded into the Beckman-Coulter Allegra 6KR centrifuge (Beckman Coulter Life Sciences, Inc, IN, USA), placing, and securing lids for each bucket. The centrifuge was run for 10 minutes at 1600xg at 4°C and followed by a natural deceleration. Once the centrifuge step was completed the streck tubes were carefully transferred to a tube rack within the class II safety cabinet and the lids removed, ensuring that the two blood layers were not disturbed. 2-3.5ml of separated plasma (volume dependant on how much clear plasma is available) was decanted from the streck tubes into a corresponding 5ml pre-labelled tube for that patient, whilst being witnessed by another operator. It was vital that the blood layer below the plasma was not disturbed and that the plasma layer was not drawn too close to the blood layer to ensure that there

was no blood cell contamination in the plasma sample. Once completed the separated blood samples were discarded into a biohazard waste container. The plasma samples were placed into the Eppendorf Centrifuge 5427 R (Eppendorf AG, DE), to run for 10 minutes at 16000xg at 4°C. Following the centrifuge step there would be a visible pellet at the bottom of the tube. The tubes were carefully transferred back to the class II cabinet. The caps removed and 1ml of plasma aliquoted into each of the corresponding 2ml micro tubes labelled for that patient, ensuring that the pellet was avoided, whilst being witnessed by a second operator. Dependant on the initial volume of the plasma, only two 2ml micro tubes may have been required at this step, however, if the initial volume were lower than 2ml, the sample would be cancelled. Once transferred the 5ml tubes with any remaining sample and pellet were discarded into a biohazard waste container.

2.2.2.2 Automated plasma isolation

Ninety-four blood samples could be run simultaneously in a single run using the Hamilton Microlab STAR (Hamilton Bonaduz AG, CH) (Figure 2.11), along with one positive and one negative control. Barcoded blood samples were centrifuged at 1600xg for 10 minutes. When completed the tubes were removed and inspected to see if they contain at least 1.5 ml of plasma. The uncapped tubes were loaded into carriers 15, 16, 17 and 18 of the Hamilton Microlab STAR, whilst witnessed by another operator into the corresponding locations which were pre-determined on a sample lab tracking form. A positive plasma control was also loaded at this stage into its corresponding position on the Hamilton Microlab STAR. The carriers were loaded into and sample barcodes read by the Hamilton

Microlab STAR. A deep well plate was labelled with 'Intermediate Plasma' along with a barcode. Another deep well plate was labelled with 'Final Plasma' along with a barcode. Both were placed into their corresponding positions on the Hamilton Microlab STAR. The programme 'Veriseq NIPT Method', was started on the computer corresponding to the Hamilton Microlab STAR to be used. The plasma isolation step was started, and batch ID and username were entered into the programme. The corresponding sample sheet for the run was uploaded to the programme, one sample control selected, and batch size determined as ‘96’ was selected on the programme. Tips and plates with barcodes facing to the right were loaded into their corresponding carrier on the Hamilton Microlab STAR. Once everything was loaded correctly, the Hamilton Microlab STAR could continue according to its pre-determined programme, it is observed throughout especially ensuring that the Hamilton Microlab STAR deck was free of any obstructions. Once this step was completed, the deck of the 'Intermediate Plasma' plate was unloaded, the plate was visually inspected to ensure there were consistent volumes in each well (this should be 1000µl), any inconsistencies were recorded when noted. The plate was sealed using Bio-rad foil and placed into the centrifuge at 5600xg for 10 minutes, acceleration set at 9 and deceleration set at 3. Once centrifugation was completed the plate could be unsealed and reloaded into the carrier. The programme at this point would progress to the next stage. The Hamilton Microlab STAR was observed through its automated steps. Once completed, the deck was unloaded, the 'Final Plasma' plate was inspected, looking for a consistent volume of 900µl, and ensuring the plasma was as expected (e.g. no visible cell pellets or hemolysis).

Figure 2.11: Image of Hamilton Microlab STAR at CooperGenomics UK.

2.2.3 DNA extraction

2.2.3.1 Manual DNA extraction

Samples that had been processed through the plasma isolation step were then ready for DNA extraction using (QIAGEN, Ltd, UK). If the plasma samples had been stored in a -20°C freezer prior to commencing the extraction procedure, they were required to be removed and thawed prior to starting the process. All samples were centrifuged for 10 minutes at 16000xg. The QIAvac 24 plus vacuum manifold would be set up before starting the extraction, sixteen VacValves, VacConnectors, mini-columns and extension tubes were connected into the first sixteen positions. An additional sixteen 5ml falcon tubes and sixteen mini-column collection tubes were also required, the mini-columns, collection

tubes and falcon tubes were all labelled 1-16. A corresponding lab tracking form would have details of which patient sample corresponded to each position (1-16). 1ml of nuclease-free water chilled to 4°C was added for at least 30 minutes to each mini-column on the QIAvac 24 plus vacuum manifold to hydrate the columns for maximum DNA recovery. 900µl of protease was added to each labelled 5ml falcon tube followed by 900µl of each plasma sample to tubes 1-15, 900µl PBS was added to tube 16 for the negative control, whilst witnessed by a second operator ensuring that the correct patient was matched up to the corresponding tube. 900µl AL buffer was added to the tubes before tightly capping the tubes and mixing 8-10 times by inversion of the tubes. The tubes were flicked downwards to ensure that there was no liquid remaining in the lids. The tubes were placed into an incubator at 56°C for 17±2 minutes. The samples were incubated for a further 5 minutes at room temperature. Once incubation was completed, 900µl of ethanol was added to each tube, the tubes were tightly recapped and mixed by inversion 5-7 times, again flicking the tubes to ensure that there was no sample in the lid of the tube. The QIAvac 24 plus vacuum was turned on and adjusted to 400mm Hg (533 mbar), the VacValves were rotated to allow the 1ml of water previously added to hydrate the mini-columns to pull through, once the water was pulled through the valves were rotated to close. The entire sample contents of each 5ml falcon tube was loaded to the corresponding extension tube on the vacuum manifold, the valves were turned once the samples had been loaded to allow the vacuum to pull the samples through the mini-columns. Once completely drained the vacuum was turned off, valves closed once again, and extension tubes discarded. The column wash steps were carried out at this point,

750µl of AW1 was added to each of the mini-columns, the vacuum was turned on and once 400mm Hg was reached, the valves were opened to draw the buffer through, once completely drained the step was repeated again using AW2. Once completed, the mini-columns were transferred to their corresponding collection tubes and placed into a centrifuge to spin for 5 minutes at 16000xg to remove any remaining supernatant from the mini-columns. Once the mini-columns had been centrifuged, DNA elution could be carried out. The mini-columns were transferred to new labelled collection tubes, 60µl EB buffer was added to the centre of the filter of each mini-column, ensuring that the pipette tip was changed between each sample. The mini-columns were incubated at room temperature for 5 minutes. Once incubation was completed the mini-columns were centrifuged for 1 minute at 16000xg. New screw-cap micro tubes were labelled 1 to 16, along with the corresponding library ID. Following centrifugation the columns were discarded and the eluted cfDNA was decanted into the corresponding labelled screw-cap micro tubes, the samples could be used straight away in the manual library preparation step or stored at -20°C if not being processed straight away. The QIAvac 24 vacuum manifold was cleaned using 70% ethanol after the DNA extraction was completed.

2.2.3.2 Automated DNA extraction

Once the plasma isolation was completed, the DNA extraction process could commence. Two full-skirt plates were required, one was labelled 'intermediate' and the other 'cfDNA Elution' and a barcode added to both plates. One deep-well plate was labelled 'Extraction Intermediate', with a barcode added. A DNA binding plate also had a barcode added. It

was important to ensure the vacuum waste on the Hamilton Microlab STAR was empty and the vacuum system was on. The 'Veriseq NIPT Method' programme would be running on the Hamilton's corresponding computer. Pipette tips were loaded onto the tip carriers on the deck, and location confirmed on the programme for the location of the first tip of each rack, the extraction box and accessory box were scanned, and all plates listed previously and the 'Final Plasma' deep well plate were loaded into the plate carrier. The DNA binding plate was loaded onto the vacuum manifold. 16ml EB and 15ml Proteinase K were placed into the reagent carriers. 200ml Wash Buffer II (which had 100 ml of 100% ethanol previously added), 125ml Wash Buffer I, 100ml 100% ethanol, 100ml Lysis Buffer, > 100ml Dnase/RNase-free water, were all poured into corresponding labelled deep-well plates and loaded onto the carriers. The Hamilton Microlab STAR at this point would run its corresponding programme, which would be observed throughout. Once the final vacuum step had been completed on the Hamilton Microlab STAR, the DNA binding plate could be removed, the bottom surface was then cleaned with 70% ethanol. The DNA binding plate was placed onto the empty 'Final Plasma' deep-well plate and centrifuged at 5600g for 10 minutes, acceleration and deceleration set at 9. Once centrifugation was completed, the DNA binding plate was loaded onto the cfDNA elution plate which was on the vacuum manifold of the Hamilton Microlab STAR, the next step on the corresponding computer would be ready to be actioned, observing the Hamilton Microlab STAR throughout its automated steps. Once the incubation step had completed, the DNA binding plate/cfDNA elution plate would have been centrifuged at 5600g for 2 minutes with acceleration and deceleration as before. Once centrifuged, the elution plate was

inspected to check for consistent volumes in each well, expected volume was approximately 65µl. The elution plate was retained ready for the library preparation step. The Hamilton Microlab STAR deck would at this point be unloaded.

2.2.4 Library preparation

2.2.4.1 Manual library preparation

Samples that had undergone the DNA extraction step and were at room temperature were at this point ready for library preparation using the VeriSeq NIPT Sample Prep Kit (Illumina, Inc, San Diego, CA, USA). All samples I.D.s were recorded on the NIPT lab tracking form. An end repair step was first carried out to convert any overhangs on the DNA from fragmentation into blunt ends ready for ligation. A multi-channel pipette was used where appropriate during the following process. Two new 96-well plates were labelled with 'Reagent' and 'Sample'. 30µl of ‘End Repair Mix’ which had been vortexed was added to each well of column 4 of the 'Reagent' plate. 48µl of each DNA sample which had been vortexed and centrifuged was added to columns 2 and 4 of the 'Sample' plate, ensuring that tips were changed between each sample. 12µl of ‘End Repair Mix’ was transferred from column 4 of the 'Reagent plate' into each well of column 2 and 4 of the 'Sample' plate, the 'End Repair Mix' was mixed using the pipette 5 times in each column, ensuring that the pipette tips were changes between samples. The 'Sample' plate was then sealed using Microseal 'F' Foil and vortexed for 5 seconds to ensure the reagent and samples were mixed thoroughly. The 'Sample' plate was centrifuged at 280 x g for 5

seconds. The 'Sample' plate was placed into the thermal cycler with the following program: 75°C preheated lid, 30°C for 10 minutes, 75°C for 5 minutes and held at 10°C. Once the thermocycler had reached 10°C the 'Sample' plate was removed from the thermocycler. The following step added a single 'A' and 'T' base to the blunted 3' end of the DNA, this was to allow a complementary overhang on the DNA fragment for the adapter to be ligated to, the 'A' base ensures that the DNA fragments do not bind to each other during ligation. Diluted A-Tailing Mix (consisting of 150µl A-Tailing Mix and 200µl of Resuspension buffer) was vortexed and centrifuged before adding 40µl to each well of column 6 in the 'Reagent' plate. The plate was sealed and centrifuged for 5 seconds ensuring all bubbles were removed. 15µl of diluted A-Tailing Mix was transferred from the reagent plate into columns 2 and 4 of the 'Sample' plate and mixed by pipette 5 times ensuring that the tips were changed between samples. The 'Sample' plate was resealed, vortexed for 5 seconds and centrifuged for 5 seconds at 280 x g. The 'Sample' plate was placed into the thermal cycler with the following program: 75°C preheated lid, 37°C for 10 minutes, 75°C for 5 minutes and held for 10°C. Once the thermocycler had reached 10°C the 'Sample' plate was removed from the thermocycler. The samples at this stage were ready for the ligation step which ligates different indexing adapter to the DNA fragments, the indexes are known to each sample that they are added to, so that the samples can be traced during sequencing. The adapter index sets were vortexed and centrifuged for 5-10 seconds. 10µl of each DNA adapter index was added to columns 11 and 12 of the 'Reagent' plate in the order outlined in Table 2.1, ensuring that the pipette tips were changed between each index:

	Column 11	Column 12
Row A	AD002	AD001
Row B	AD005	AD003
Row C	AD007	AD008
Row D	AD012	AD010
Row E	AD013	AD020
Row F	AD014	AD022
Row G	AD018	AD025
Row H	AD019	AD027

Table 2.1: DNA adapter indexes for NIPT library preparation.

8% ligation mix (consisting of 60µl Ligation Mix 2 and 680µl Resuspension Buffer) was vortexed and centrifuged before adding 37.2µl to each well of columns 8 and 9 of the 'Reagent' plate. 3µl of the indexes from each well of column 11 was added to each well of column 8 of the 'Reagent' plate and mixed 5 times, then 3µl of the indexes from each well of column 12 was added to each well of column 9 of the 'Reagent' plate and mixed 5 times, ensuring that the pipette tips were changed between indexes. The 'Reagent' plate was sealed, vortexed and centrifuged at 280 x g for 10 seconds. The seals were removed from both the 'Reagent' plate and the 'Sample' plate. 8µl of the Index/Ligation mix from column 8 of the 'Reagent' plate was added and mixed into column 2 of the 'Sample' plate and this repeated from column 9 of the 'Reagent' plate to column 4 of the 'Sample' plate,

ensuring the pipette tips were changed between each index. The 'Sample' plate was sealed, vortexed and centrifuged at 280 x g for 5-10 seconds. The sealed 'Sample' plate was placed onto the thermocycler set with the following program: Lid preheated to 75°C, 30°C for 10 minutes, 75°C for 5 minutes, held at 10°C. Once the thermocycler had reached 10°C the 'Sample' plate was removed from the thermocycler. The 'Reagent' plate was no longer required and discarded. The samples at this stage went through the following process to clean up the ligation products. The 'Sample' plate was unsealed, 2.1ml of vortexed Sample Purification Beads were added to a clean split trough and 7ml 70% ethanol added to another clean split trough. The Sample Purification Beads were pipetted up and down 4 times before adding 83µl to column 2 of the 'Sample' plate, this was mixed 30 times using the pipette. The process was repeated for column 4 of the 'Sample' plate, ensuring the pipette tips were changed between wells. It was important to avoid making bubbles during this process, if bubbles did occur, the 'Sample' plate was sealed and centrifuged for 5 seconds to remove the bubbles. Once the Sample Purification Beads had been added and mixed into the 'Sample' plate, the plate stood at room temperature for 8 minutes. The plate was then placed onto a magnetic stand for 10 minutes at room temperature ensuring that the liquid became clear in the plate. The supernatant was removed and discarded from the 'Sample' plate whilst not disturbing the beads. Whilst the 'Sample' plate remained on the magnetic stand, 200µl of 70% ethanol was added to each well of columns 2 and 4. The 'Sample' plate was incubated at room temperature for 1 minute before the supernatant was removed and discarded, this step of adding and removing the ethanol was repeated again. Any remaining ethanol was removed and then

the plate was left to stand for 10 minutes at room temperature on the magnetic stand to dry. Whilst the 'Sample' plate was still on the magnetic stand 30µl QIAGEN EB Buffer was added to each well. The 'Sample' plate was removed from the magnetic stand and the beads re-suspended by pipetting up and down 15 times. The 'Sample' plate was incubated for a further 5 minutes at room temperature, then the 'Sample' plate was replaced on the magnetic stand for a further 5 minutes.

This next step enriched those DNA fragments that had adapters on both ends of the DNA fragments. This process also amplified the DNA in the library. A new 96-well plate was labelled 'PCR', 60µl of PCR mix (consisting of 400µl Enhanced PCR Mix and100µl Primer PCR Cocktail mix) was added to each well of column 10 and 25µl of PCR mix added to each well of columns 2 and 4 of the 'PCR' plate. 25µl of each sample in column 2 of the 'Sample' plate was transferred to column 2 of the 'PCR' plate and mixed by pipetting 5 times. This process was repeated with new tips from column 4 of the 'Sample' plate to column 4 of the 'PCR' plate. The 'PCR' plate was sealed with Microseal 'F' Foil, then vortexed for 5 seconds followed by centriugation at 280 x g for 10 seconds. The 'PCR' plate was placed onto a thermal cycler set with the following programme: Lid pre-heated to 100°C, 95°C for 3 minutes, 13 cycles of 98°C for 20 seconds, 60°C for 15 seconds, 72°C for 30 seconds, 72°C for 5 minutes, then after the 13 cycles the programme held at 10°C. Once the thermal cycler programme had completed, the 'PCR' plate was removed from the thermocycle, foil removed, and the plate retained for the next step. A new 96 well plate was labelled 'Elution'. 1.4ml of Sample Purification Beads which had been vortexed to mix

were added to a 25ml split trough and 7ml of 70% ethanol was added to another 25ml split trough. 50µl of Sample Purification Beads were added to columns 2 and 4 of the 'PCR' plate ensuring the beads were mixed up and down using the pipette 30 times. The 'PCR' plate was incubated for 8 minutes at room temperature. The 'PCR' plate was then placed onto the magnetic stand and left to incubate for a further 5-10 minutes at room temperature, ensuring that the solution had become clear. The supernatant was removed and discarded whilst the 'PCR' plate remained on the magnetic stand. 200µl of 70% ethanol was added to each well of columns 2 and 4 of the 'PCR' plate and the 'PCR' plate was incubated for a further 1 minute at room temperature before removing the ethanol and discarding, this process of adding and removing the ethanol was repeated again. Once the remaining ethanol had been removed and discarded the 'PCR' plate was left to stand at room temperature for 10 minutes for the beads to air-dry. 120µl of QIAGEN EB Buffer was added to each well of columns 2 and 4 of the 'PCR' plate. The 'PCR' plate was then removed from the magnetic stand and the beads were re-suspended by pipette mixing the bead up and down 15 times. The 'PCR' plate was incubated at room temperature for 5 minutes, then the plate was placed back onto the magnetic stand to incubate for a further 5 minutes at room temperature until the solution was clear. 100µl of supernatant from column 2 of the 'PCR' plate was transferred to column 2 of the 'Elution' plate and this step repeated for the supernatant from column 4 of the 'PCR' plate to column 4 of the 'Elution' plate. The 'Elution' plate was sealed using Microseal 'F' Foil and retained for the library quantification and normalization step.

2.2.4.2 Automated library preparation

Following DNA extraction, library preparation was carried out. The following reagents and plates were required for library preparation: End repair mix, Resuspension buffer, Hybridisation buffer, VeriSeq NIPT DNA adapter plate, Sample purification beads, 80% ethanol, cfDNA elution plate, A-Tailing Mix (1800µl of A-Tailing Mix and 2400µl of Resuspension buffer in a labelled 5ml eppendorf tube), Ligation Mix (440µl of Ligation Mix and 3278µl of Resuspension buffer in a labelled 5ml eppendorf tube), all reagents and plates were thawed and vortexed prior to commencing the library preparation step. A new full-skirt plate was required, labelled with the library I.D. and a barcode. The Hamilton Microlab STAR thermal control was required for this step and needed to be on. The 'Veriseq NIPT Method' was open on the corresponding computer, with the correct batch I.D. The checkboxes in the programme were selected and the programme was continued by selecting 'ok'. The Library Prep box barcodes were scanned ensuring that the box was not expired. The initials of the operator that prepared the reagents were entered, and the barcode for the Accessory Box scanned. 50µl and 300µl tips are loaded into their corresponding positions on the carrier of the Hamilton Microlab STAR, once these were loaded, ‘ok’ was selected on the programme. Further, 1000µl, 300µl and 50µl tips were loaded into their corresponding positions on the carrier. The location of the first tip for each tip rack was entered into the programme. The following plates were loaded onto the plate carriers into their corresponding positions, the cfDNA elution plate from the end of the DNA extraction step, a DNA adapter plate with barcode, new 96-well full-skirt plate with barcode and labelled 'Library_GGPE_(number of the run)’, 2 x new 96-well full-skirt

plates labelled SPB1 and SPB2. A deep-well reservoir was loaded onto the carrier containing 50ml of 80% ethanol, 4 x new 96-well full-skirt plates labelled as 'ERM', 'ATM', 'LIG' and 'ELU'. The following reagents were labelled and added into reagent tubs on their corresponding carrier positions, 'ERM' (with 2.5ml end repair mix added), 'ATM' (with the total volume of prepared A-Tailing Mix), 'LIG' (with the total volume of prepared ligation mix), 'ELU' (with 10ml sample purification beads added) and 'HT1' (with 12ml hybridisation buffer). Once everything was loaded, the programme was continued by selecting 'ok'. Having confirmed everything previously listed was aligned correctly 'ok' was selected again on the programme. The automated reagent check was carried out. Then the Hamilton Microlab STAR was observed throughout its automated steps, watching especially for carry over between wells. Once the programme was completed, the deck was unloaded. The library 96 well plate was checked to ensure that the volumes were consistent in each of the wells.

2.2.5 Library preparation for sequencing

2.2.5.1 Manual library preparation for sequencing

The samples were at this point ready for a quantification and normalization step before pooling of the cfDNA samples. This was to ensure a consistent optimum cluster density across all the samples and each lane of the flow cell at sequencing. First the lambda DNA standards were prepared. A new 8-well strip tube was labelled 1-6 across the top, in a new 50ml centrifuge tube 20ml of 1xTE was prepared (19ml of MgH2O and 1ml of 20x TE

buffer), this tube was then vortexed for 5 seconds. 9950µl of the 1xTE was decanted into a new 15ml tube labelled 'Dye'. 60.8µl of 1xTE was added to tube 1 of the strip tube and 40µl into tubes 2 to 6. 19.2µl of mixed Lambda DNA was added to tube 1 of the strip tube and mixed by pipetting 25 times, 40µl was transferred from tube 1 to tube 2 and again mixed 25 times, this was repeated again in sequence through the subsequent tubes up to tube 6. A new 96-well plate was labelled 'Dilution' and 25µl of MgH2O was added to all wells of columns 2 and 4. The 'Elution' plate was uncovered and 25µl of sample from column 2 transferred to column 2 of the 'Dilution' plate, this was repeated from column 4 of the 'Elution' plate to column 4 of the 'Dilution' plate. The 'Dilution' plate was sealed with microseal foil and vortexed and centrifuged for 10 seconds. A new white 96-well luminometer plate was retrieved and 90µl of 1x TE buffer added to each well of columns 1-9. 10µl of standard DNA from each of tubes 1-6 of the 8-well strip tube was added to wells B-G of column 1, 2 and 3 of the luminometer plate. 10µl of 1x TE buffer was added to wells 1A, 2A and 3A. 10µl of each sample from column 2 of the the 'Dilution' plate was added to columns 4, 5 and 6 of the luminometer plate. 10µl of each sample from column 4 of the 'Dilution' plate was added to columns 7, 8 and 9 of the luminometer plate. 50µl of premixed quantifluor DNA dye was added to the 15ml tube already labelled 'Dye', this step was completed with the lights off, the tube was vortexed for 5 seconds. 100µl of the 'Dye' mix was added to each row of the luminometer plate, mixing 25 times in each well (the pipette tips were changed between each well). The luminometer plate was incubated in the dark for 5 minutes. The luminometer plate was loaded onto a plate reader, the reader would have had the Quantiluor dsDNA protocol selected and standard

measurements for the first 3 wells of columns B-G entered into the programme. The wells containing individual patient samples were also outlined in the programme. The plate was then read by the plate reader, the output data were transferred to a USB and opened into instinct software where the analysis of the plate was automated. The file was exported from instinct to an excel file, displaying the data for each well and a standard curve. This file was saved and the results copied into a normalization spreadsheet including the patient sample details, this spreadsheet automatically generated a dilution amount for each sample, this was EB added to each column of 11 and 12 of the 'Elution' plate. Once the corresponding volume of EB has been added to all wells of column 11 and 12 of the 'Elution' plate, 10µl from each sample of column 2 was added to column 11, this was repeated from column 4 to column 12 ensuring the pipette tips were changed between samples. The 'Elution' plate was sealed and vortexed and centrifuged for 5 seconds. A new lo-bind tube was labelled 1.7nM and the batch I.D. written on the side of the tube. A volume of EB which had been pre-determined on the previously mentioned spreadsheet was added to the lo-bind tube, followed by 10µl of each of the samples from columns 11 and 12 from the 'Elution' plate, ensuring that the pipette tips were changed between each sample, the 'Elution' was then sealed and stored at -20°C. The lo-bind tube was vortexed and centrifuged for 5 seconds on each. In a new lo-bind tube, 7.4µl of the eluted library was combined with 7.4µl of 0.2N NaOH (800µl Rnase/DNase free water and 200µl stock 1.0N NaOH), the lo-bind tube was vortexed for 5 seconds and centrifuged at 280 xg for 10 seconds. The tube was incubated at room temperature for 5 minutes (this denatured the DNA into single strands). Then 7.4µl of 200mM Tris-HCl (800ul Rnase/DNase free water

and 200ul 1.0 N Tris-HCl) was added to the lo-bind tube, the tube was vortexed for a further 5 seconds and centrifuged for 10 seconds at 280 xg. 977.8ul of HT1 was added to the lo-bind tube and vortexed for another 5 seconds. A new lo-bind tube was labelled 'Final library' and 160µl of the library solution from the previous lo-bind is combined with 1340µl HT1, the tube was inverted 5 times and pulsed for 5 seconds in the centrifuge. The library could be stored at 4°C until ready to load for sequencing.

The pooled library was then prepared for sequencing on the NextSeq 500 (Illumina, Inc, San Diego, CA, USA), one TG NextSeq® High output kit v2 reagent cartridge (Illumina, Inc, San Diego, CA, USA) (Figure 2.12) was required for preparation for sequencing. 3ml of bleach NaOCl was added to position 28 of the reagent cartidge. 650µl of the pooled library was added to position 10 on the cartridge. The pool tube was recapped and put into storage at -25°C to -15°C. The NextSeq 500 to be used for sequencing the library was prepared as follows for sequencing, flow cell I.D. was entered into experiment name, single-end run with 36 cycle reads, one index with 6-cycle index reads and output directory confirmed. The reagent cartridge which had the library pool added was loaded into the NextSeq and a new clean flow cell was loaded. Sequencing at this point could commence.

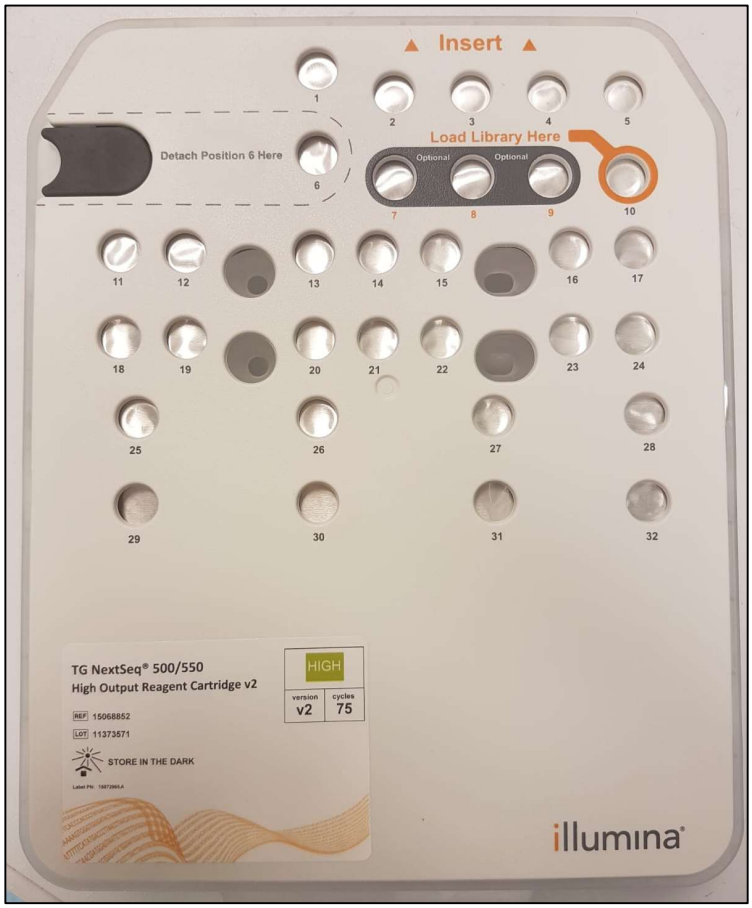


Figure 2.12: Image of TG NextSeq® High output kit v2 reagent cartridge at CooperGenomics UK.

2.2.5.2 Automated library preparation for sequencing

A new 34-well plate and a new full-skirt plate were required with a new barcode attached to each. The programme 'Veriseq NIPT Method', was required to be open on the computer corresponding to the Hamilton Microlab STAR, with the batch I.D. for the run and username entered. The Accessory Box barcode was scanned into the programme, followed by the username or the initials of the person who prepared the reagents. 300µl and 50µl tips were loaded into their corresponding positions on the tip carrier of the Hamilton Microlab STAR. Once this had been done the programme could be continued by

selecting 'ok'. The following plates were then loaded onto the multiflex carrier on the Hamilton Microlab STAR; barcoded full-skirt plate, barcoded 384-well plate, barcoded libraries plate as well as 2 x new unbarcoded 96-well full-skirt plates. When the plates were positioned, the programme was continued by selecting 'ok'. The DNA Quantitation Reagent and DNA Quantitation Standard were vortexed and centrifuged prior to loading uncapped into their corresponding positions on the tube carrier of the Hamilton Microlab STAR, clicking ‘ok’ on the programme once loaded. It was important to protect the DNA Quantitation Reagent from the light. Reagent tubs were loaded onto the reagent carrier, one was labelled 'RSB' (with 16ml of Resuspension buffer added), another was labelled 'DYE' which was empty, once loaded 'ok' was selected on the programme. If the Hamilton Microlab STAR had been stopped and unloaded after the library preparation step new tips were added at this stage, if not, the process could carry onto the next stage. 1000µl, 300µl and 50µl tips were required in their corresponding positions on the Hamilton Microlab STAR, the location of each first tip in each rack was entered into the programme and 'ok' selected. When everything was loaded correctly the programme was proceeded on the quant deck verification screen. The reagent volumes were checked by the Hamilton Microlab STAR, observation of all the automated steps continued throughout. Once completed the deck was unloaded. Inspecting the libraries plate for consistent volumes in each well afterwards. The libraries plate was sealed and stored at room temperature until after fluorometric data analysis had completed. At the time of unloading the 96-well plate and 384-well plate, the volumes were checked for consistency in each well of each plate. The 384-well plate was put onto the Gemini XPS microplate reader and left to incubate at

room temperature for 9 minutes. The SoftMax Pro 7.0 software was required on the corresponding computer to the microplate reader. The VeriseqNIPT_spectramax _template was loaded onto the software, plate size selected and the plate name from the 384-well plate entered. Once the 9 minute incubation was completed the plate was read by selecting 'read' on the software. After the read was completed it was exported to a USB as a raw XML file. The exported file was loaded to the corresponding computer of the Hamilton Microlab STAR which had been used previously for that batch run. The file was opened from the scanner information screen to review the fluorometric data in the form of standard curves and sample concentration results. Any comments about affected wells were recorded if applicable. If the fluorometric data passed specification the process proceeded to library pool, if the fluorometric data failed, could be run again once, but as soon as possible.

The libraries were now pooled together. The libraries plate was retrieved and placed onto a thermocycler which was pre-set to denature with the following programme: preheated lid to 102°C, reaction volume at 50µl, ramp rate set at 4°C per second, incubate at 96°C for 10 minutes and then 0°C for 5 seconds, then held at 4°C. On the corresponding computer to the Hamilton Microlab STAR in use, the pool concentration was required to be set at 5.00, once confirmed the software was continued to the next step. The 'Use default sample sheet' was selected. 50µl tips were loaded onto the corresponding carrier of the Microlab STAR. Once the libraries had finished its denaturing step on the thermocycler it was spun to ensure the samples were at the bottom of their wells, unsealed and loaded

into the corresponding position on the Multiflex carrier on the Hamilton Microlab STAR. Once completed the programme was continued. Two new 2ml tubes were required labelled with 'Pool A' and 'Pool B', these were loaded uncapped onto the corresponding carriers. Once completed the programme was continued. A new reservoir was labelled with 'HT1' with 3ml of Hybridisation Buffer added and loaded onto its corresponding position on the carrier. Once completed the programme was continued. The following tips were loaded onto the corresponding positions on the carrier: 1000µl, 300µl and 50µl. The first location of each tip was entered before continuing the programme. Once everything was loaded the Hamilton Microlab STAR carried out its automated steps, it was observed throughout. Once completed the deck was unloaded. 'Pool A' and 'Pool B' tubes were capped, vortexed and centrifuged quickly.

The pooled libraries were then prepared for sequencing on the NextSeq 500, two TG NextSeq® High output kits v2 reagent cartridges were required for preparation for sequencing. 3ml of bleach NaOCl was added to position 28 of both reagent cartidges. 450µl of each pooled libraries were added to position 10 on the cartridge (Pool A in cartridge one and Pool B in cartridge two), along with 900µl hybridisation buffer into each, once added a pipette was used to mix. The Pool tubes were recapped and put into storage at -25°C to -15°C. The NextSeq 500 (Figure 2.13) to be used for sequencing the libraries (two in this case) were prepared as follows for sequencing, pool tube barcode entered as a run name, paired-end run with 36 x 36 cycle reads, dual indexing with 8-cycle index reads and output directory confirmed. The reagent cartridge which had the library pool

added was loaded into the NextSeq and a new clean flow cell was loaded. Sequencing at this point could commence.

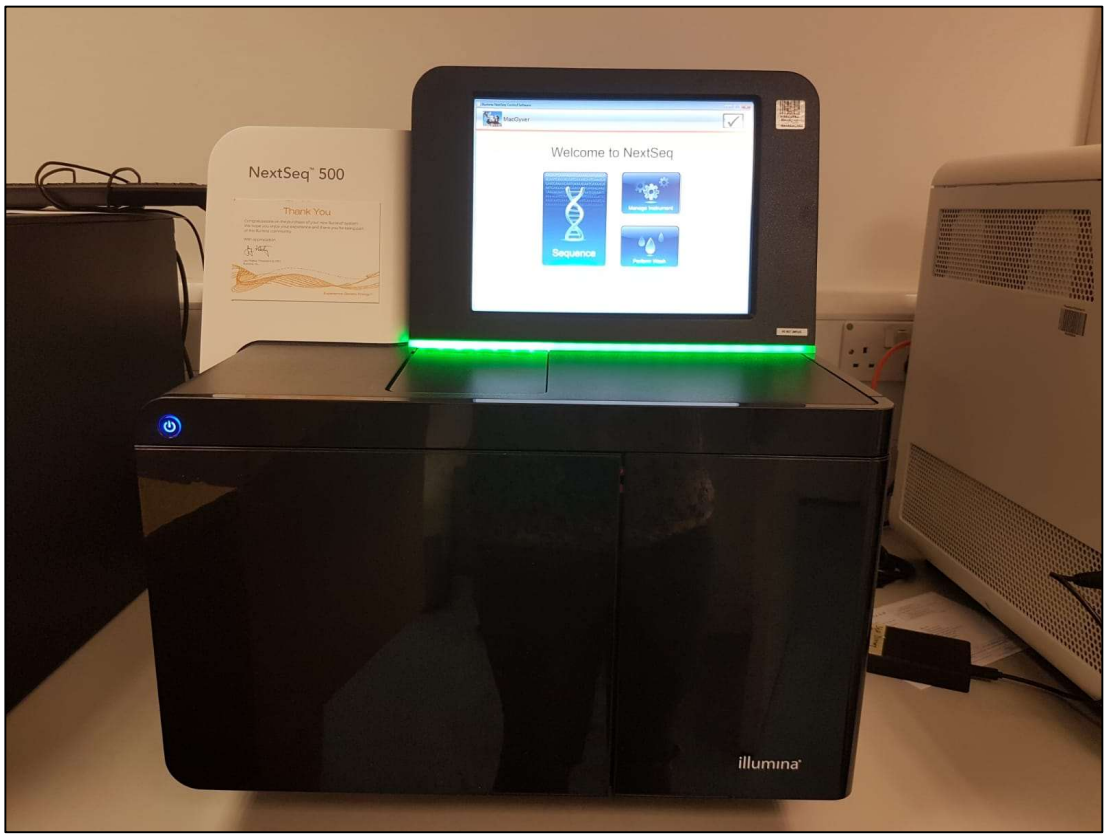


Figure 2.13: Image of the NextSeq® 500 at CooperGenomics UK.

2.2.6 Sequencing, interpretation software and reporting

Like the process described for PGT-A samples undergoing NGS, NIPT had a very similar process from this step. The following is a description of the process undertaken by the NextSeq 500 to sequence the NIPT samples:

Before the sequencing commences the library indexes which were added to the fragmented DNA become bound to the flow cell at complimentary locations and the fragments were amplified through bridge amplification. This process provides a template

for sequencing. During the sequencing step, each single base is detected as it is added to the template DNA strand. As the DNA is fragmented, multiple fragments are sequenced simultaneously. Early NGS was carried out as single-end sequencing, that is the DNA stand fragments are sequenced in a singular direction, this later changed to paired-end sequencing from February 2017, which meant the DNA fragments would be sequenced from both ends of the fragment, there is a degree of overlap between each end of the paired end read to align the sequencing data during data analysis, this allows paired-end sequencing to have a greater level of accuracy.

When the NextSeq 500 finished its sequencing run, the data were exported to a dedicated server. The sequencing data, indexes and patient information are matched up. The sequencing fragments are aligned alongside a reference genome and the number of reads for each chromosome are counted. Fetal DNA is smaller in fragment size to maternal DNA, helping to identify the relevant DNA for analysis. This aids in the estimate of the fetal fraction of cfDNA. Aneuploidy in the fetus can be detected by an increased fragment count matching the fetal fraction, that is, for example, trisomy 21 would see a slightly elevated read count on chromosome 21 when compared to the other chromosomes. The data were imported into VeriSeq NIPT Assay software, where the results from sequencing can be seen and interpreted. All cases were read independently by two trained scientists to ensure that they are read and reported correctly.

CooperGenomics offered a 24 chromosome NIPT analysis called Serenity24 from January 2018. This analysed the raw sequencing data from the NextSeq 500 to analyse in the same

way mentioned above for all other chromosomes.

2.2.7 Follow up of results

Where a detected or suspected aneuploidy was reported, I followed up these cases with the referring centres. The information requested, if available, was: the method of investigation for follow up of the pregnancy for example amniocentesis, CVS, abnormalities confirmed through ultrasound scan; whether the follow-up was confirmatory or not with the NIPT result and if there were any additional information that could be provided. False negative results were reported voluntarily by providers. Limited information was obtained from the process of following up with the referring centres, due to patients being lost to follow up and limited availability of time for the clinics to provide this information. Once CooperGenomics had decided to offer NIPT no longer it was decided that we would no longer pursue clinics for this information.

3 Specific Aim 1: How often is only a mosaic embryo available for transfer? And what proportion of these would be considered for transfer when reviewed using the CoGEN position statement?

3.1 Introduction

Mosaicism detected in preimplantation embryos has been documented in the literature for nearly 30 years and has long been established to be present in embryos reaching the blastocyst stage (Delhanty *et al.*, 1993; Munné *et al.*, 1994a, 1994b; Magli *et al.*, 2000). However, technological improvements with PGT-A in the form of NGS and its high level of sensitivity has highlighted the prevalence of these mosaic embryos over the previous aCGH technique. A higher proportion of trophectoderm biopsied that were previously reported as either euploid or aneuploid are now more accurately being detected as mosaic (Maxwell *et al.*, 2016). This has resulted in a reduction in the number of embryos available for transfer (Friedenthal *et al.*, 2018), and has raised questions regarding the accuracy of trophectoderm sample diagnosis when compared with the karyotype of the whole embryo and inner cell mass. This leaves clinicians and embryologists in some doubt regarding the safety and effectiveness of transferring mosaic embryos.

Mosaicism is the presence of 2 or more distinct cell lines within the embryo. These can occur in aneuploid/aneuploid or diploid/aneuploid forms, the latter form being the type of mosaicism called into question regarding its viability. Mosaicism can arise from meiotic

errors followed by postzygotic trisomy rescue (Robberecht *et al.*, 2012), however, it is generally accepted that most cases of mosaicism arise due to mitotic errors post-zygotically during the first few cell divisions after the formation of the zygote. These errors can occur during mitotic cell division from a variety of mechanisms including, non-disjunction, tripolar mitosis from tripolar spindle formation and anaphase lag resulting in micronuclei or chromosome loss (Bean *et al.*, 2002; Chatzimeletiou *et al.*, 2005; Vázquez-Diez *et al.*, 2016). Unlike meiotic errors, mitotic error and subsequently mosaicism has been shown to not be associated with maternal age (McCoy *et al.*, 2015; Nakhuda *et al.*, 2018; Popovic *et al.*, 2018). When a mosaic embryo reaches the blastocyst stage, there can be a variety of distribution patterns of cell types across the embryo, ranging from total or true mosaicism, where a mixture of diploid and aneuploid cells are found in both the trophectoderm and the inner cell mass, to confined placental mosaicism, where either the inner cell mass or the trophectoderm possess aneuploid cells (Figure 3.1). The frequency of mosaic embryos detected following PGT-A varies in the literature between 4% and 21% of all embryos biopsied (Greco *et al.*, 2015; Munné *et al.*, 2017; Simon, 2017; Coll *et al.*, 2018). It is thought that this variation could be due to factors in referring fertility clinics causing mosaicism in the embryos that they produce or through biopsy technique, although it is more probable that this is related to individual genetic testing laboratories; the sensitivity levels of their PGT-A technology and thresholds that are set for calling mosaics.

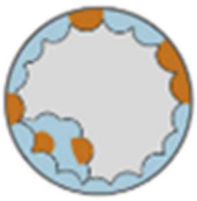
Mosaicism type	Possible TE biopsy	Diagnoses accuracy
Total Mosaic 	Euploid	Misdiagnosis
	Mosaic	Accurate
	Aneuploid	Misdiagnosis
ICM Mosaic 	Euploid	Misdiagnosis (Mosaicism never detectable)
TE Mosaic 	Euploid	Misdiagnosis
	Mosaic	Accurate
	Aneuploid	Misdiagnosis
ICM/TE Mosaic Type I 	Euploid	Misdiagnosis (Mosaicism never detectable)
ICM/TE Mosaic Type II 	Aneuploid	Misdiagnosis (Mosaicism never detectable)

Figure 3.1: Types of mosaicism in the blastocyst and possible causes of misdiagnosis from trophetoderm biopsy. Image from (Vera-Rodriguez & Rubio, 2017)

These mosaic embryos only become an issue for clinical decision makers and patients when there are no euploid embryos available for transfer. In some instances, where there is one euploid embryo that does not result in a live birth a mosaic embryo can subsequently be queried for its viability. An increasing number of studies report normal live births after the transfer of mosaic embryos, but nonetheless, the issue of knowingly transferring an embryo with a detected abnormality is an ethically fraught issue. This has led to various parties producing guidance statements to aid clinicians' decision making on the transfer of mosaic embryos (PGDIS, 2016; CoGEN, 2016; Grati *et al.*, 2018a, 2018b; Murtinger *et al.*, 2018). We particularly liked the guidelines set out in the CoGEN statement; they stress that euploid embryos regardless of morphology are always the first choice for embryo transfer. When a euploid embryo is not available they recommend that lower levels of mosaicism present in the trophectoderm are preferential and that those with multiple chromosome mosaicism are not transferred. They also detail a preference order to the chromosomes involved with mosaicism based on known adverse outcomes associated. Along with ensuring patients are appropriately counselled and suitable pregnancy and live birth follow up is offered (CoGEN, 2016). PGDIS guidelines on the other hand suggested a prioritisation towards mosaics involving monosomies, we felt that this was not suitable advice as where an embryo has a monosomic error it is possible that another cell possesses a monosomic error. Monosomic errors are rarely detected in early pregnancy and are likely to be lethal to embryonic development. Therefore, we feel it is not advantageous to select mosaic monosies over triosmies. Some may argue that a mosaic embryo that is morphologically superior to its euploid counterpart could be

preferentially selected, due to the fact that the euploid of poorer morphological quality could possess some level of undetected aneuploidy, that the aneuploidy was just not present within the biopsy sample, and therefore the mosaic may be preferential for transfer when keeping this in mind. It is our stance however that euploid embryos be selected for transfer first over counterparts which have had mosaicism detected.

To the best of our knowledge no studies have yet, in a sufficiently large cohort, audited the extent to which clinicians encounter this predicament and the extent to which it is related to maternal age.

3.1.1 Specific aims

With the above in mind, the purpose of this study was therefore to address these questions using a large data set from CooperGenomics:

1. What is the frequency that clinicians will encounter a patient with no euploid embryos for transfer, but one or more mosaic embryo available?
2. To what extent is the above related to maternal age?
3. What is the impact of the CoGEN position statement when applied to patient cases? By identifying what proportion of mosaic embryos would be considered for transfer.

3.2 Methods

Under University of Kent ethical regulations, this project did not require further ethical approval. This project was reviewed internally within CooperGenomics, it was determined that no further ethical approval was needed, and that patient confidentiality and General Data Protection Regulations should be observed throughout.

PGT-A cases that were received into CooperGenomics, Houston, TX, from multiple fertility clinics between December 2015 and December 2017 were included for retrospective data analysis. Samples were processed in accordance with standard protocols at CooperGenomics (refer to section 2.1). NGS has been validated by CooperGenomics to detect mosaic aneuploid cells between 20-80%. Mosaic embryos were not routinely reported during this period, low level mosaic embryos with no other full aneuploid chromosomes were reported as euploid, and high-level mosaic embryos were reported as full aneuploids. However, the mosaic nature of all embryos was captured on CooperGenomics internal databases.

Anonymised embryo biopsy data were first cleaned for errors, e.g. the number of biopsy samples submitted and reported were equal, any duplicates removed, and that all meiotic and mitotic classifications had been recorded correctly. Re-biopsy and sex identification only cases were excluded. The data were interrogated to identify those mosaic embryos of interest. Any embryos containing full aneuploid chromosomes and mosaic aneuploidy were classed as aneuploid for this study. Once the embryos of interest had been

identified, the biopsy cases were reviewed for types of biopsy results; number of euploids, aneuploids, mosaics and no result. Those cases which had no euploid embryos available but one or more mosaic embryo were identified. These have been categorised as mosaic only cases (that is only mosaic embryo/s are available for transfer and no euploid embryos are available for transfer).

Categorical variables are presented as numbers and percentages, continuous variables are expressed with means± standard deviation. Case results were categorised for statistical analysis. These result categories are as follows:

- Mosaic only: patient cases, where no euploid embryos were detected, but one or more mosaic embryo was identified.
- Aneuploid only: patient cases, where only aneuploid embryos were detected.
- Euploid available: patient cases, where 1 or more euploid embryo was detected.

Maternal age along with result category were entered into the Statistical Package for the Social Science database (SPSS) (SPSS, Chicago, IL, USA) to obtain statistical analysis. Chi2 analysis was carried out to test the null hypothesis that there was no difference in the rates of result categories associated with maternal age. Statistical significance was considered at P values of <0.05. Where multiple tests were carried out across the referral categories, an adjusted Bonferroni P value was calculated to avoid type 1 statistical errors, the adjusted P value is presented where it has been applied.

Following the CoGEN position statement recommendation, we categorised mosaic embryos into 3 groups: high, medium, and low priority for transfer:

- Highest priority: aneuploidy detected in <40% of the biopsy sample and only 1 affected chromosome involving chromosomes 1, 3, 4, 5, 6, 8, 9, 10, 11, 12, 17, 19 & 20.
- Medium priority: aneuploidy detected in >40% of the biopsy sample and only 1 affected chromosome involving chromosomes 1, 3, 4, 5, 6, 8, 9, 10, 11, 12, 17, 19 & 20.
- Lowest priority: mosaic biopsy result involving 2 or more chromosomes; or only 1 chromosome involving chromosomes 2, 7, 13, 14, 15, 16, 18, 21, 22, X & Y.

The categories were applied to those patients that had been classed as mosaic only to establish the percentage of cases which might be considered for transfer.
The above categorisation is also presented when applied to all mosaic embryos individually within the mosaic only cases. This is also applied to all mosaic embryos to demonstrate if the mosaics in the mosaic only cases are representative of the general population of mosaic embryos.

My own personal contribution was that I carried out all data analysis. I also worked with a bioinformatician who designed a Perl script to run on the excel database to identify the mosaic embryos more efficiently and to quantify the number of euploids, aneuploids, mosaics and no result biopsy results for each case.

3.3 Results

6,748 PGT-A cases were carried out at CooperGenomics Houston TX, between December 2015 and December 2017. 134 re-biopsy cases were excluded. 446 donor oocyte cases were not included for maternal age analysis as we did not know the maternal age of the donor.

6,614 cases involving 27,390 biopsies were included for further analysis. 692 (10.5%) cases had no euploid embryo but 1 or more mosaic embryos available (mosaic only) (Table 3.1). 1,384 (20.9%) cases only had aneuploid embryos (aneuploid only), 4,538 (68.6%) cases had one or more euploid embryos (euploid available). When looking for cases that only had 1 euploid embryo available, we found that 670 (10.1%) cases would have the option of a mosaic embryo if the first euploid transfer did not result in a live birth.
The percentage of mosaic only cases was found to be statistically significant for the <35 and 38-40 age groups ($Chi2=17.47$, $P<0.001$, $Chi2=11.49$, $P<0.001$, respectively), the adjusted Bonferroni P value for this analysis was 0.0063.

6,168 cases were considered for further maternal age analysis. The rates of euploids, aneuploids and mosaics by maternal age are presented in Figure 3.2. As might be expected, with increasing maternal age, there is a decrease in the rate of euploids and aneuploids increase rapidly. Mosaics on the other hand do see a down-ward trend but not to the same degree as the other two types of classification. It is likely that the rate of mosaics is decreasing since more embryos become aneuploid with increasing maternal

age. Chi2 analysis found there is a significant difference in the proportion of mosaic only cases, when assessed by age (Chi2 = 23.66, P<0.001). The <35 age group was significantly lower than the other age groups (P<0.001), the 38-40 age group was significantly higher than the other age groups (P<0.001), but not significant for the 35-37 and >40 age groups (P=0.617, P=0.043, respectively), the adjusted Bonferroni p value in this analysis is 0.00625. It is likely that the whilst the percentage of mosaic only cases did vary by age and reach statistical significance for the <35 and 38-40 age groups, the difference observed between these age groups is due to the maternal age effect on the rate of euploid and aneuploid embryos. When Chi2 analysis was carried out on all patient categories by maternal age following PGT-A: aneuploid only, mosaic only and euploid available. There was found to be a significant difference in category by maternal age (Chi2 = 1231.34, P<0.001). Post-hoc analysis looking at these categories in detail, found all age groups to be statistically significant for aneuploid only results and euploid available results, whereas in the mosaic only category 35-37 and >40 were not significant (P = 0.617 and P = 0.043, respectively), (adjusted Bonferroni p value in this analysis was 0.00417).

	<35	35-37	38-40	>40	**Totals**	Including donated oocytes
Total number in cohort	2177	1430	1447	1114	**6168**	6614
Total number of biopsy samples	10954	6029	4985	2851	**24819**	27390
Mosaic only	8.54% * (n=186)	10.42% NS (n=149)	13.20% * (n=191)	12.48% NS (n=139)	**10.78% (n=665)**	10.46% (n=692)
Euploid available	84.93%* (n=1849)	75.66%* (n=1082)	58.95%* (n=853)	31.87%* (n=355)		

Table 3.1: Breakdown of PGT-A cases by maternal age. Number and percentage of cases that are in the mosaic only and euploid available categories. Chi2 analysis *=statistically significant, NS=Not signficant

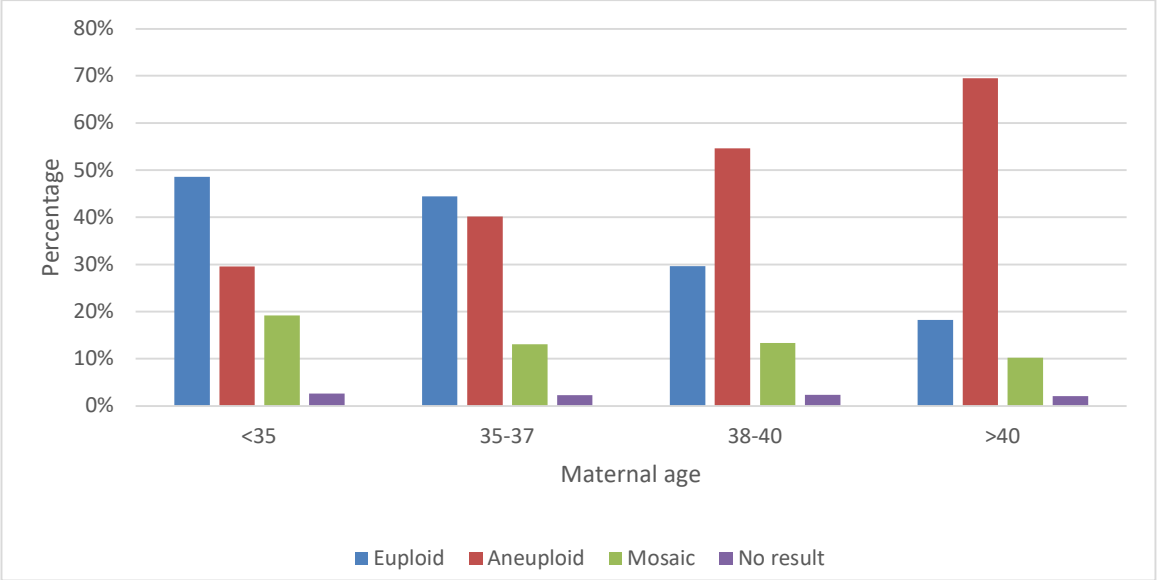


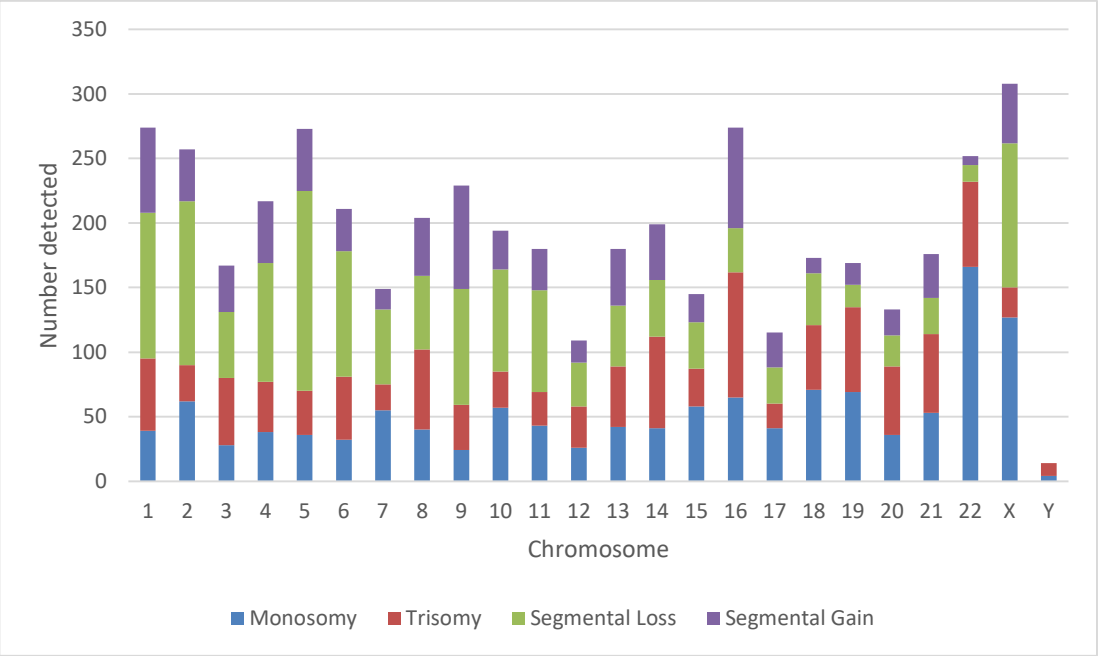
Figure 3.2: Percentage of embryos found to be euploid, aneuploid, mosaic and no result following PGT-A by maternal age (donor oocytes excluded).

4,585 (16.74%) embryos were classified as mosaic of the 27,390 embryos biopsied. 3,704 mosaic embryos were identified in cycles that had a euploid embryo available and therefore those mosaics would not be the first choice for transfer. Of the 4,585 mosaic embryos, 573 (12.5%) were considered complex abnormal (that 3 or more chromosomes were affected in the biopsy), 3,422 (74.6%) involved a single chromosome abnormality and 590 (12.9%) involved 2 chromosome abnormalities. 50.1% of the chromosomes affected were whole chromosome abnormalities, 27.2% being monosomy and 22.9% being trisomy. 49.9% were segmental errors, with a greater proportion being losses 31.6% vs gains 18.3% (Table 3.2). The most frequently affected chromosomes for all types of error were X, 1, 16, 5 and 2 and the least affected were chromosomes Y, 12, 17, 20 and 15. Chromosomes 22, 16, X, 19 and 18 were the most common whole chromosome mosaics, these were the most frequent monosomies, whereas in triomsies the most

common were 16, 14, 22, 19 and 8. For segmental abnormalities, chromosomes 5, 1, 9, 2 and X were the most affected. The distribution of the chromosomes affected by mosaicism can be seen in Figure 3.3.

The 692 mosaic only cases consisted of a total of 881 mosaic embryos being potentially considered for transfer. 129 (14.6%) embryos were considered complex abnormal, 628 (71.3%) had 1 chromosome affected and 129 (14.6%) had 2 chromosomes affected. The chromosome distribution of the remaining mosaic embryos can be seen in Figure 3.3. 55.0% were whole chromosome mosaics (31.6% and 23.4%, monosomy and trisomy, respectively) and 45.0% were mosaic segmentals (27.1% and 18.0%, loss and gain, respectively). Chromosome 22 had the highest mosaic aneuploidy rate (9.1%), followed by the X chromosome (7.2%) and chromosome 1 (6.6%). Chromosome 1 also saw the highest rate of mosaic segmentals, whereas chromosome 22 had the highest rate of mosaic monosomies and chromosome 19 had the highest rate of mosaic trisomies.

A.



B.

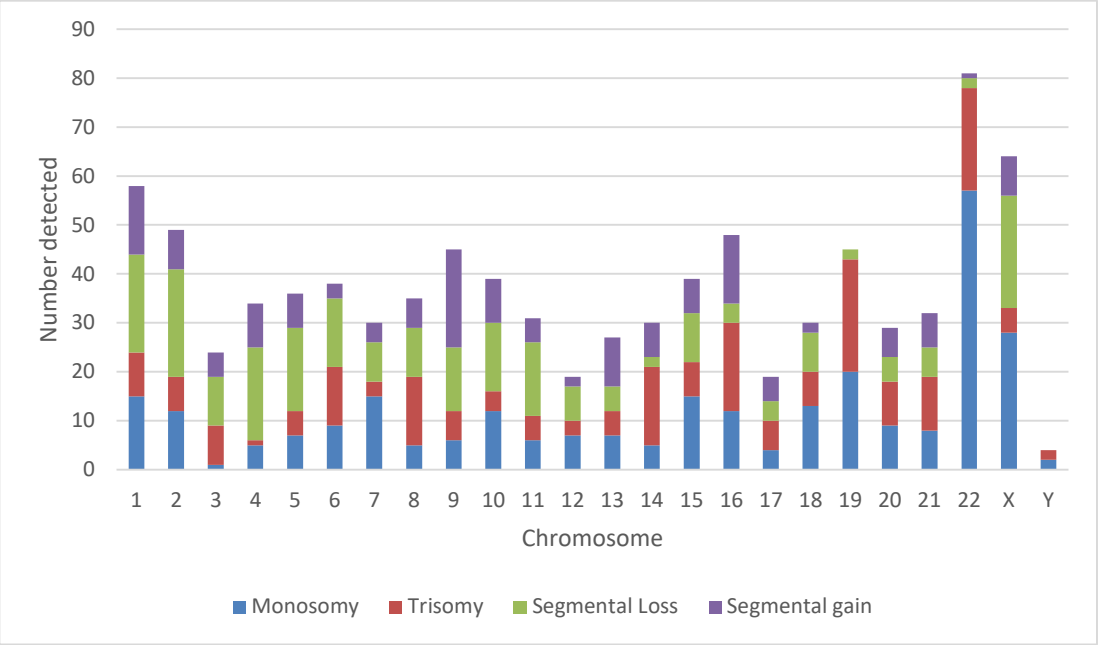


Figure 3.3: Number and type of error per chromosome. A: For all mosaic embryos; B: For mosaic embryos in mosaic only category.

	Whole chromosome			Segmental chromosome			Total for chromosome	% of all chromosomes
	Total	Monosomy	Trisomy	Total	Loss	Gain		
1	95	39	56	179	113	66	274	5.95%
2	90	62	28	167	127	40	257	5.58%
3	80	28	52	87	51	36	167	3.63%
4	77	38	39	140	92	48	217	4.72%
5	70	36	34	203	155	48	273	5.93%
6	81	32	49	130	97	33	211	4.58%
7	75	55	20	74	58	16	149	3.24%
8	102	40	62	102	57	45	204	4.43%
9	59	24	35	170	90	80	229	4.98%
10	85	57	28	109	79	30	194	4.22%
11	69	43	26	111	79	32	180	3.91%
12	58	26	32	51	34	17	109	2.37%
13	89	42	47	91	47	44	180	3.91%
14	112	41	71	87	44	43	199	4.32%
15	87	58	29	58	36	22	145	3.15%
16	162	65	97	112	34	78	274	5.95%
17	60	41	19	55	28	27	115	2.50%
18	121	71	50	52	40	12	173	3.76%
19	135	69	66	34	17	17	169	3.67%
20	89	36	53	44	24	20	133	2.89%
21	114	53	61	62	28	34	176	3.82%
22	232	166	66	20	13	7	252	5.48%
X	150	127	23	158	112	46	308	6.69%
Y	14	4	10	0	0	0	14	0.30%
Complex Abnormal							573	

Table 3.2: Number of errors per chromosome for all mosaic embryos.

526 (76.01%) of the mosaic only cases, had at least one mosaic embryo where only one chromosome was affected. 87 (12.57%) had at least one mosaic embryo with 2 chromosomes affected and 79 (11.42%) only had complex abnormal mosaic embryos available. The mosaic only cases were further analysed applying the CoGEN guidelines regarding the types of chromosomes involved in the mosaic embryos (Figure 3.4). 6% of all PGT-A cases would fall into a low priority for transfer mosaic group. Whilst 4.5% would fall into a high or medium priority for transfer groups. All the mosaic embryos were analysed using the CoGEN guidelines and saw a similar percentages of mosaic embryos being categorised into high, medium, and low priority for transfer as seen in the mosaic only group (14.8%, 25.4% and 59.8%) (Figure 3.5).

4,089 mosaic embryos had maternal age information available for them. Analysis of the number of chromosomes involved with the mosaic embryo in relation to maternal age can be seen in Table 3.3. There appears to be a slight decline in the amount of embryos with one mosaic chromosome and a reciprocal increase in the complex mosaic embryos, however, Chi2 analysis found that this relationship was not significant (Chi2 = 4.29, P = 0.637).

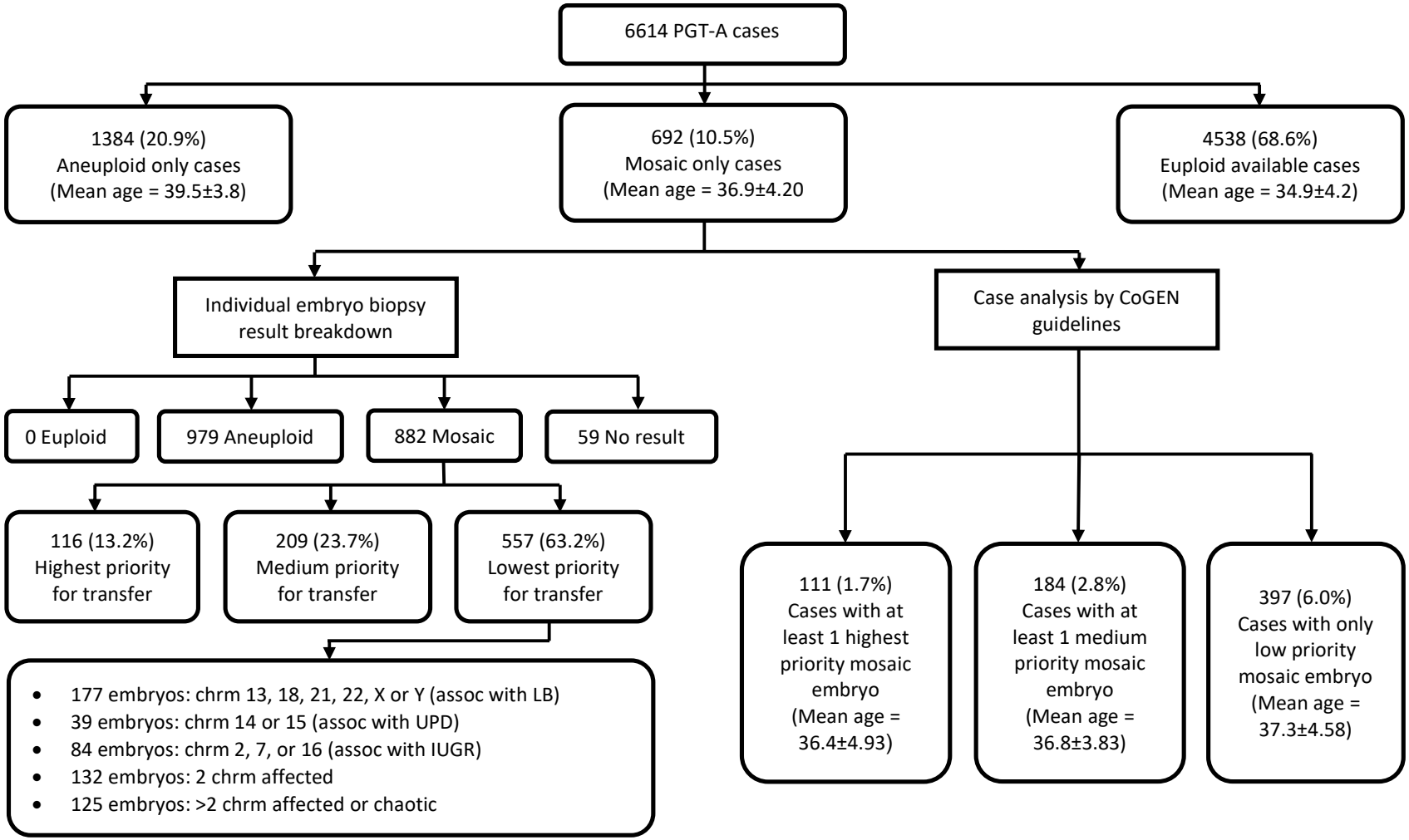


Figure 3.4: Mosaic only cases broken down into priority for transfer categories. PGT-A category distribution into aneuploid only, mosaic only and euploid available cases included. Breakdown of individual mosaic embryos also detailed.

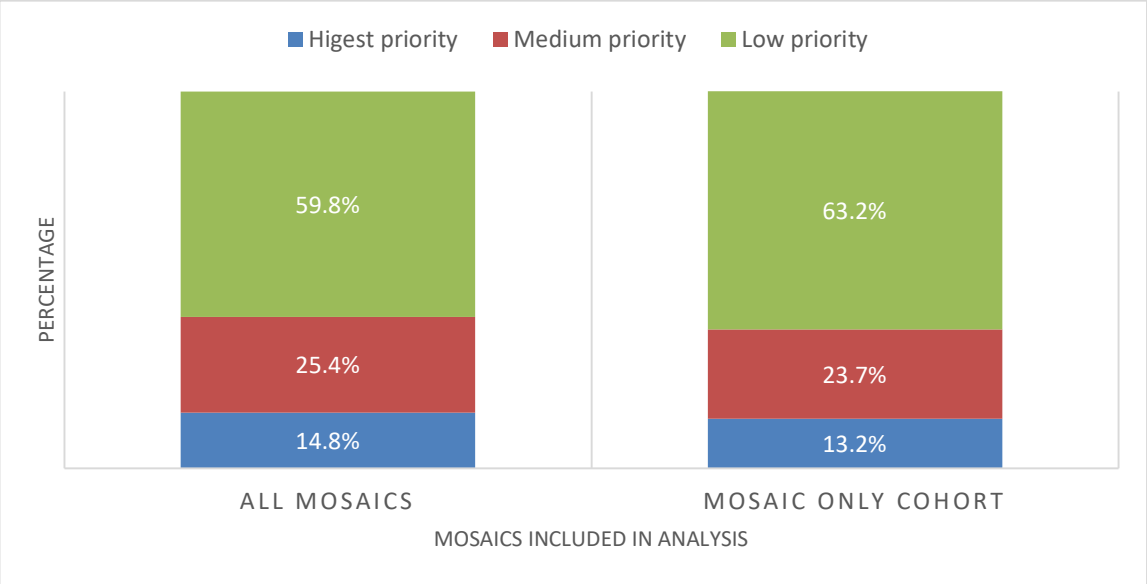


Figure 3.5: Distribution of all mosaic embryos vs mosaic embryos of the mosaic only group in priority for transfer categories.

Age	1 mosaic chromosome	2 mosaic chromosomes	Complex mosaic chromosomes	Total embryos
<35	1574 (75.0%)	259 (12.3%)	267 (12.7%)	2100
35-37	772 (75.0%)	132 (12.8%)	126 (12.2%)	1030
38-40	490 (73.5%)	96 (14.4%)	81 (12.1%)	667
>40	208 (71.2%)	46 (15.8%)	38 (13.0%)	292

Table 3.3: Number of chromosome errors in mosaic embryos by maternal age.

3.4 Discussion

In the present study the specific aims were largely addressed. We aimed to assess the frequency that clinicians will encounter a patient with no euploid embryos for transfer but have one or more mosaic embryo available for transfer. Our findings indicate that 10-11% of cases only have a mosaic embryo available for transfer, and a further 10% of cases only have 1 euploid embryo and a further mosaic embryo available for double embryo transfer or a subsequent frozen embryo transfer. Conversely, 80% of cases would not present such a predicament with, 21% of cases only having aneuploid embryos reported and 59% of cases having one or more euploid embryos available (excl. our 1 euploid and 1 or more mosaic group).

This study also aimed to evaluate the extent that only having a mosaic embryo available for transfer is related to maternal age. Whilst, the incidence of overall euploidy vs aneuploidy is clearly an age-related phenomenon on both an individual embryo and per case basis, the incidence of mosaic only cases does not appear to vary to the same degree seen in the euploid available and aneuploid only groups. Statistical significance was seen in two mosaic only age groups however, this is likely due to the maternal age effect on the rate of both euploidy and aneuploidy, for example fewer younger patients would fall into our mosaic only category as they are more likely to have a euploid embryo available, and fewer of our oldest patients would be included in our mosaic only group as the embryos are more likely to possess meiotic errors leading to fully aneuploid embryos, leaving less embryos available to be classed as mosaic.

This study further aimed to assess the impact of the CoGEN position statement when applied to patient cases. We found that when the mosaic only cases are reviewed using the CoGEN mosaic prioritisation guidelines, 63% of the mosaic embryos would be low priority for transfer, and unlikely to be transferred. When reviewed on the by case basis, 57% of the mosaic only group (6% of all cases) would be very unlikely to have a mosaic embryo that would be considered for transfer, as these embryos would either have a chaotic chromosome composition or involve a chromosome which is known to be associated with an adverse outcomes. Most of these embryos (177, 32%) had mosaic forms which involved a chromosome associated with live birth abnormalities (in trisomic, monosomic and segmental forms). It has been observed that 5% of live birth trisomy 21 cases are mosaic in origin, whereas most live birth forms of trisomy 13 and trisomy 18 and 50% of sex chromosome monosomy live births are mosaic. It is thought that the presence of a normal cell line is what permits these pregnancies to reach term (McFadden & Friedman, 1997). With the lowest priority mosaic only cases not considered for transfer, in reality this leaves only those cases with high priority and medium priority mosaic embryos for consideration, which is only in 4.5% of all the PGT-A cases observed in this study.

In the present study we found that mosaic embryos occur in 16.74% of trophectoderm biopsies, a similar rate of mosaicism was reported in a study by by Nakhuda *et al,* 2018 (17.5%), although the rate of mosaicism does vary in the literature as mentioned previously between 4% and 21% of all embryos biopsied (Greco *et al.*, 2015; Munné *et al.*, 2017; Simon, 2017; Coll *et al.*, 2018). The distribution we observed between whole

chromosome mosaicism and segmental mosaicism was roughly even at 50.1% and 49.9%, a previous study has reported a higher rate of whole chromosome mosaicism at 64%, however, in our mosaic only category, the distribution was trending towards whole chromosome mosaics (Nakhuda *et al.*, 2018). We saw a slightly higher proportion of monosomies than trisomies, which is different to that reported in the Nakhuda *et al* paper, they reported significantly more trisomy than monosomy, conversely a previous study by McCoy *et al*, supports the findings in the current study that chromosome loss is more frequent than chromosome gain with mitotic errors (McCoy *et al.*, 2015). Further comparison regarding the type of chromosomes affected reveal more discrepancies with the Nakhuda *et al* paper, we saw chromosomes, X, 1, 16 5 and 2 to be the most commonly affected, whereas they reported 21, 22 and 2. Interestingly from our analysis chromosome 21 was towards the lower end of the scale. Our least affected were Y, 12 and 17, theirs was 10, 12 and Y. However, chromosome 10 made up 4.22% of chromosomes affected in our study. The variance seen between our study and that previously reported could be due to the much larger cohort included in our study (4,585 vs 270 mosaic embryos), the present study also benefitted from involving multiple centres. If we had more time available, it would have been interesting to investigate the difference in the number of mosaic chromosome errors when assessed by clinic. When comparing our distribution of mosaic chromosome errors with that reported in the literature for non-mosaic aneuploidy there are clear differences, chromosomes 15, 16, 21, 22 and 19 are repeatedly reported to be the most common chromosomes affected, which was not seen with our mosaic errors, although chromosome 16 was one of our most common and chromosome 22 ranked 6th

(Franasiak *et al.*, 2014; Rubio *et al.*, 2017; Girardi *et al.*, 2020). However, some similarities can be drawn, chromosomes 22 and 16 are regularly reported with the highest frequencies of monosomies, which agrees with the present study (Franasiak *et al.*, 2014; Rubio *et al.*, 2017). There is a trend towards the rate of segmental errors in relation to chromosome size, which has been previously reported with non-mosaic aneuploidy (McCoy *et al.*, 2015; Girardi *et al.*, 2020). McCoy *et al*, reported that complex mitotic errors are selected against prior to blastulation, that is a lower rate of complex mosaic errors are seen at the blastocyst stage when compared to the cleavage stage (McCoy *et al.*, 2015). We saw a higher rate of single mosaic chromosomes errors over 2 or more errors which could be explained by this hypothesis.

A limitation of this study as mentioned previously is that further breakdown into clinics has not been carried out and this information could have been useful to identify to what degree the types and rates of mosaicism vary between clinics. This study also does not have details of confounding factors such as, ovarian stimulation, culture media and insemination technique, which may contribute to variation rates and types of mosaicism. An increased rate of mosaicism has previously been reported with IVF vs ICSI (Palmerola *et al.*, 2019). The mosaic status of embryos was not originally reported, instead low level mosaics were routinely reported as euploid and high level mosaics reported as aneuploid, it is possible that there may have been variation in the level of accuracy of recording of mosaic findings as this was not essential for final reporting to clinics. If this study were

carried out prospectively the mosaicism and more specifically the percentage of the biopsy affected could be verified.

Inner cell mass concordance with those embryos determined to be euploid or aneuploid following PGT-A has been shown to be high (Johnson *et al.*, 2010; Capalbo *et al.*, 2013; Chuang *et al.*, 2018; Victor *et al.*, 2019a, 2019a; Navratil *et al.*, 2020; Sachdev *et al.*, 2020). Although it has been reported that there is a great deal of variance across the embryo with both full segmental aneuploidy and mosaic results (Johnson *et al.*, 2010; Popovic *et al.*, 2018; Victor *et al.*, 2019a, 2019a; Girardi *et al.*, 2020; Navratil *et al.*, 2020). Therefore, deciding whether to transfer or discard an embryo which has been reported as mosaic is a challenge. The reported percentage of mosaicism from an initial trophectoderm biopsy should be taken with caution as the distribution of euploid to aneuploid cell lines may not be uniformly distributed throughout the embryo (Kushnir *et al.*, 2018; Lin *et al.*, 2020).

Without PGT-A analysis mosaic embryos are routinely transferred unknowingly in IVF clinics. Although it is not completely understood if the transfer of these embryos results in healthy live births, implantation failure or adverse outcomes. More reports of the outcomes following the transfer of mosaic embryos are becoming available in the literature. Whilst they all agree that mosaic embryos have a lower ongoing pregnancy and live birth potential than euploid embryos (Fragouli *et al.*, 2017; Lledó *et al.*, 2017; Kushnir *et al.*, 2018; Victor *et al.*, 2019b; Zhang *et al.*, 2019). There is a lot of disagreement on the potential of different types of mosaic embryo. A common theme is to assess the potential

of mosaic embryos based on the percentage of the biopsy sample that is affected (high vs low percentage of mosaicism). A number of studies have reported that there is no difference in ongoing pregnancy rates (OPR) and live birth rates (LBR) between these two groups (Kushnir *et al.*, 2018; Victor *et al.*, 2019b; Lin *et al.*, 2020), whilst others have reported better OPR and LBR with low level mosaics (Munné & Wells, 2017; Spinella *et al.*, 2018; Munné *et al.*, 2020). The number of chromosome errors has also been addressed and it has been shown that lower success rates with are associated with more complex mosaic forms. Although these complex mosaics do sometimes achieve pregnancy and live birth but at very low rates (4-9%) (Fragouli *et al.*, 2017; Munné *et al.*, 2017, 2020; Spinella *et al.*, 2018, 2019). However, there is disagreement on the success rates associated with the different types of chromosome abnormalities, no difference has been demonstrated between whole, segmental, monosomy and trisomies (Munné *et al.*, 2017, 2020; Lin *et al.*, 2020). Whereas one study has indicated that mosaic monosomies may have an advantage over trisomies (Spinella *et al.*, 2019). Two studies have reported improved outcomes with segmental mosaics (Fragouli *et al.*, 2017; Victor *et al.*, 2019b). Some of these studies which have reported on mosaic transfers, have included details of chromosome number and type transferred. In some instances those transferred are those deemed lowest priority for transfer in the present study (those associated with live birth abnormalities or adverse pregnancy outcomes), however, there is very little follow up information available for these pregnancies if they do result in pregnancy. It is therefore difficult to draw significant conclusions regarding the safety of such transfers, especially when considering the often-small numbers involved in these studies. Interestingly, there has been one case

of a mosaic monosomy 2 embryo being transferred which resulted in the healthy live birth of a child with a mosaic monosomy 2 karyotype, there was no intrauterine growth retardation evident during this pregnancy (Kahraman *et al.*, 2020). Given these findings reported in the literature, the priority categorisations used in our current study still seem valid, it appears if there is a choice between an embryo of high or low level mosaicism, to select the lower level in the first instance. Avoiding those with multiple errors, especially 2 or more. And approaching those mosaics with chromosome errors associated with adverse outcomes with caution. It may be advantageous to prioritise segmental mosaics over those of whole chromosome mosaics if the option is available.

Since CoGEN released their statement offering guidance on the transfer of mosaic embryos, there have been further recommendations produced by other parties. One such scoring system has been offered by Grati *et al*, who reviewed mosaicism detected in CVS samples, along with the associated impact on the pregnancy (Grati *et al.*, 2018b). The benefit of using CVS data for inferences in the selection of mosaic embryos detected following PGT-A, is that cells tested during CVS will have originated from the trophectoderm which is tested for PGT-A. They make similar suggestions regarding priority for transfer based on the chromosome number involved, however they break these down further into 5 priorities, based on risk of miscarriage, UPD, and live birth abnormalities. This scoring system has been criticised as the fate of mosaic embryos based on CVS outcomes is not definitively correlated (Grati *et al.*, 2018a; Murtinger *et al.*, 2018). However, without extensive information on the outcome of mosaic embryos especially

based on the chromosomes involved, CVS data along with rates of miscarriage associated with mosaic pregnancies is valuable for both clinicians and patients when making informed decisions. PGDIS have since updated their recommendations on the transfer of mosaic embryos, they too have recommended low-level mosaic embryos over high-level mosaic embryos, and one chromosome involvement over multiple chromosomes, and refer to the Grati *et al* paper on the type of chromosome involvement (Cram *et al.*, 2019). Therefore, the recommendations outlined in the current study are still in line with more recent recommendations that have been published.

Advances with PGT-A testing in the form of NGS has resulted in a higher level of accuracy of detection with trophectoderm biopsy. Identifying mosaic embryos which may have been classed as euploid with aCGH resulting in lower rates of implantation and higher rates of miscarriage, or conversely categorising mosaic embryos as aneuploid when they may have had a chance of success. This could explain the improved ongoing pregnancy rates associated with NGS over aCGH (Munné *et al.*, 2020). Whilst, knowing how to deal with mosaic embryos may present a challenge to clinicians, this additional information might be aiding in the improvement in success rates for ART with PGT-A. For those patients that only have a mosaic embryo available for transfer, clinical and genetic counselling involvement would be required to aid in decision making as to whether a mosaic embryo is to be transferred. Patients may wish to undertake another fresh cycle with PGT-A in the hope of obtaining a euploid embryo for transfer. Where the cost implication or maternal age are limiting the option for additional fresh cycles, the patient

should be adequately informed of the lower success rates and risks associated with the transfer of a mosaic embryo and offered appropriate clinical management to continue to monitor the ongoing pregnancy (such as prenatal screening) and live birth outcome.

3.5 Conclusion

The conclusion of this study is that 10.5% of cases have only a mosaic embryo available for transfer and no option of a euploid embryo transfer. When these cases are further assessed for type of mosaic embryo available, only 4.5% of all cases are likely to be considered for transfer. Conversely, 89.5% of all cases will not be faced with this predicament. This study also demonstrates that mosaic embryos do not vary with maternal age, but instead the chance of only having a mosaic available is linked to the decreasing availability of euploid embryos with maternal age. It is important that we continue to monitor pregnancies and live birth outcomes where mosaic embryos are transferred to gather more information regarding those which are more likely to result in live births and low levels of pregnancy loss.

4 Specific Aim 2: Preimplantation genetic testing for aneuploidy, a retrospective multicentre study to determine efficacy and inter-clinic variation

4.1 Introduction

Aneuploid embryos which result in pregnancy are the leading cause of pregnancy loss and live birth developmental disabilities (Hassold & Hunt, 2001). The link between aneuploidy and an increase in meiotic errors (most commonly meiosis I) with advanced maternal age is well documented in the scientific literature (Hassold & Hunt, 2001, 2009). One large retrospective study of PGT-A following IVF involving more than 35000 embryos has demonstrated that the likelihood of having a euploid embryo available for transfer declines with maternal age (Demko *et al*., 2016), in this study they showed that in under 35s, 35% of blastomere biopsies to 55% of trophectoderm biopsies are euploid and this declined to 0% by the age of 44. The types of aneuploidy become more complex with increasing maternal age, that is the number of chromosomes involved in aneuploidy increase with maternal age (Franasiak *et al*., 2014). Implantation failure and miscarriage rates following embryo transfer in ART are mostly attributed to aneuploidy within the embryos chosen for transfer. Whereby, live birth rates are seen to decrease with maternal age. HFEA statistics for 2018 present live birth rates per fresh embryo transfer ranging from 30% in the under 35s, dropping to 3% for the over 44s, however across all age groups using donated oocytes the live birth is above 25% (HFEA, 2020).

Morphological assessment has been the primary method for embryo selection for transfer since the beginning of ART (Edwards *et al*., 1984). Many different methods of assessing embryos at their various time points were developed, in 1999 the Gardner blastocyst scoring system was presented along with subsequent publications which demonstrated that there was improved implantation and pregnancy rates associated with higher scoring blastocysts (Schoolcraft *et al*., 1999; Gardner *et al*., 2000). However, a variety of scoring systems were developed and adopted leading to an array of morphological scoring methods being used in ART centres. In 2011, a consensus was established to standardise embryo grading across ART centres (ASRM & ESHRE, 2011), in line with the evidence for improved implantation and pregnancy rates. Over the last decade clinics have moved towards culturing embryos to the blastocyst stage and selecting a blastocyst for transfer at day 5 of embryo development. With blastocyst transfer there is an improvement in live birth over that of transfer at the cleavage stage (ASRM, 2018), embryos which do not successfully develop to the blastocyst stage and arrest are more likely to have been aneuploid (Qi *et al*., 2014), therefore waiting until day 5 to transfer will avoid some aneuploid cleavage stage embryos which would have led to implantation failure. However, research looking at embryo quality and ploidy status is mixed, studies have found that there is a link between euploid embryos and higher graded blastocyst embryos with up to 60% of top graded embryos being euploid (Schoolcraft *et al*., 2009). Whilst, other publications have highlighted that despite a proportion of aneuploid embryos failing to blastulate, the association between aneuploidy and blastocyst grade is weak, with a significant proportion of the top scoring blastocysts being aneuploid and lower scoring

blastocyst being euploid (Rubio *et al*., 2007; Alfarawati *et al*., 2011). Developments in time-lapse technology has allowed groups to develop algorithms focusing on the timing of cell divisions to select embryos for transfer (Campbell *et al*., 2013; Basile *et al*., 2015; Fishel *et al*., 2018), as time-lapse technology offers continuous embryo assessment, unusual events can be detected which previously may have been missed (Ottolini *et al*., 2017). Time-lapse technology has the additional benefit of being able to assess embryos without removing them from the incubator and thus potentially avoiding exposure to the environment outside of the incubator which may damage embryos on-going developmental potential. Although the reliability of time-lapse algorithms to predict the ploidy status has been variable across different groups (Campbell *et al*., 2013; Kramer *et al*., 2014; Rienzi *et al*., 2015; Minasi *et al*., 2016).

Using genetic analysis of the polar body from the oocyte or a small number of cells from embryo to determine the ploidy status as a means for selecting the appropriate embryo for transfer has been used since the 1990s. The early form of PGT-A was fluorescent in situ hybridisation (FISH) which was most often carried out on polar bodies or cleavage stage blastomeres. The technique focused on a small number of chromosomes associated with live birth aneuploidies relating to live birth defects and recurrent miscarriage (Griffin, 1991; Munné *et al*., 1994). Although there were publications which indicated improvements in live birth rates associated with FISH (Gianaroli *et al*., 2005), one randomised controlled trial showed that there could be a reduction in live birth success following embryo biopsy and FISH (Mastenbroek *et al*., 2007). Over the last 10 years PGT-

A has moved towards 24 chromosome copy number analysis, firstly in the form of aCGH and more recently NGS due to technological improvements. Further research has been carried out evaluating the benefit of these newer versions of PGT-A with a number of studies showing that PGT-A offers an advantage over control groups for achieving live birth (Scott *et al*., 2013; Rubio *et al*., 2017; Anderson *et al*., 2019). However, a multi-centre RCT carried out by Munne *et al*, in 2019 showed that there was no benefit seen in the PGT-A group on a per embryo transferred basis or by intention to treat, particularly in patients under 35 (Munné *et al*., 2019).

To date, there have been no large-scale multi-centre retrospective studies carried out to determine the effectiveness of PGT-A in the UK. Therefore, this study intends to assess the effectiveness of the transfer of euploid embryos following PGT-A over conventional IVF without genetic screening.

4.1.1 Specific aims

With the above in mind the purpose of this study is by retrospective analysis of the largest cohort to date:

a) To determine if there is an improvement in live birth following PGT-A compared to conventional IVF, with particular regard to “intent to treat” criteria
b) To test the hypothesis that there is inter-clinic variation between these figures.

4.1.2 Methods

Under University of Kent ethical regulations, this project did not require further ethical approval. This project was reviewed internally within CooperGenomics and CARE Ferility and it was determined that no further ethical approval was needed, and that patient confidentiality and General Data Protection Regulations should be observed throughout.

This study consisted of retrospective analysis of patients that underwent PGT-A using aCGH and NGS at four reproductive clinics across the UK between 2011 and 2017. This was a retrospective study performed on existing clinical data which was compiled and de-identified before analysis.

Cases include patient cycles using aCGH and NGS for PGT-A and controls include patient cycles not using PGT-A or any other genetic diagnostic testing. Embryo transfers of cryopreserved embryos up to December 2019 were included. Follow up from embryos transferred were recorded up until December 2019.

aCGH was the genetic analysis method available in the first instance, NGS was offered from August 2015 at which point all clinics began uptake of this method for PGT-A. Not all clinics offered PGT-A from 2011 but information was collected from the point at which they began providing PGT-A, the breakdown for PGT-A testing and aCGH uptake for each clinic is as follows:

- Clinic A carried out PGT-A cycles between 2011 and 2017, they moved from aCGH to NGS around August 2015.
- Clinic B carried out PGT-A cycles between 2012 and 2017, they moved from aCGH to NGS around August 2015.
- Clinic C carried out PGT-A cycles between 2014 and 2017, they began moving from aCGH to NGS around August 2015, however a small number of cycles used aCGH in 2016 to allow for fresh embryo transfer.
- Clinic D carried out PGT-A cycles between 2014 and 2017, they moved from aCGH to NGS around August 2015.

Patient demographics and basic clinical information were collected where available, this included patient age (oocyte donor age, where applicable), previous number of cycles and outcome of previous cycles, infertility diagnosis, BMI, AMH. Exclusion criteria included patients undertaking genetic analysis for PGT-M or PGT-SR purposes, in the control group (non PGT-A) patients who used oocyte donation (due to time involved to match recipient to donor to establish donor maternal age), those undertaking oocyte storage only and those who had no ovarian stimulation.

Each clinic performed controlled ovarian stimulation and ovulation trigger hCG in accordance with their own protocols. Gonadotropin used, duration of ovarian stimulation, gonadotropin doses, hCG dose and type were recorded where available. Gonadotopins used were grouped into recombinant FSH “Gonal-F” (Merck-Serono), or recombinant FSH

plus hMG “Menopur” (Ferring Pharmaceutical), Combination “Gonal-F” and “Menopur” or Other.

Intracytoplasmic sperm injection (ICSI) was performed on the majority of cases undergoing PGT-A between 2011 and 2014, from 2015 In Vitro Fertilisation (IVF) or ICSI was performed in accordance to each clinics protocols (for example, for poor sperm counts or previous poor or failed fertilisation), the fertilisation method was recorded for each cycle. Embryos were routinely cultured until day 3 prior to embryo biopsy until 2015 where embryos were cultured until day 5 before biopsy, the type of biopsy; blastomere or trophectoderm were recorded for all cases. Biopsy was carried out on day 3 or day 5 or 6 (depending on rate of embryo blastulation) of embryo development using a LASER to perforate the zona pellucida, followed by biopsy of a single blastomere from each embryo for day 3 or a small cluster of cells from the trophectoderm of day 5 or 6 embryos. Each clinic used their own protocols to determine if an embryo or blastocyst was suitable for biopsy. Biopsy technique varied across the study period and each clinic performed biopsy procedures in accordance with their own protocols. The biopsied cells were washed in clean biopsy wash media before being placed into a transport tube to be sent to the genetics laboratory for analysis.

All genetic analysis was performed by CooperGenomics between 2011 and 2016 in accordance with their standard operating procedures (refer to section 2.1). During 2017 clinic B and C also used a second genetics provider Source Bioscience, the genetic provider

for these clinics was recorded for each case. Mosaic embryos were not routinely called during this study period, generally high-level mosaics were reported as aneuploid and low-level mosaics were reported as euploid.

Fresh and frozen embryo transfers were carried out over the study period, single embryo transfers and double embryo transfers were also carried out. Outcomes were recorded on a per embryo transferred and per embryo transfer event basis. Biochemical pregnancies were detected as a positive hCG level on day 14 to 16 days after embryo transfer. Biochemical pregnancies were followed up with a transvaginal ultrasound scan between 4 to 6 weeks following embryo transfer, clinical pregnancies were confirmed by detection of a fetal heart. Pregnancy loss prior to confirmation of clinical pregnancy were recorded as either biochemical pregnancy only, 0 fetal heart or early miscarriage (normally detected at the time of scan). Miscarriages were recorded when they occurred following clinical pregnancy confirmation. Live birth information and adverse outcomes such as terminations, stillbirth and neonatal death were collected, it was recorded if any of these events were due to aneuploidies (for example, Trisomy 21).

Number of sequential transfers until the achievement of a healthy live birth were collected for patients in this study period. This included multiple cycles where applicable to achieve embryos for transfer. Some patients in this study period would batch embryos for PGT-A analysis, therefore there could be multiple full cycles involving controlled ovarian stimulation before euploid embryos were transferred. Patients that switched

between transfers from non-PGT-A embryos and PGT-A embryos were excluded from this part of the analysis, however if a patient switched from one group to the other and continued with this method they were included as a separate entry in each group, embryos that were biopsied and subsequently resulted in no result then transferred without further genetic testing were also excluded at this stage.

Categorical variables are presented as numbers and percentages, continuous variables are expressed with means± standard deviation. Most statistical analysis was carried out based on live birth outcomes and is stated where this is presented on a per embryo transfer or per patient basis. Data were entered into Statistical Package for the Social Science database (SPSS) (SPSS, Chicago, IL, USA) to obtain statistical analysis. Chi2 analysis was carried out to compare the clinics, study groups and biopsy practitioners. Statistical significance was considered at P values of <0.05.

My own personal contribution was that I developed a database to include relevant information to answer the research aims. I audited CooperGenomics work lists to establish all patients from the study clinics during the study period that had undergone PGT-A, I updated the database with all these patients including the genetic results of all embryos biopsied for each cycle. I reviewed each patient cycle individually using the clinics in house database to complete patient demographics, stimulation protocols, cycle information, embryo transfer and outcome information and obtaining information which was not available on the in-house database I collected from patient files. Ensuring to the best of

my ability that I knew which euploid embryo was transferred and from which cycle it originated, this was particularly important for frozen embryo transfer events. With the assistance of staff at the two clinics which used a secondary genetics company in the form of accessing laboratory records of PGT-A cases carried out in 2017, I identified the further patients who had PGT-A and pulled genetic reports to add their embryos to the database. I compiled the control data, following export of all cycles from the clinics in house databases by in-house IT, I excluded the PGT-A cases plus any further exclusions noted in the methods. I organised the data for each patient's sequential cycles and transfers and audited if all cycles and subsequent frozen transfers were included manually, for frozen embryo transfers carried out after 2017 this required manual data collection from the clinics databases. I carried out all data analysis.

4.2 Results

A total of 2961 fresh cycles were started in the PGT-A group across all four clinics, with a total of 10,293 embryos biopsied. 14,292 fresh cycles were started in the non PGT-A group during the same period. A breakdown of the distribution of patients in each arm for each clinic can be seen in Figure 4.1, clinic D has the largest proportion of patients referred for PGT-A with clinic A having the least. 9808 (95.3%) embryos had a result after genetic analysis, 2523 (25.7%) of those embryos were found to be euploid, 7208 (73.5%) were aneuploid, 77 (0.8%) were mosaic (although mosaics were not routinely reported throughout the study period, therefore this figure will not be representative of the whole

cohort). 2371 (32.9%) of the aneuploid embryos possessed a single aneuploid chromosome, 1420 (19.7%) had a double chromosome aneuploidy and 3417 (47.4%) were complex.

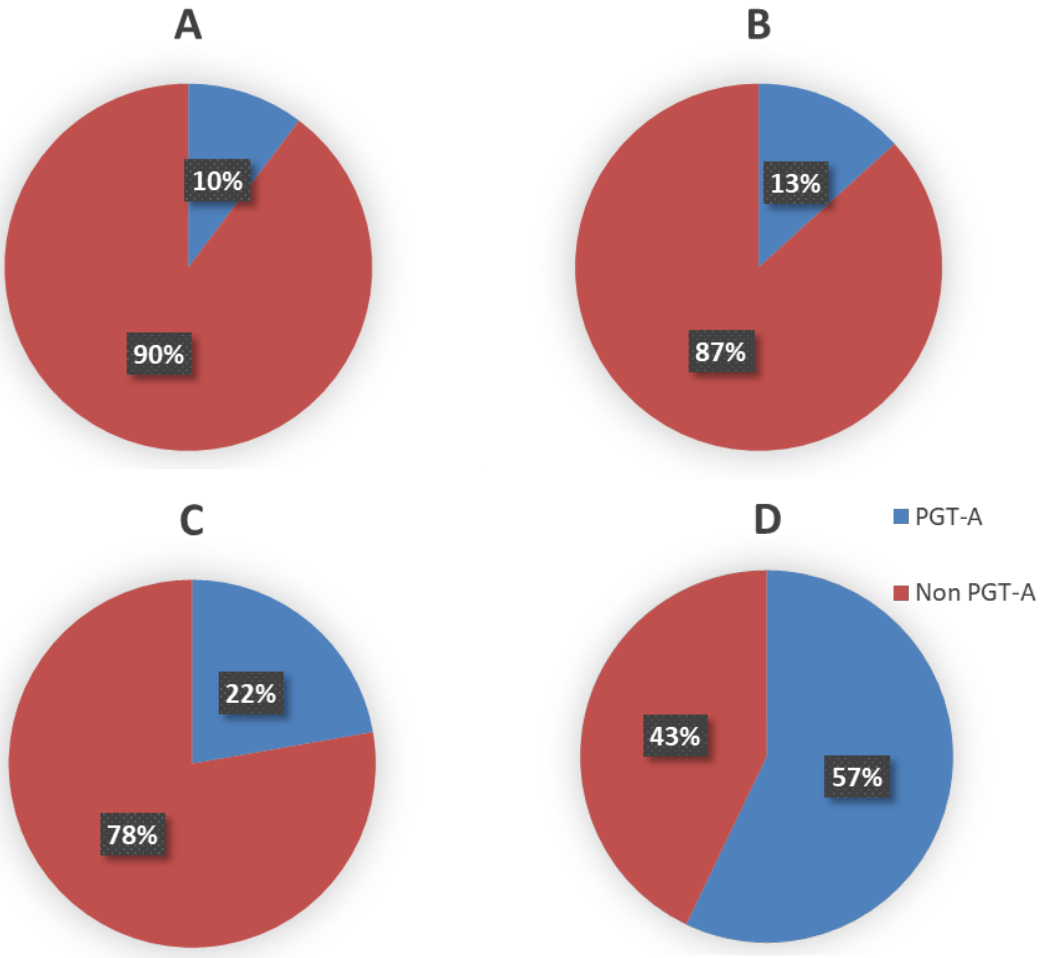


Figure 4.1: PGT-A and control distribution by clinic. Distribution of cases that underwent PGT-A and cases that did not have any genetic testing by clinic.

Table 4.1 shows the mean age of patients in the PGT-A group and non-PGT-A group for each clinic, along with the mean number of oocytes collected, oocytes fertilised and

embryos that were available for biopsy. There are similar numbers between the PGT-A group and non-PGT-A groups of each clinic, however, there is some variation between clinics. In Table 4.1, it is presented what percentage of patients have their cycles abandoned prior to embryo biopsy for PGT-A and embryo transfer or embryo cryopreservation, and at what stage these cycles are abandoned. We see similar rates of cycles being abandoned prior to egg collection, with no eggs or immature eggs collected and failed fertilisation between the PGT-A and non-PGT-A group within individual clinics. We also saw, that the proportion of patients in the PGT-A arms that either had embryo biopsy or did not proceed to biopsy due to poor quality embryos or low embryo numbers is similar to the proportion of patients in the non-PGT-A arm that either have embryo transfer or embryo freezing. This is expected, as despite low embryo numbers or poor quality, if an embryo is available transfer it will most likely still go ahead in the non-PGT-A group. The exception to this is clinic C which does see variation between the PGT-A group and non-PGT-A group for cycles abandoned at each stage. This clinic also had a much lower rate of cycles abandoned due to low embryo numbers or poor embryo development. A large proportion of patients in the PGT-A group that abandoned embryo biopsy due to low embryo numbers or poor embryo quality did proceeded to embryo transfer, although this does vary between clinics, with clinic B seeing 91% of these patients having a subsequent transfer whereas clinic D only had 16% of these patients have an embryo transfer. There are a number of patients in each clinic that despite having a euploid embryo available following PGT-A dod not proceed to transfer, this is for a

variety of reasons, including, spontaneous pregnancy, storing embryos for fertility preservation, breakdown of relationships, illness and in rare instances death.

	Clinic A		Clinic B		Clinic C		Clinic D	
	PGT-A	Non PGT-A	PGT-A	Non PGT-A	PGT-A	Non PGT-A	PGT-A	Non PGT-A
Total cycles started	624	5448	823	5383	847	2960	667	501
Age mean ± SD	39.5 (4.25)	35.0 (4.59)	38.2 (4.48)	34.5 (4.67)	39.1 (3.88)	37.6 (4.33)	39.5 (3.63)	36.6 (3.84)
Oocytes retrieved mean ± SD	9.1 (5.39)	9.2 (5.87)	9.2 (5.70)	9.2 (5.65)	13.2 (7.59)	9.9 (7.27)	10.9 (6.74)	10.0 (6.27)
Oocytes fertilised mean ± SD	5.3 (3.64)	5.2 (3.82)	5.4 (3.93)	5.0 (3.74)	8.7 (5.26)	5.9 (5.04)	6.4 (4.54)	5.6 (4.55)
% fertilised	49.9	56.0	57.8	54.2	65.4	59.2	58.7	55.0
Embryos biopsied mean ± SD	4.6 (2.58)	N/A	3.9 (2.33)	N/A	5.5 (3.24)	N/A	3.6 (2.45)	N/A
% biopsied (of fertilised)	62.3	N/A	44.9	N/A	62.2	N/A	46.0	N/A
N. cases cancelled before oocyte retrieval (%)	15 (2.4)	123 (2.3)	5 (0.6)	45 (0.8)	23 (2.7)	129 (4.4)	40 (6.0)	40 (8.0)
N. cases no oocytes retrieved, immature and oocyte freeze (%)	7 (1.1)	33 (0.6)	12 (1.5)	28 (0.5)	4 (0.5)	24 (0.8)	9 (1.3)	7 (1.4)
N. cases failed fertilisation (%)	24 (3.8)	186 (3.4)	38 (4.6)	235 (4.4)	3 (0.4)	133 (4.5)	24 (3.6)	16 (3.2)
N. cases no embryo biopsy (%)	134 (21.5)	N/A	267 (32.4)	N/A	9 (1.1)	N/A	81 (12.1)	N/A
N. cases failed cleavage/arrested (%)	N/A	77 (1.4)	N/A	33 (0.6)	N/A	35 (1.2)	N/A	14 (2.8)
N. cases had PGT-A (%)	444 (71.2)	N/A	501 (60.9)	N/A	808 (95.4)	N/A	514 (77.1)	N/A
N. cases had at least 1 euploid	229 (36.7)	N/A	274 (33.3)	N/A	450 (53.1)	N/A	301 (45.1)	N/A
N. cases had no euploids	215 (34.5)	N/A	226 (27.5)	N/A	358 (42.3)	N/A	213 (31.9)	N/A
N. cases that had transfer	209 (33.5)	N/A	257 (31.2)	N/A	401 (47.3)	N/A	268 (40.2)	N/A
N. cases that had embryos available for transfer or freezing	N/A	5029 (92.3)	N/A	5042 (93.7)	N/A	2639 (89.2)	N/A	424 (84.6)

Table 4.1: Description of cycle outcomes of patients at each stage of the process up to embryo transfer. N=Number, SD=Standard deviation

The percentage of biopsied embryos found to be euploid following PGT-A was seen to decline with maternal age for all clinics (Figure 4.2), from 42% for <35 to 2.6% for patients >44 for all cases. A higher rate of euploids was detected with NGS than aCGH, for example, 46.6% vs 33.5% in the under 35s (NGS, aCGH, respectively). There is a significantly higher rate of euploids seen with clinic D when reviewing all cases, however, this clinic carried out a much larger proportion of NGS cases than all the other clinics (Figure 4.4), and when NGS only was tested for statistical significance clinic D was only statistically higher than the other clinics in the 35-37 age category (chi2=18.58, p<0.001).

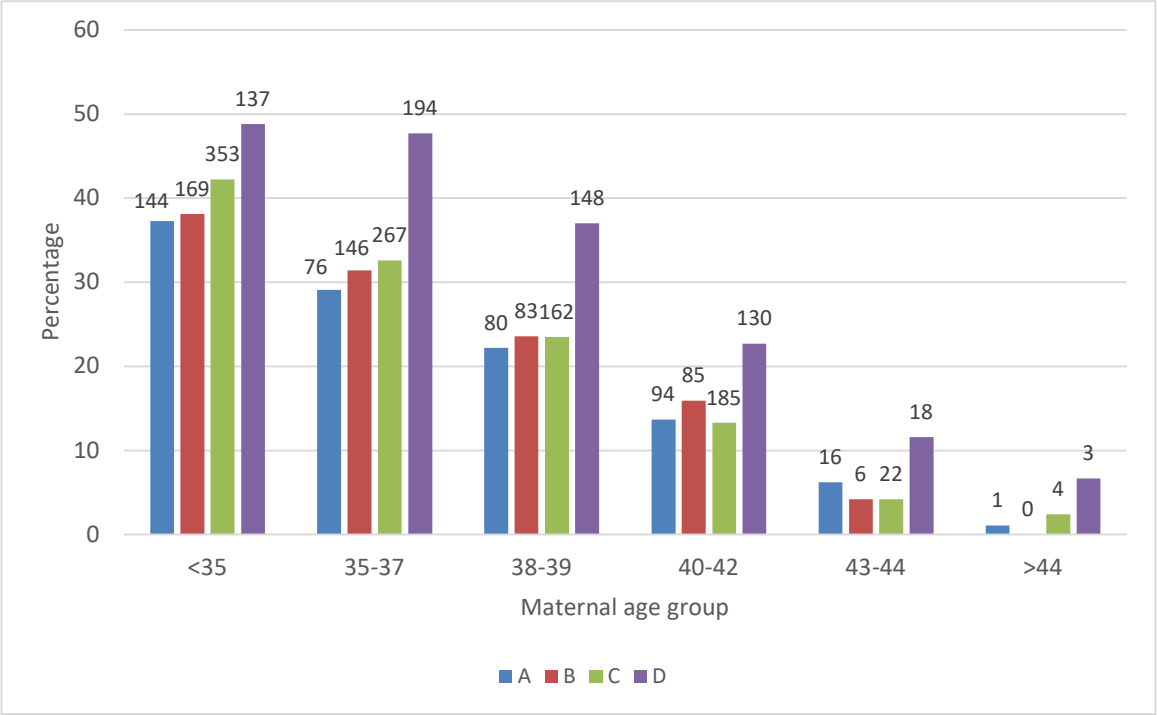


Figure 4.2: Percentage of embryos biopsied found to be euploid by clinic and maternal age

We can also see from Figure 4.3 that the chance of a patient having a euploid embryo available for transfer after PGT-A declines rapidly with increasing maternal age. The proportion of patients who also had embryos of good enough quality available for biopsy varied by clinic Table 4.1.

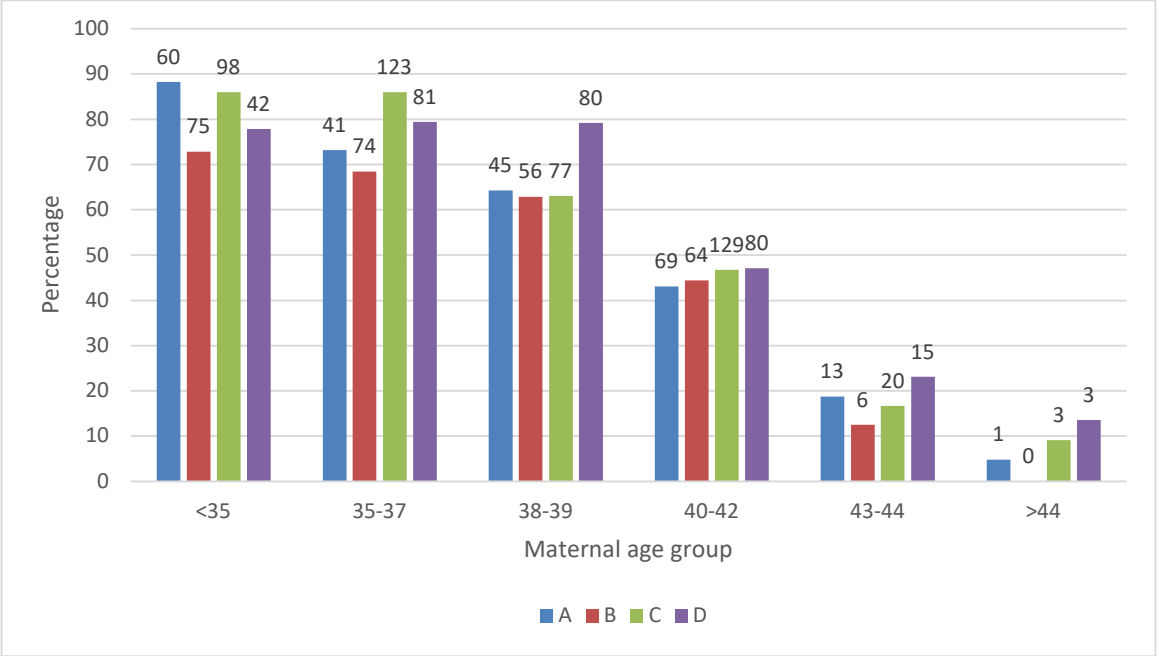


Figure 4.3: Percentage of cases that have at least one euploid embryo available after PGT-A by clinic and maternal age.

The proportion of NGS vs aCGH cases per clinic can be seen in Figure 4.4, clinic B and D performed NGS on the highest proportion of cases whilst clinic A mostly performed aCGH and clinic C was almost half and half. This also impacts on the proportion of blastomere and trophectoderm biopsy cases performed by per clinic as well as fresh vs frozen embryo transfers. To begin with it was more common for aCGH to be carried out on day 3

embryos, performing a blastomere biopsy then transferring euploid embryos fresh at the blastocyst stage on day 5. Whilst some NGS cases will have resulted in fresh transfers, the majority were frozen embryo transfers. The proportion of blastomere vs trophectoderm biopsies by clinic can be seen in Figure 4.5. Clinic D carried out almost all PGT-A cases at the blastocyst stage (99.8%), whereas clinic A carried out a larger percentage at the cleavage stage (61.9% vs 38.1%).

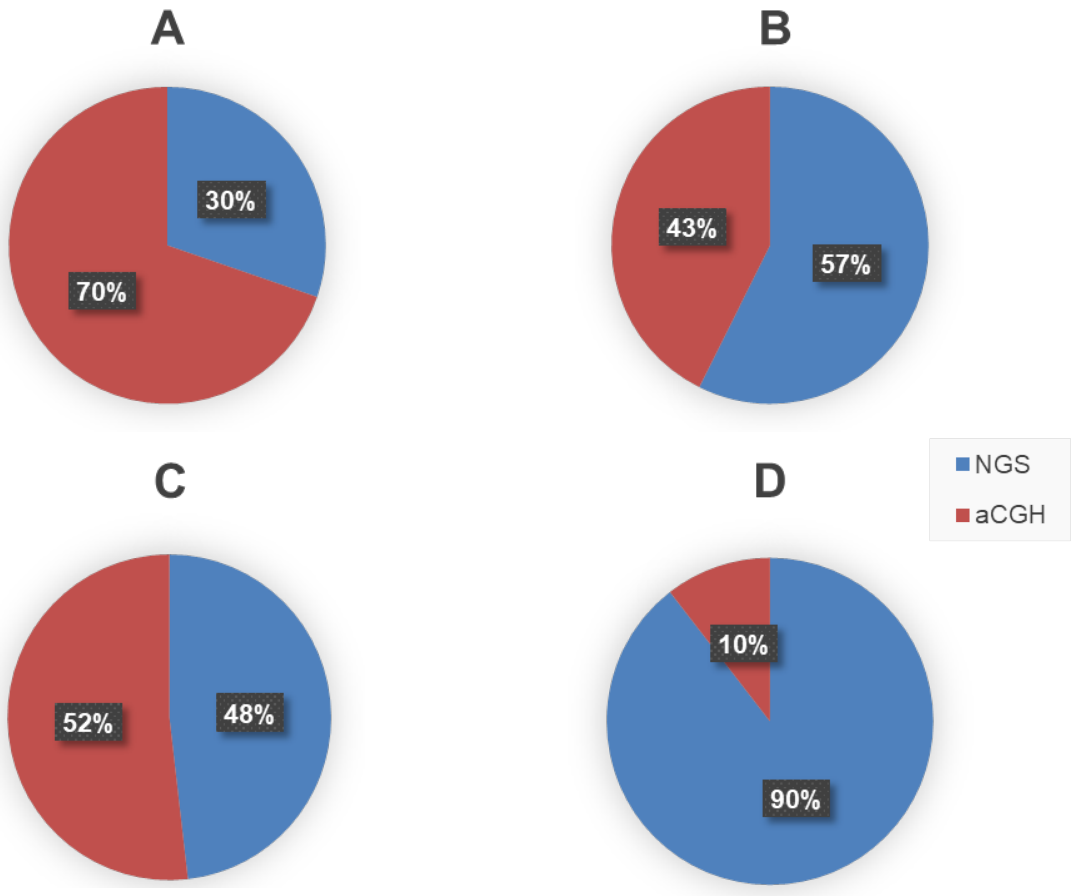
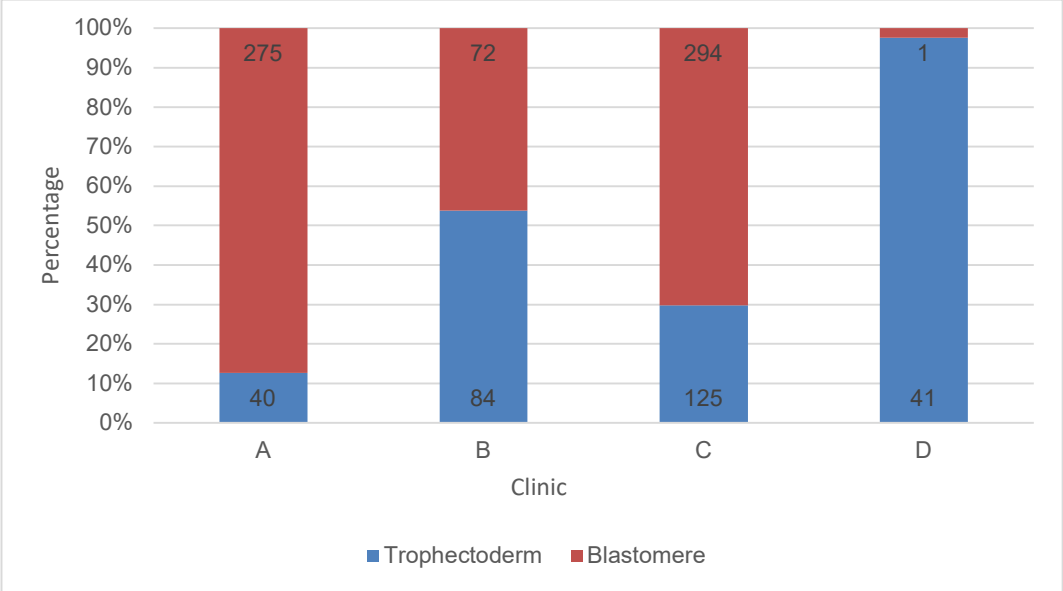


Figure 4.4: Proportion of PGT-A cases which had NGS or aCGH by clinic.

A. aCGH



B. NGS

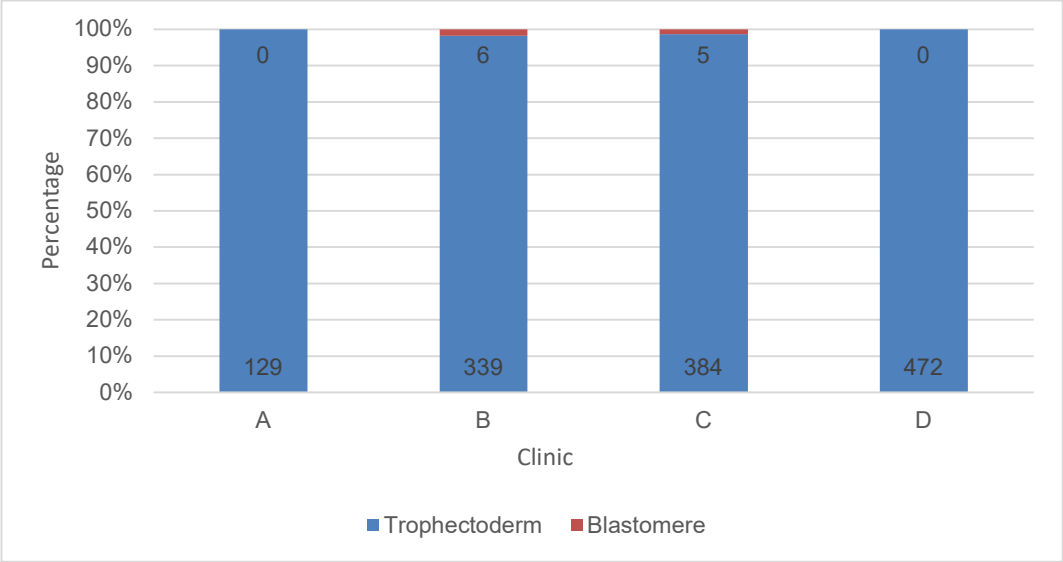
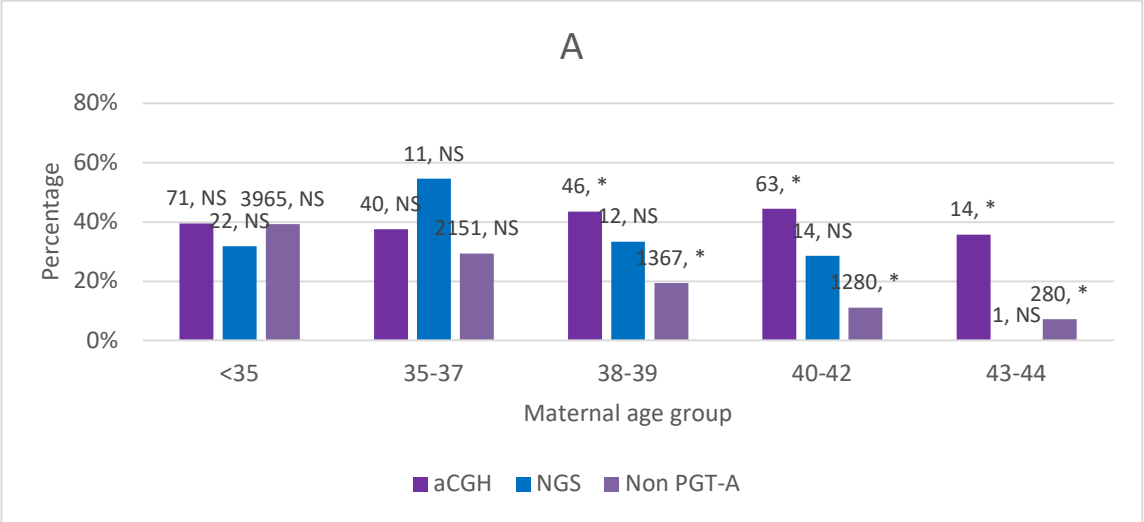


Figure 4.5: Percentage of PGT-A cases which were carried out on the cleavage stage embryo vs the blastocyst embryo, by clinic.

When reviewing live birth percentages per embryo transferred, by maternal age for each clinic (Figure 4.6), we can see that results vary by clinic. For all clinics there is a statistically higher percentage of live births associated with transfers in older patients over 35 (for clinic A, this does not achieve statistical significance in the 35-37 age group, but is trending towards a higher live birth rate). However, all clinics with the exception of clinic D, there is no statistical difference between PGT-A (both aCGH and NGS) over the non PGT-A group in the under 35s. Clinic D does have a statistically higher percentage of live births with NGS for the under 35s, although the NGS live birth rate of clinic D is similar to the live birth rate seen for the non PGT-A groups for clinic A and C.

NGS consistently achieved a significantly higher percentage of live births than the non PGT-A group in all but clinic A, however, from Figure 4.4 we can see that this clinic carried out a much lower proportion of NGS cases, therefore this is likely to not be statistically significant due to the low number of NGS cases performed. aCGH also has an improvement in live birth percentage over non PGT-A, especially for clinic A (which performed a higher proportion of aCGH cases), however it does not achieve a significantly higher percentage of live births across as many age groups as that seen with NGS.

There were very few transfers carried out in the >44 age group across all clinics, making it not possible to carry out statistical analysis for this age group.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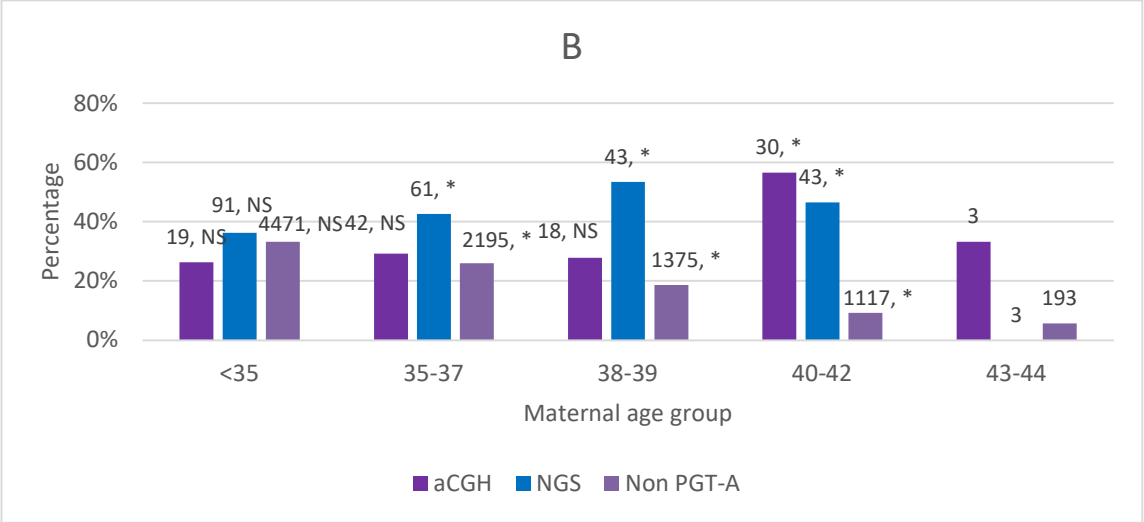
43-44
Maternal age group
aCGH
NGS
Non PGT-A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Maternal age group
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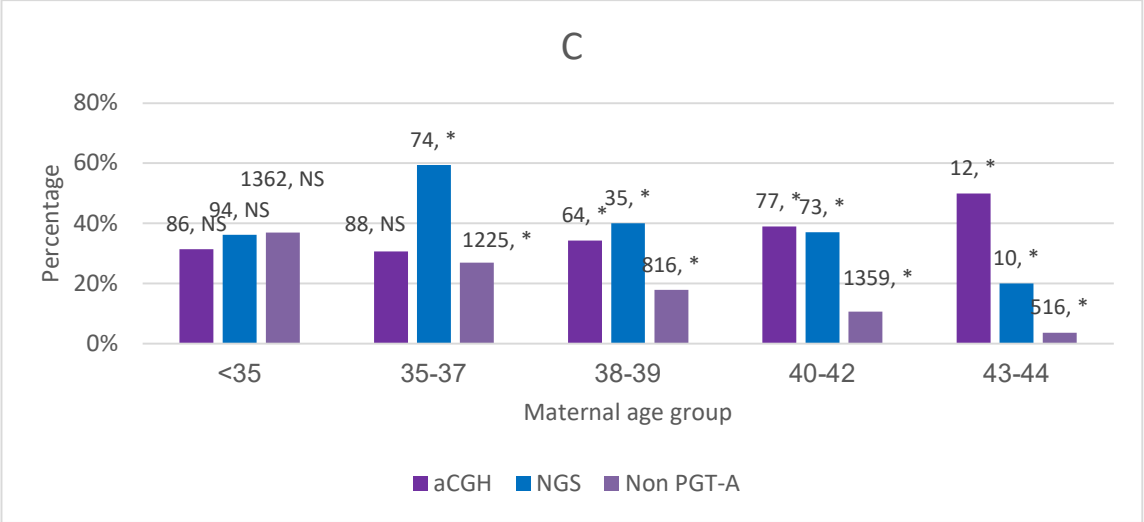
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1359, *
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Maternal age group
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Non PGT-A

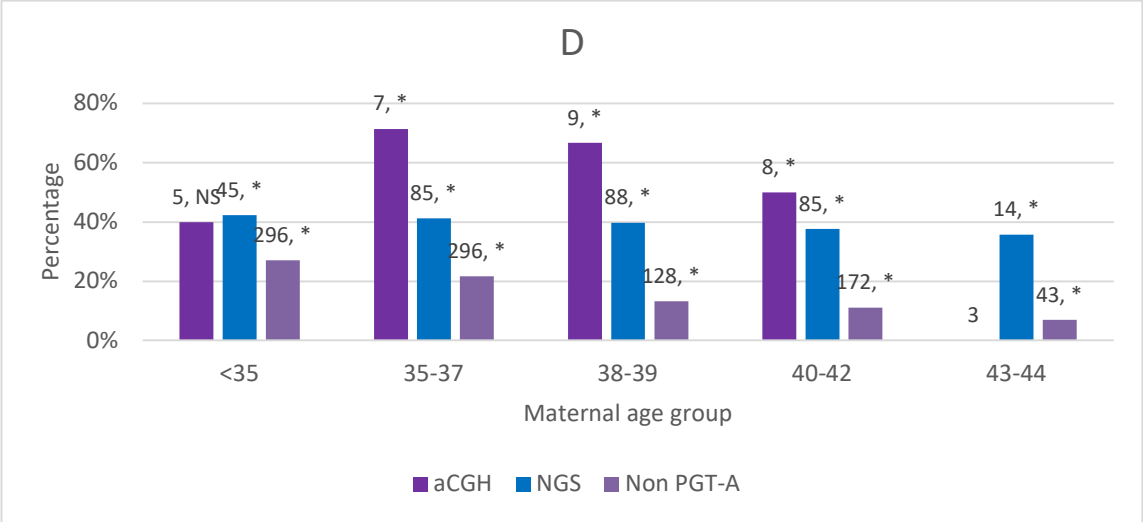


Figure 4.6: Percentage of live births per embryo transferred by age, NGS, aCGH and non PGT-A, for each clinic. N=number of embryos transferred, Chi2 test: *=statistically significant P<0.05, NS=Not significant. (Chi2 test, tests observed frequencies against expected frequencies (mean of all observed frequencies), therefore any that are statistically significant will be statistically different from the expected mean of all groups either above or below.)

As might be expected when comparing all PGT-A cycles and non PGT-A cycles, PGT-A had a statistically higher proportion of the embryos which implanted that continued to clinical pregnancy, 75.3% vs 69.1% (Chi2=16.55, P<0.001). PGT-A was also associated with statistically fewer miscarriages following clinical pregnancy, 9.1% vs 12.4% (Chi2=7.05, P=0.008).

We observed that more oocytes collected in the non PGT-A group across all clinics eventually make it to embryos that are transferred than in the PGT-A group, as would be expected (Figure 4.7). This is most likely due to those embryos which are reported as aneuploid not being considered for transfer in the PGT-A group. Therefore, a higher proportion of non PGT-A embryos which are transferred, fail to implant, or make it to live

birth, such as was previously discussed and seen in Figure 4.6. However, it can be seen that with sequential embryo transfers a significantly greater proportion of oocytes (that go on to become embryos for transfer), result in live birth in the non PGT-A group in all age groups except >44 (Figure 4.8).

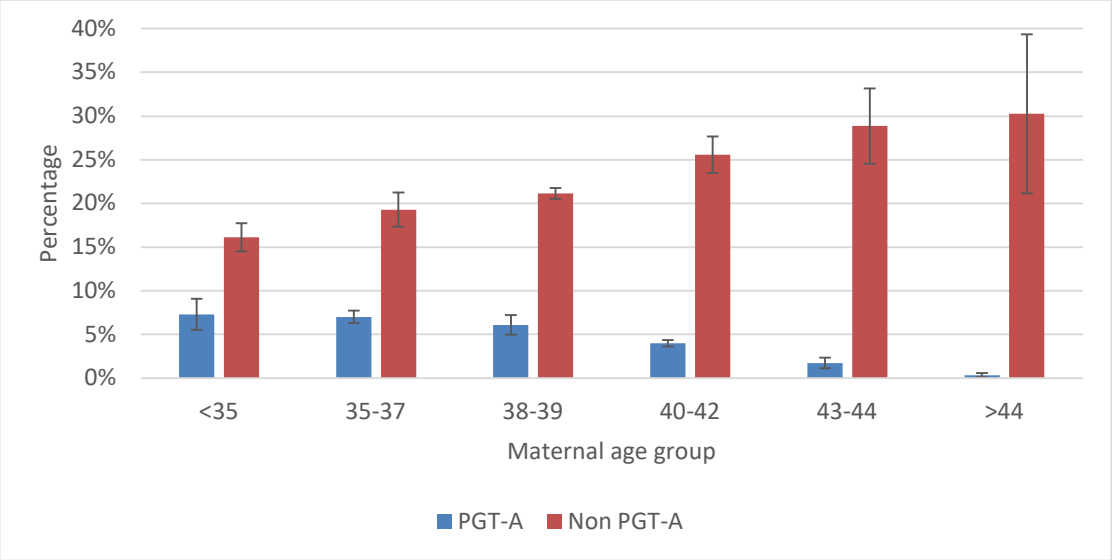


Figure 4.7: Percentage of oocytes collected that are transferred, by maternal age, all clinics combined, including error bars.

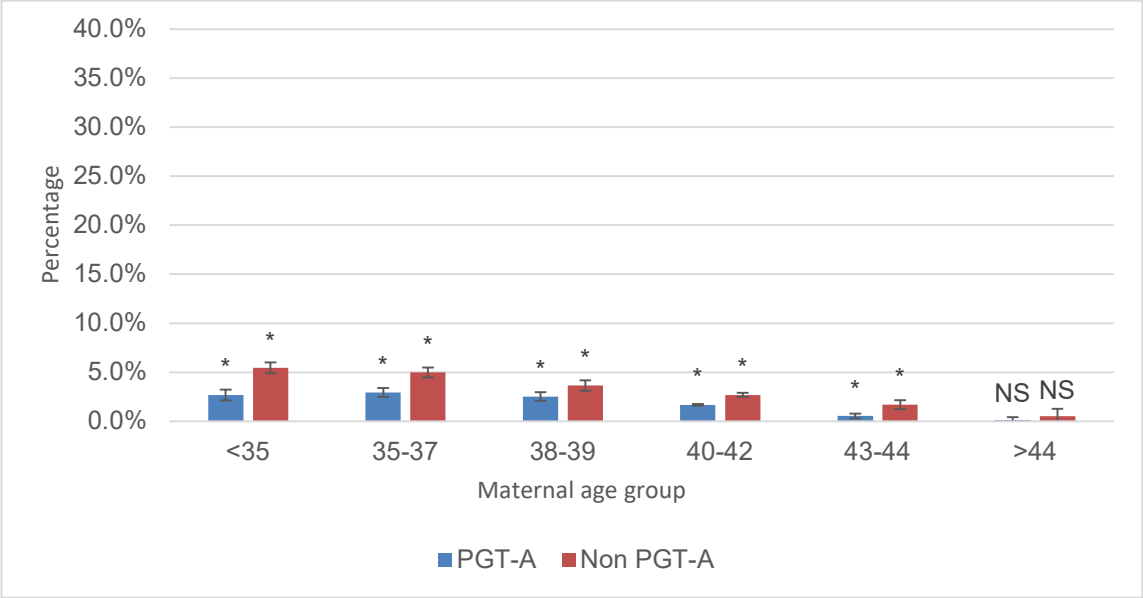
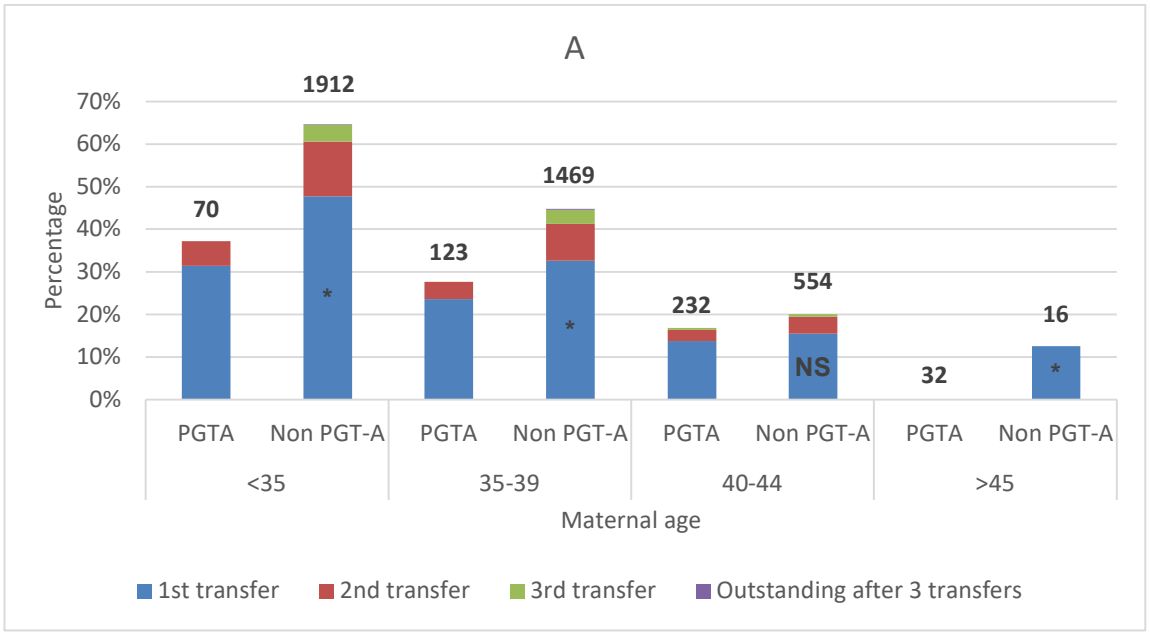


Figure 4.8: Percentage of oocytes collected that ultimately result in live birth, by age group, all clinics combined. Chi2 test * = statistically significant, p<0.05, NS= not significant. (Chi2 test, tests observed frequencies against expected frequencies (mean of all observed frequencies), therefore any that are statistically significant will be statistically different from the expected mean of all groups either above or below.)

The data were reviewed on a per patient basis to first live birth for each clinic and by age category (Figure 4.9), attempts for a second live birth and those that did not have any euploid embryos transferred (but had euploid embryos available) were excluded from this analysis. 1946 patients underwent cycles with PGT-A and 10493 patients underwent cycles without PGT-A across all clinics. Some patients had multiple cycles started prior to getting to their 1st, 2nd, or 3rd transfer. For the PGT-A patients, it was common to see embryo batching carried out prior to the transfer of a euploid embryo (that is undergoing multiple fresh cycles to obtain a larger number of embryos for PGT-A analysis). The average number of cycles carried out for the PGT-A patient's vs non PGT-A was 1.4 and 1.3, respectively. Whilst the average number of embryo transfers per patient PGT-A vs non

PGT-A was 0.7 vs 1.5, respectively. As expected the percentage of patients that had a 1st, 2nd and 3rd transfer was less in the PGT-A group: 53.6%, 14.2%, 4.6% and 96.7%, 34.9%, 12.4% (1st,2nd and 3rd transfers; PGT-A, non PGT-A, respectively). From Figure 4.9 we can see that for the under 35 patients, the only clinic that saw an improvement at the first transfer with PGT-A was clinic D, although this did not reach statistical significance (Chi2=2.85, P=0.091). The 35-39 age group saw an improvement in live birth rate at clinic C and D but was only statistically significant for clinic D (Chi2=2.91, P=0.088 and Chi2=4.18, P=0.041, respectively). All clinic data were combined to assess aCGH vs NGS by maternal age, there was a slight improvement with NGS only in the 35-39 age group, 34.2% vs 39.8% (aCGH, NGS, respectively) but this was not statistically significant (Chi2=0.24, P=0.621).

The variation in live birth percentage per embryo transferred by biopsy operator for those that had 30 or more euploid embryos transferred was found to range from 33.1% to 49.0%, although the variation by biopsy operator was not significant (P=0.630).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Maternal age
1st transfer
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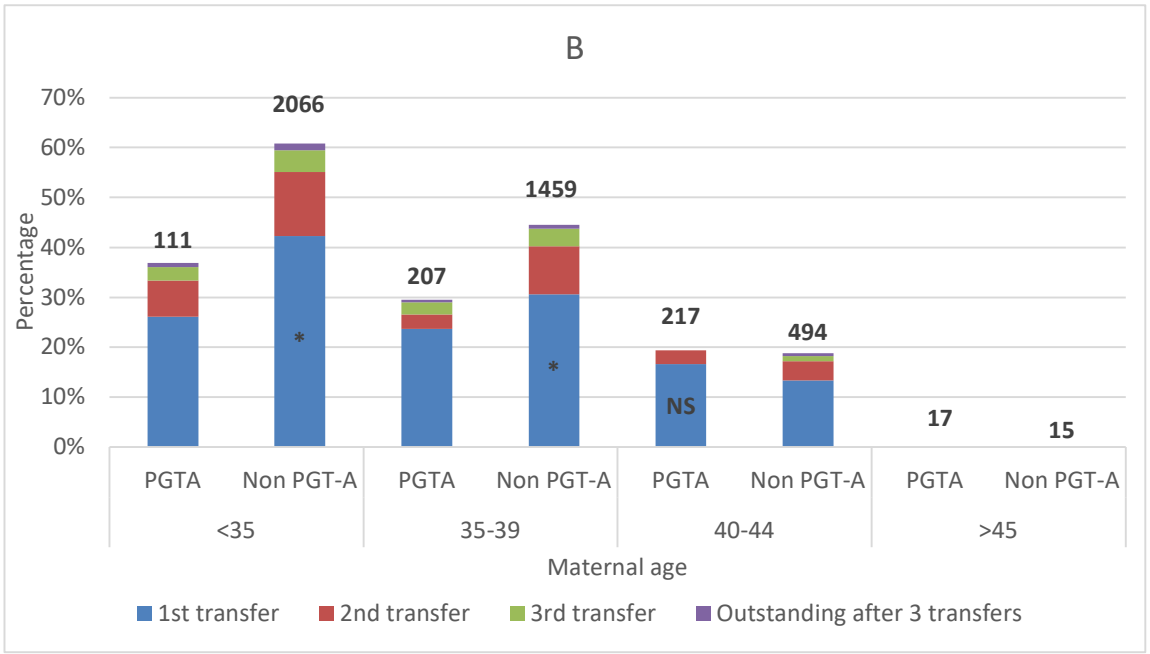
3rd transfer
Outstanding after 3 transfers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Maternal age
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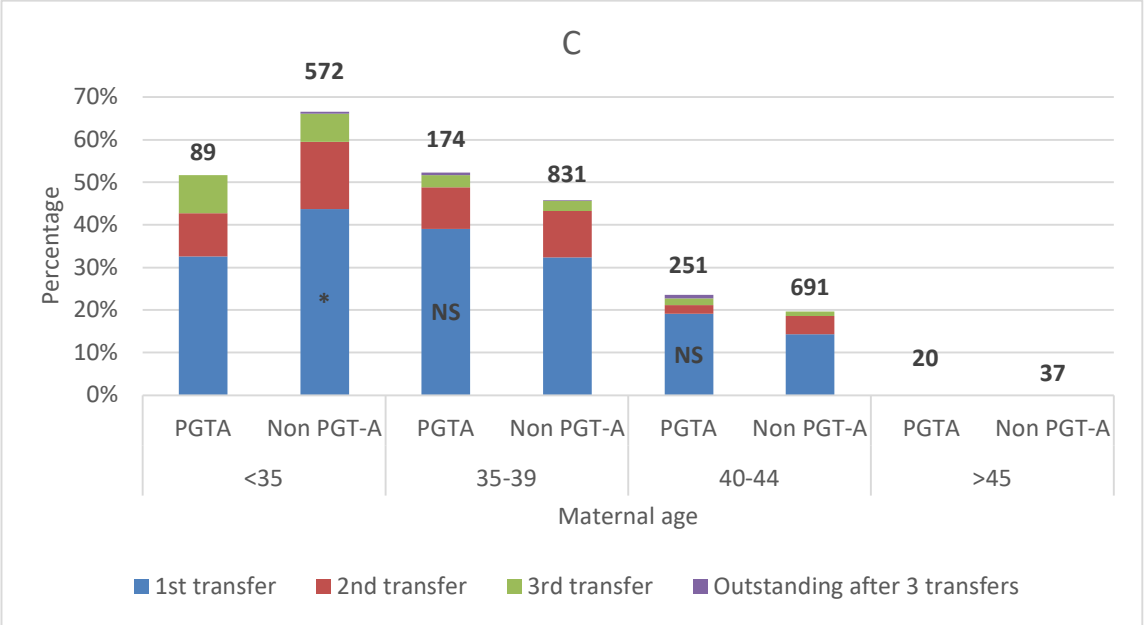
3rd transfer
Outstanding after 3 transfers



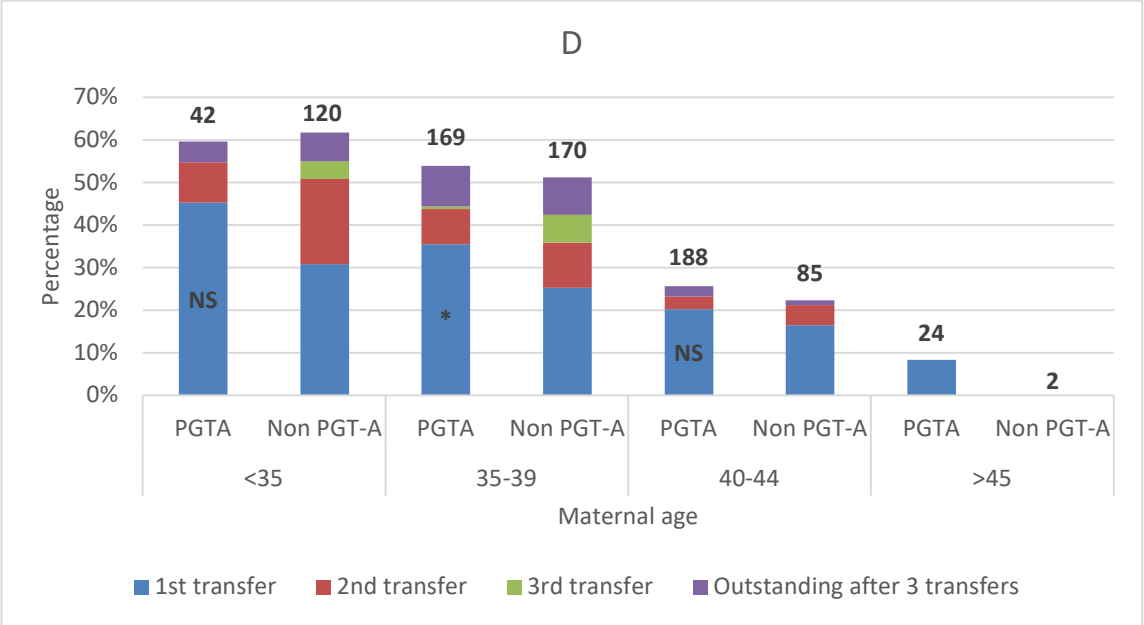


Figure 4.9: Percentage of live births after each embryo transfer up to 3 transfers, per patient that started treatment (multiple cycles may have been required to achieve an embryo transfer), by maternal age, by clinic. Statistical analysis provided for first transfer only, Chi2 test *=statistically significant p<0.05, NS=not significant, N=number of patients that underwent treatment in each group.

4.3 Discussion

This study aimed to assess if there was an improvement in live birth rate with PGT-A compared to conventional IVF without PGT-A, and to what degree this varies between fertility clinics. Our findings indicate that there was an improvement in live birth with PGT-A over conventional IVF on a per embryo transferred basis for patients over 35 years old. However, with patients under 35 years there was not a statistically significant improvement associated with PGT-A over conventional IVF. We also found the extent of improvement in live birth rates found with PGT-A varied considerably across different fertility clinics.

On a per patient transfers to live birth basis, clinics A and B did not see an improvement with PGT-A over conventional IVF for all age groups. Clinic C saw an increased rate of live births for the first embryo transfer in the 35-39 and 40-44 age groups but did not reach statistical significance. Clinic D saw an improvement across all age groups for the first embryo transfer, although only achieved statistical significance in the 35-39 age group.

The variability between clinics with regards to success rates appears to be due to several factors. Firstly, the clinics that refer the greatest proportion of patients for PGT-A experience the most benefit from PGT-A particularly on a per patient basis following the first embryo transfer. Here, we are seeing variation in clinic approaches regarding who they recommend taking up PGT-A as part of their treatment. For those referring the fewest, they are likely to be referring patients of poorer prognosis, e.g. recurrent

miscarriage, severe male factor infertility and recurrent implantation failure. It is therefore feasible that for these patients on an individual basis, they are seeing increased success than they may have experienced through conventional IVF without PGT-A. Secondly, there was variation between the clinics in management of patients prior to reaching embryo biopsy. The stages at which cycles are abandoned prior to embryo biopsy were presented in this study, clinics C and D had the highest proportion of patients intending to undergo PGT-A that proceeded to embryo biopsy. Whilst those patients that do not have PGT-A due to low embryo numbers or poor embryo quality may still proceed to have a transfer and some cases a live birth, these will impact on the overall figures for live births per patient undergoing PGT-A. Ultimately if there are low embryo numbers or poorer quality embryos, it is the patients decision whether to proceed to embryo biopsy. However, the management of patient treatment cycles to yield a higher number of oocytes, fertilisation rates and blastulation rates will provide more embryos available for biopsy and an increased chance of finding a euploid embryo available for transfer, such as that seen in with clinics C and D. It is often suggested that variability in the skill level of the biopsy practitioner is likely to be impacting success rates of the transfer of subsequent euploid embryos. Whilst there was variability seen between biopsy practitioners this was not statistically significant, and was mostly due to number of transfers achieved in relation to live birth, e.g. those biopsy practitioners who had fewer euploid embryos transferred could have much higher or lower rates of live birth, due to the difference of one or two transfers.

For clinic D, the argument can be made that the overall improvement in success rates with PGT-A seen, is due to this clinic predominately carrying out NGS cycles and trophectoderm biopsy. Indeed, previous research has confirmed that trophectoderm biopsy is superior to blastomere biopsy (Harton *et al*., 2013; Rubio *et al*., 2013; Coll *et al*., 2018). It has also been reported that NGS is more accurate than aCGH as well as, resulting in higher live birth rates (Friedenthal *et al*., 2018, 2020). NGS therefore, could be contributing to the increased success rate documented in this study, especially for clinic D. We too noted an improvement in live birth rates per embryo transferred with NGS over aCGH for clinic B and C. However, as clinic D performed very few aCGH cases and conversely clinic A performed much fewer NGS cases, this trend cannot be confirmed here. When data for all clinics was combined to assess aCGH vs NGS on a per patient basis there was a slight improvement with the 35-39 age group, but was not statistically significant, indicating that the variation in individual clinics may be playing a bigger role here than the type of PGT-A performed. Interestingly, there was an increase in the rate of euploid embryos detected with NGS over aCGH, this offers an explanation why the two clinics that performed the most NGS cycles had a higher proportion of patients with at least 1 euploid embryo available for transfer, although is likely to represent the shift from blastomere biopsy to trophectoderm biopsy and the higher frequency of euploids detected at the blastocyst stage (Adler *et al*., 2014). It should also be considered that a link between poorer prognosis patients and increased rates of aneuploidy have been reported, therefore as previously mentioned the clinics referring the fewest patients for PGT-A are likely to see fewer patients with at least 1 euploid embryo available for transfer (Kort *et al*., 2018).

This study does have limitations predominately due to its retrospective design. Not all data desired to be examined for its confounding effect was available for all patients, e.g. BMI, number of previous cycles. It would have been advantageous to have infertility diagnosis available for all patients, along with other factors to compare like for like prognosis, as these have been shown to be compounding factors in achieving live birth with PGT-A (Boynukalin *et al*., 2020). Biopsy method and the embryo quality threshold had varied by clinic and over the duration of the study period in question, which could have a considerable impact on the success of a subsequent euploid transfer. Clinics have also moved towards single embryo transfer in recent years, over double embryo transfer which did occur near the beginning of the time period studied and in some instances a euploid embryo and a no result embryo were transferred simultaneous, therefore we cannot be sure of the outcome had a single euploid embryo been transferred. It was an aim of this study to obtain outcomes for sequential embryo transfers, however, the timescale required for these transfers to have taken place and to obtain an outcome can be considerable. Especially as some PGT-A patients may delay the transfer of euploid embryos for a variety of reasons e.g. ill health. During the time taken to obtain these outcomes, the technology and approach to management of PGT-A patients has progressed e.g. the reporting and transfer of mosaic embryos. It was also not possible to obtain outcomes for all pregnancies and this varied considerably between clinics.

Whilst we can see from the data that more embryos are transferred in the non PGT-A group, and this results in lower implantation rates and clinical pregnancies when compared to the transfer of euploid embryos. It was interesting to see that given sequential embryo transfers, more oocytes which develop to embryos that are transferred in the non PGT-A group result in live birth than the PGT-A group. There are many factors which could be affecting the outcomes of both the PGT-A and non PGT-A patients. Firstly, mosaic embryos during the study period may have been reported as aneuploid. It has recently been shown that mosaic embryos do have the potential to achieve a live birth, although at lower rates than euploid embryos (Victor *et al*., 2019; Lin *et al*., 2020; Munné *et al*., 2020). It has also been noted more recently that up to 49% of embryos reported as segmental aneuploid could be mosaic but possess a euploid ICM (Girardi *et al*., 2020). Ultimately in the non PGT-A group, the embryos that are transferred, will have a variety of ploidy status, the true aneuploid embryos are the least likely to implant and when they do are most likely to result in early pregnancy loss, however, the mosaic embryos which have been transferred in the non PGT-A group will have a potential (albeit lower than euploid) to achieve a healthy live birth, whereas in the PGT-A group these embryos would most likely have been discarded. This factor could result in a slightly elevation of embryos making it to live birth in the non PGT-A group. This is especially important when you consider some PGT-A patients will have had no euploid embryos available for transfer but may have had a mosaic embryo available. Conversely, some of the embryos which had been reported as euploid especially through aCGH, may have had a low level of mosaicism

that was not detected, which impacted on the viability of these embryos following transfer (Maxwell *et al*., 2016).

During the study period, time-lapse morphological assessment had gained in popularity and algorithms developed to predict the best embryo for transfer (Campbell *et al*., 2013; Basile *et al*., 2015; Fishel *et al*., 2018). It is likely that this will have impacted on the success rates of patients in the non PGT-A group. Within the organisation involved in this study, they have shown improvements in live birth rates associated with time-lapse of around 19% for patients under 38 years (Fishel *et al*., 2017). And indeed, the use of time-lapse algorithms could be avoiding aneuploid embryos for transfer for patients undergoing conventional IVF without PGT-A. Especially in patients under 35 who from our findings will have between 40 and 50% of their embryos being euploid, it is therefore, likely with improved morphological assessments that these embryos will be selected for transfer. It also, cannot be denied that the invasive nature of biopsy for PGT-A could be impacting on the implantation viability of embryos, whilst we could not find a link between biopsy practitioner and live birth rates from the euploid embryos transferred, it is possible that not all euploid embryos that are transferred will survive the biopsy procedure (Rubino *et al*., 2020).

Like that previously reported by Munne *et al* 2019 for patients under 35 we have not seen an improvement in live birth with PGT-A, even with the best performing clinic (Munné *et al*., 2019). However, for patients over 35 there is some improvement seen with PGT-A

over non PGT-A, especially on a per embryo transferred basis. PGT-A appears to be most beneficial for patients with an indication for such treatment, such as, recurrent miscarriage and severe male factor infertility. However, in this study the clinic which outperformed the others with PGT-A was the clinic which referred most patients. Therefore, there could be an argument to providing PGT-A for all patients over 35. Anderson *et al* 2019, have reported higher than average live birth rates per cycle started for all age groups by carrying out PGT-A for all patients (Anderson *et al*., 2019). It is possible that by offering PGT-A to all patients both poor and good prognosis, you see the biggest improvement in live birth rates. Opponents to PGT-A argue that the same live birth outcomes on an intention to treat basis would be achieved by sequential embryo transfers, and that this method would avoid the potential damage to embryos from biopsy. However, although this may be true to an extent, this does require patients to endure the potential disappointment of multiple failed embryo transfers, and increased chance of miscarriage, the effect of which, for the individual patient is difficult to quantify. Also, for older patients the time taken to progress through multiple embryo transfers could delay them from progressing to a subsequent fresh cycle required to find an embryo which will result in live birth (Neal *et al*., 2018). In this study, we found that PGT-A patients did on average have more oocyte collection cycles to find a suitable euploid embryo but on average had fewer embryo transfers. Therefore, the time taken to get to the point of transfer of an embryo which could result in live birth is reduced. However, the extra cost and disappointment at not having a euploid embryo for transfer can prevent patients from progressing to a subsequent PGT-A cycle.

Previously, multicentre PGT-A studies have combined the outcomes from all centres to carry out analysis, whilst this is advantageous to provide a large enough cohort for analysis, it doesn't take into account the degree to which centres will vary. There are many variables which a prospective study will aim to control for, however they will not be able to control for all. Each centre is unlikely to manage patients the same way throughout their treatment and this ultimately could impact on success rates seen with PGT-A.

In the future, it is important for clinics offering PGT-A to continuously monitor their protocols for treatment and review their success rates to ensure they are offering the best treatment for their patients. It would be advised to approach offering PGT-A to patients under 35 with caution, and only do so where indicated. With improvements in NGS and reporting of mosaic embryos, and potentially considering these mosaic embryos for transfer when no euploid is available, there could be an increase in success rates for patients under-going PGT-A. In addition, developments in non-invasive PGT-A is becoming more promising. Non-invasive PGT-A could offer an alternative to embryo biopsy, avoiding the potential damage to embryos, whilst allowing clinics to select euploid embryos for transfer (Rubio *et al*., 2020).

4.4 Conclusion

The work described in this study, suggest that PGT-A improves live birth rates for patients over 35 on an embryo transfer basis, however this same benefit is not seen for patients under 35. The degree of improvement seen with PGT-A varies significantly between clinics.

It is important for clinics to consistently review their PGT-A cases to ensure that they are offering PGT-A to the most appropriate patients and that the process is optimised to achieve the best outcomes. This study also provides additional evidence that NGS is superior to aCGH for the detection of euploid embryos and results in a higher proportion of transferred embryos achieving live birth.

5 Specific Aim 3: To determine which of the many referral indications are more likely to return a result of "aneuploid" following NIPT

5.1 Introduction

America was one of the first countries where NIPT was available clinically, high risk patients and those of advanced maternal age were predominately offered NIPT from the outset. Given the high positive predictive values (PPV) reported for NIPT, naturally the next step was to offer screening to more pregnant women (Futch, 2013; Dar *et al.*, 2014). However, there was concern that there would be an increase in false positive results in low risk patients, leading to unnecessary invasive procedures, such as amniocentesis and CVS being subsequently offered. With enhanced screening in high risk patients more of these affected pregnancies are detected, however, not offering NIPT to low risk patients has resulted in there being a shift towards more affected live births in younger mothers (Bunt & Bunt, 2014). In 2015, The American College of Obstetricians and Gynecologists recommend that high risk patients be referred for NIPT with the following indications: AMA, ultrasound findings indicative of increased risk of aneuploidy, history of pregnancy affected by trisomy, positive biochemical screening results, parental balanced translocation. They also recommended that NIPT not be offered to low risk patients or those with multiple gestations (ACOG, 2015). This position was not supported by the American College of Medical Genetics and Genomics, who recommend that all pregnant

women are informed that NIPT is the most sensitive screening option for common aneuploidies (Gregg *et al.*, 2016). As there is no consensus in the USA, who receives NIPT is largely dependent on what is offered by medical insurance companies. However, NIPT is now widely available in the USA, with estimated uptake of 25%-50% of pregnant women (Gadsbøll *et al.*, 2020).

The uptake of NIPT globally has grown in recent years, being offered privately and as part of national prenatal screening programmes. A recent study by Gadsbøll *et al*, 2020 demonstrated the inconsistency between countries in the selection of patients to be offered NIPT and uptake of testing. Figure 5.1 taken from this study, shows the European countries that have a national offer of NIPT and to which patients this is available. These range from all pregnant women to no national offering. Australia was also observed in this study where NIPT is available as a self-funded option.

One country that has offered NIPT to all pregnant women is The Netherlands. Their experience has been published with the study name TRIDENT-2, in this study 42% of all pregnancies opted for NIPT, with a mean maternal age of 31.7. Like that observed by previous studies, despite the inclusion of younger and lower risk patients they still report high PPVs for the common trisomy's (Song *et al.*, 2013; Bianchi *et al.*, 2014; McLennan *et al.*, 2016; Guy *et al.*, 2019). They experienced much lower PPVs with RATs which is to be expected (please see chapter 6 where I have discussed RATs in more detail). The rate of aneuploidies detected was 0.75% in this general population cohort whereas in their

previous study involving only high-risk patients this was 2.66%. Whilst they did report that more aneuploidies were detected in older women, the age range of patients with aneuploidy detected was 20-48 (Oepkes *et al.*, 2016; Meij *et al.*, 2019).

Red = No national offer including NIPT

Yellow = NIPT as an option if intermediate-high risk at combined screen

Green = NIPT for all

Figure 5.1: Non-invasive prenatal testing (NIPT) as a national prenatal offer in Europe.
Image from (Gadsbøll *et al*, 2020)

The benefits of providing a screening test such as NIPT that leads to less invasive procedures being carried out and lower rates of false negatives are easy to appreciate. It cannot be denied that offering NIPT as a first-tier screening method to all patients would be far costlier. From a cost-benefit perspective however, it has been suggested that NIPT

would be most beneficial as a contingent test, that is only offered to patients who are deemed high risk using current screening methods (ultrasound and biochemical screening in the first and second trimester) and therefore reduce the number of unnecessary invasive tests performed in this group (Bayón *et al.*, 2019).

The association of increased rates of aneuploidy and advanced maternal age is widely reported in the literature, this age effect is mirrored in the results seen in NIPT (Meij *et al.*, 2019). Most papers have focused on a cohort of patients who are deemed to be high-risk or low-risk when referred for NIPT. To the best of our knowledge there have been no studies carried out investigating rates of aneuploidy detected following NIPT associated with referral indication for a mixed cohort of low and high-risk patients.

5.1.1 Specific aims

With the above justification in mind, the purpose of this chapter was to determine which of the many referral indications (e.g. maternal anxiety, advanced maternal age, positive biochemical screen, abnormal ultrasound, history of affected pregnancy) are more likely to return a result of “aneuploid” following NIPT.

5.2 Methods

Under University of Kent ethical regulations, this project did not require further ethical approval. This project was reviewed internally within CooperGenomics, it was determined

that no further ethical approval was needed, and that patient confidentiality and General Data Protection Regulations should be observed throughout.

A total of 53,685 NIPT cases reported between September 2015 and the end of July 2018 were reviewed for NIPT referral indication. Samples were processed in accordance with standard protocols at CooperGenomics UK (refer to section 2.2).

Rates of aneuploidy were calculated for each type of referral indication category. The data were analysed in relation to maternal age in addition to referral category. Age groups were categorised as: <20; 20-25; 26-30; 31-34; 35-37; 38-40; 41-44 and 45-49.

Data for maternal age, referral indication category and result of NIPT were entered into the Statistical Package for the Social Science database (SPSS) (SPSS, Chicago, IL, USA) to obtain statistical analysis. Chi2 analysis was carried out to test the null hypothesis that there is no difference in rates of aneuploidy associated with referral category. Statistical significance was considered at P values of <0.05. Where multiple tests were carried out across the referral categories, an adjusted Bonferroni P value was calculated to avoid type 1 statistical errors, the adjusted P value is presented where it has been applied. For referral category analysis within age groups, where the Chi2 test expectation of <20% having a count less than 5 is violated the likelihood ratio is presented instead of the Chi2 statistic. Categorical variables are also presented with percentages and continuous variables are expressed with means ± standard deviation.

NIPT cases with more than one referral category were defined as “Multiple indications”, these were further broken down into “Multiple indications (incl. positive biochemical screen)” and “Multiple indications”, as we were interested in the effect this would have with and without biochemical screening referrals.

My own personal contribution was that I organised the NIPT data from CooperGenomics UK and carried out the data analysis. I carried out some plasma isolation and DNA extraction of samples at end of 2016 and beginning of 2017.

5.3 Results

53,685 NIPT cases were carried out at CooperGenomics UK between September 2015 and the end of July 2018. 51 cases were excluded due to the patients being over 50, it was not clear if these patients had undergone IVF with oocyte donation, however this is quite likely and could affect data analysis relating to age. A further 37 cases were excluded as the number in these referral categories were very small in comparison to the other referral categories making analysis of these categories unreliable. The number and types of referrals excluded are as follows: 6 had been referred because of a family history of aneuploidy, 20 were pregnancies conceived following IVF, 8 were IVF using donor oocytes and a further 3 for recurrent miscarriage. Cancelled and failed NIPT results were excluded

when carrying out chi2 analysis, however they were included in the count for each referral or age category.

A total of 53,597 NIPT cases were included for further analysis. The number of referrals for each category in descending order was as follows: 18,426 (34.4%) maternal anxiety, 17,977 (33.5%) advanced maternal age, 10,131 (18.9%) positive biochemical screen, 2600 (4.9%) abnormal ultrasound, 1398 (2.6%) history of affected pregnancy, 1217 (2.3%) cases with an unknown referral reason, 1063 (2.0%) multiple indications and 785 (1.5%) multiple indications (incl. positive biochemical screen).

Across all referral categories, a total of 1296 (2.4%) positive NIPT results were reported. 682 (52.6%) were trisomy 21, 179 (13.8%) were trisomy 18, 96 (7.4%) were trisomy 13, 151 (11.7%) were Monosomy X, 131 (10.1%) were other sex chromosome abnormalities (SCAs), 18 (1.4%) were microdeletions, 21 (1.6%) were RATs and 18 (1.4%) had multiple aneuploidies.

The number and percentage of euploids vs aneuploids reported following NIPT for each referral category can be seen in Table 5.1, the mean maternal age is also presented here for each referral category. Abnormal ultrasound had the highest proportion of positive NIPT cases at 5.9%, followed by multiple indication (incl. positive biochemical screen) with a positive NIPT rate of 5.7%, 38/45 cases were referred for both abnormal ultrasound and positive biochemical screen, the remaining cases were 7 positive cases were positive

biochemical screen and history of affected pregnancy. Maternal anxiety and history of affected pregnancy both had the lowest proportion of positive NIPT cases with 1.4% (Figure 5.2). The rate of aneuploidy associated with the referral categories was found to be statistically significant (Chi2 = 298.318, P<0.001). Further Chi2 analysis of each referral category showed that abnormal ultrasound, multiple indications (incl. positive biochemical screen) and positive biochemical screen were all significantly higher than the other referral categories. Maternal anxiety was also statistically significant, however, as might be expected this was significantly lower than the other referral categories Table 5.1.

The rate of aneuploid results increase with maternal age as expected (Figure 5.3). The rates of aneuploidy associated with maternal age was found to be statistically significant (Chi2 = 178.406, P<0.001). Patients 38 and over had a higher rate of aneuploid result which is statistically significant in these age groups, conversely those patients under 35 had lower rates of aneuploidy, which was statistically significant between 26 and 34. The <20 and 20-25 age groups were not statistically significant, however these 2 groups did have lower referral numbers than most of the other age groups (Table 5.2).

The distribution of maternal age for NIPT referrals can be seen in Figure 5.4. The ages ranged from 14 to 49 years (Mean = 34.2, SD = 5.14). The ages were grouped for analysis of referral categories independent of maternal age, the under 20s had the lowest number of referrals with a total of 341 cases, followed by 45-49 with 498. The highest number of referrals at 13,713 was in the age group 31-34.

	N. referrals	Maternal age mean ± SD	Euploids (N, %)	Aneuploids (N, %)	Chi2 value	P value
Maternal anxiety	18426	31.8 ± 4.43	17848 (96.9%)	262 (1.4%)	119.68	<0.001 *
Advanced maternal age	17977	38.1 ± 2.96	17170 (95.5%)	421 (2.3%)	0.61	0.435 NS
Positive biochemical screen	10131	33.0 ± 5.39	9585 (94.6%)	332 (3.3%)	39.31	<0.001 *
Abnormal ultrasound	2600	30.8 ± 5.28	2388 (91.9%)	154 (5.9%)	143.04	<0.001 *
History of affected pregnancy	1398	33.8 ± 4.98	1346 (96.3%)	19 (1.4%)	6.76	0.009 NS
Unknown	1217	33.9 ± 5.01	1146 (94.2%)	23 (1.9%)	1.23	0.267 NS
Multiple indications	1063	36.7 ± 4.37	1007 (94.7%)	40 (3.8%)	8.12	0.004 NS
Multiple indications (incl. biochem screen)	785	29.6 ± 4.68	732 (93.3%)	45 (5.7%)	36.24	<0.001 *

Table 5.1: Demographics of each referral category: number of NIPT cases, mean maternal age, number of euploids & aneuploids (number of cancelled & failed cases not included). Chi2 values and P values, adjusted Bonferroni P value calculated at sig <0.003125. NS: Not significant, * statistically significant

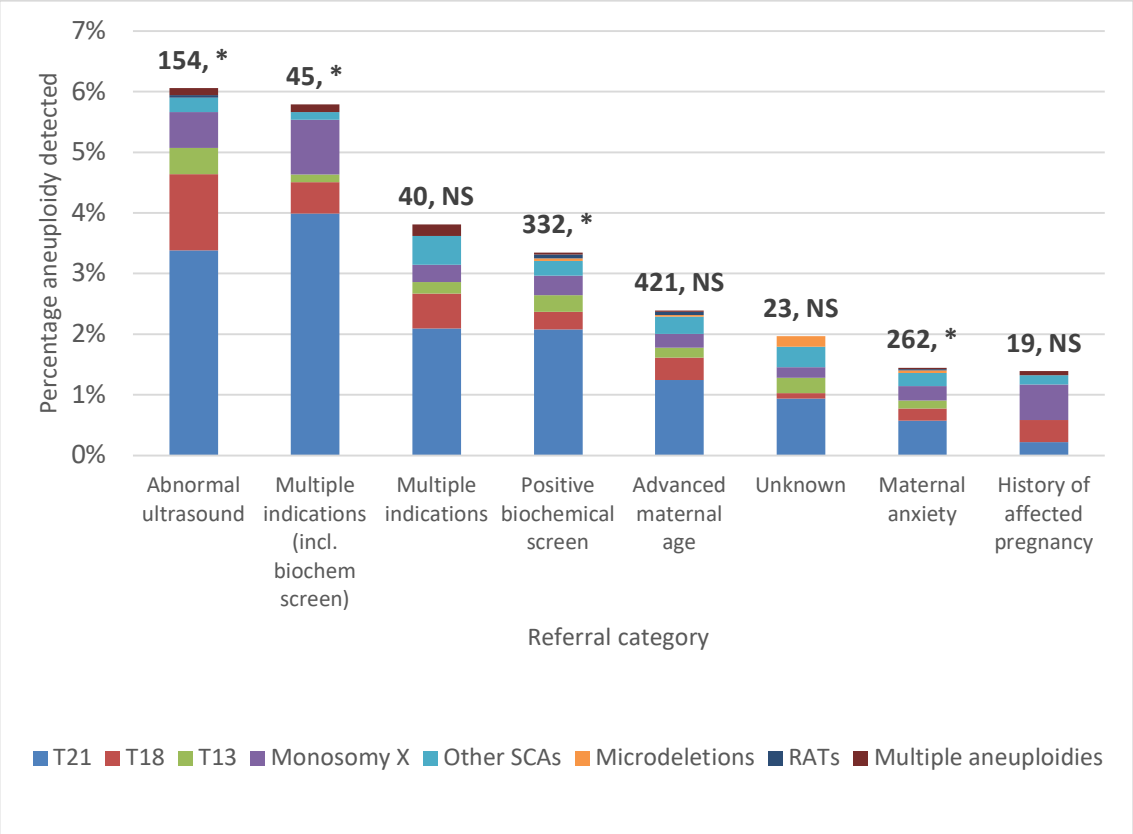


Figure 5.2: Percentage and type of aneuploidy detected by referral indication. Data label: total number of aneuploidies detected. NS: Not significant, Chi2 test *statistically significant P<0.05, NS = Not significant. SCAs = Sex chromosome aneuploidies.

	N. referrals	Euploids (N, %)	Aneuploids (N, %)	Chi2 value	P value
<20	341	329 (96.5%)	4 (1.2%)	2.22	0.136 NS
20-25	2769	2672 (96.5%)	57 (2.1%)	1.72	0.190 NS
26-30	8881	8542 (96.2%)	155 (1.8%)	20.34	<0.001 *
31-34	13713	13204 (96.3%)	247 (1.8%)	29.92	<0.001 *
35-37	12771	12220 (95.7%)	280 (2.2%)	3.53	0.060 NS
38-40	1003	9519 (94.9%)	316 (3.2%)	27.88	<0.001 *
41-44	4590	4277 (93.2%)	211 (4.6%)	101.81	<0.001 *
45-49	498	459 (92.2%)	26 (5.2%)	17.06	<0.001 *

Table 5.2: Demographics of each age group: number of NIPT cases, number of euploids & aneuploids (number of cancelled & failed cases not included). Chi2 value and P values, adjusted Bonferroni P value calculated at sig <0.003125. NS: Not significant, * statistically significant

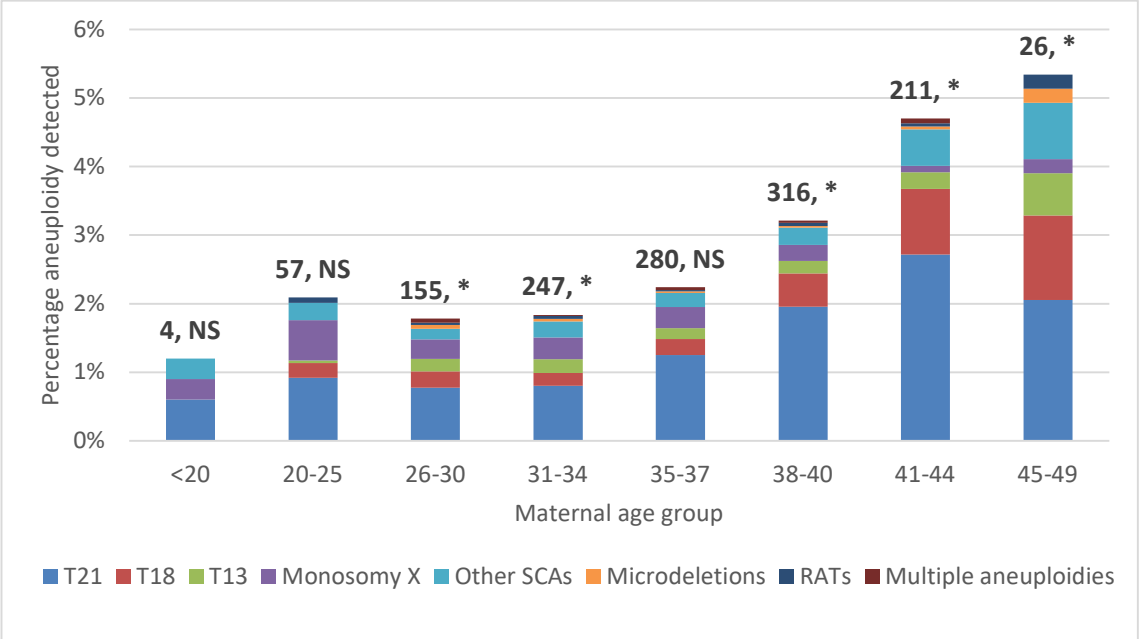


Figure 5.3: Percentage and type of aneuploidy detected by age group. Data label: total number of aneuploidies detected. Chi2 test: NS: Not significant, *statistically significant, <0.05. SCAs = Sex chromosome aneuploidies.

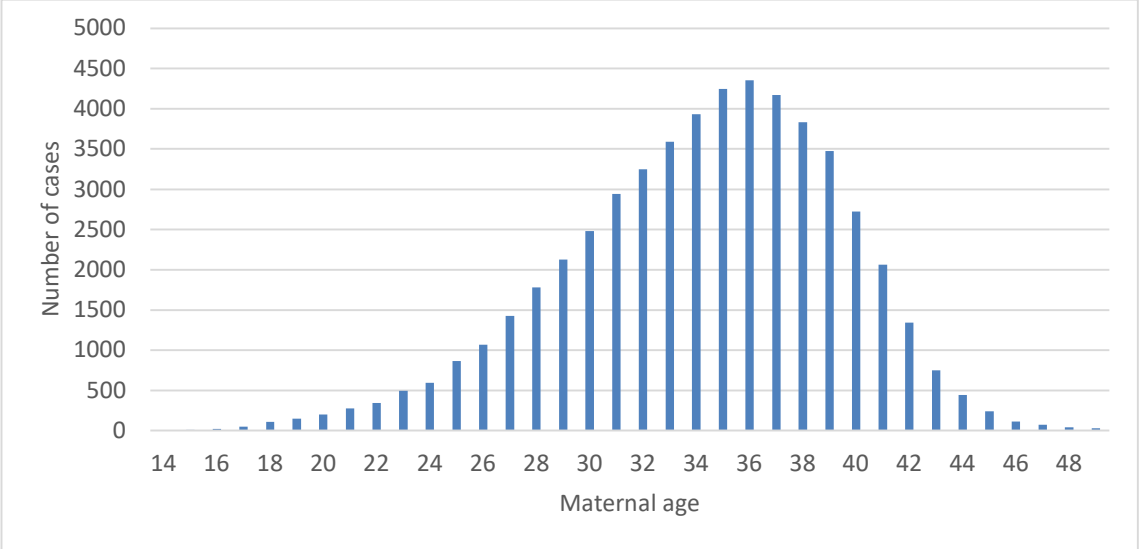


Figure 5.4: Distribution of NIPT cases by maternal age.

As might be expected, as the patient age increases the type of referral indication changes; the younger patients are predominately being referred for maternal anxiety, abnormal

ultrasound, and positive biochemical screens. Whereas, over 50% of the patients referred over the age of 35 are being referred based on their advanced maternal age alone, followed by positive biochemical screens and maternal anxiety (Figure 5.5).

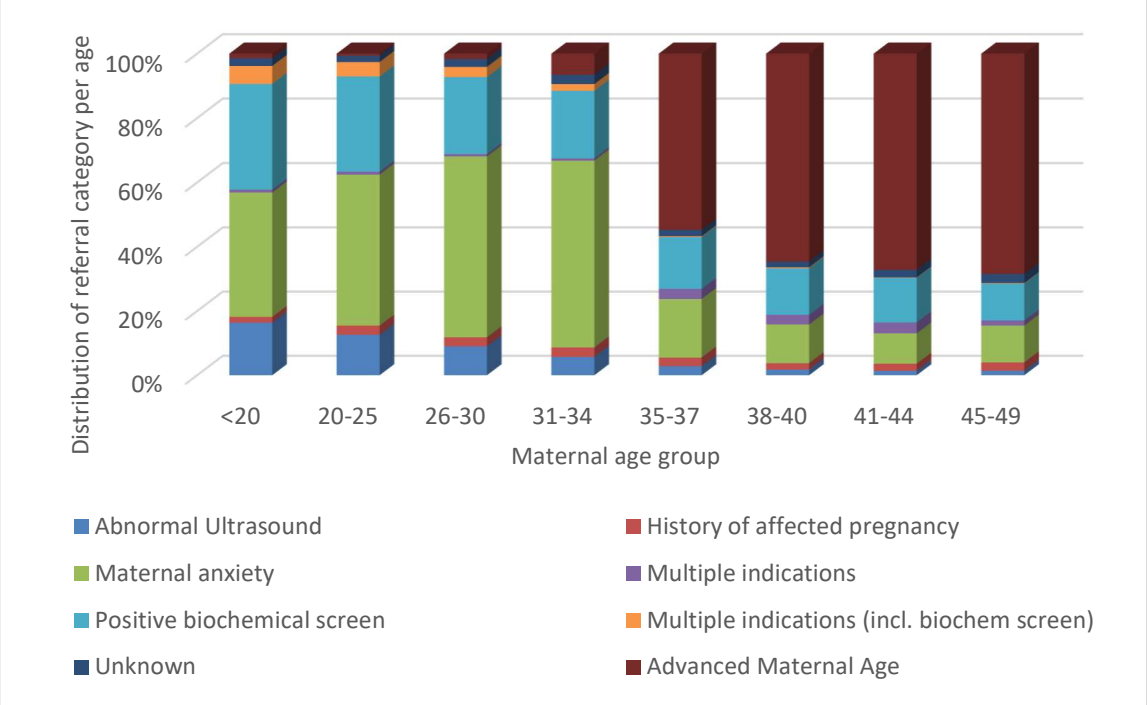
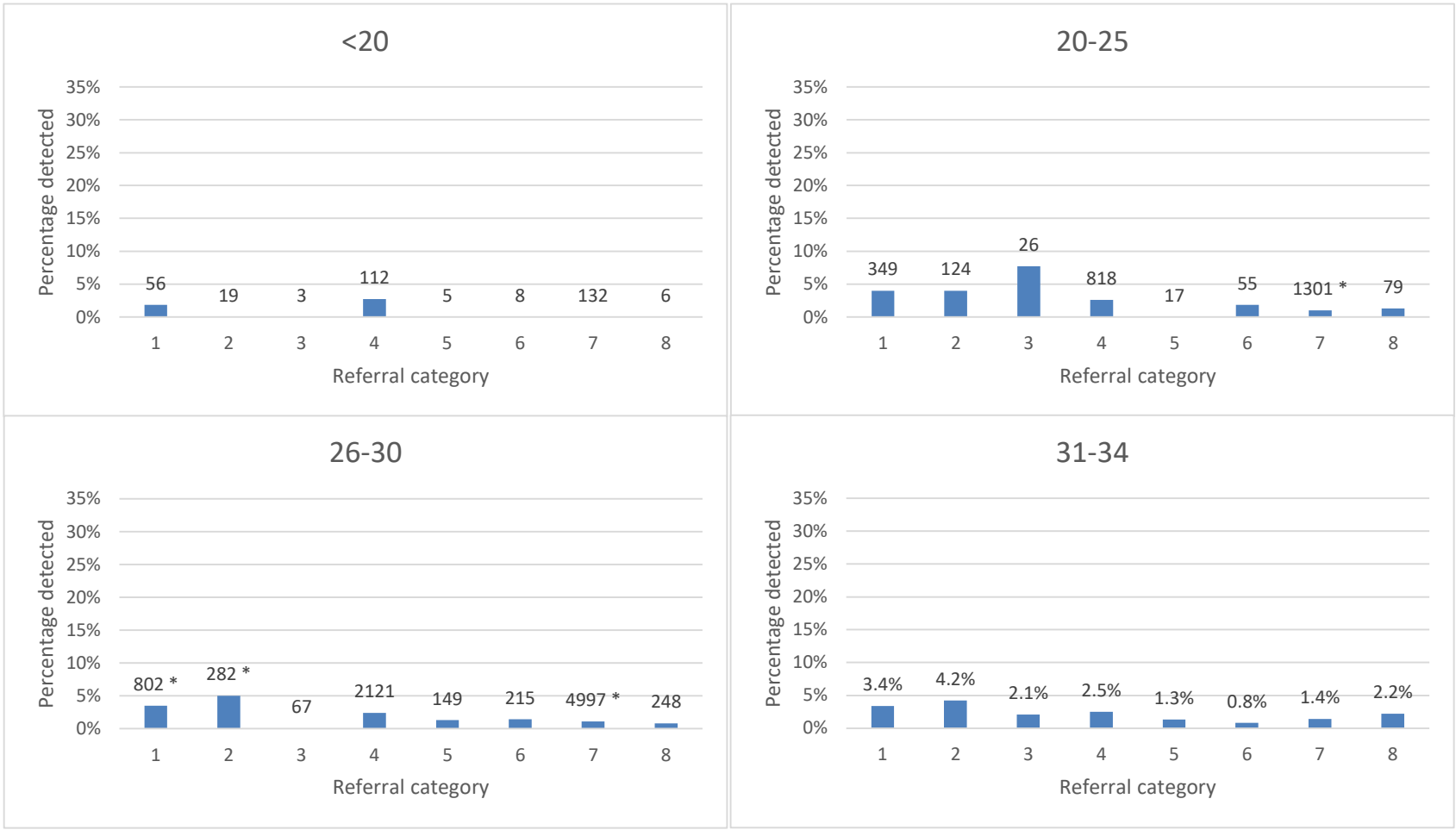


Figure 5.5: Distribution of type of referral category by age group.

Abnormal ultrasound had the highest percentage (5.92%) of aneuploid NIPT results and was found to be statistically significant in the age groups between 26 and 44. However, in the 20-25 and 45-49 age groups, abnormal ultrasound did have a higher percentage of positive NIPT results than the other referral categories, it is likely that these were not statistically significant due to the low numbers within these age groups (Table 5.3). This was followed by the referral category multiple indications (incl. positive biochemical screen) (5.73%), which was significantly higher in the age ranges 26-34 and 38-40. Similarly, to abnormal ultrasound, this category also had high rates of positive NIPT results

in the other age groups (20-25; 35-37 & 41-44) but did not reach statistical significance likely due to low overall case numbers in these categories for the age groups. The total percentage of positive NIPT results drops down to 3.76% for the subsequent referral category multiple indications and was not statistically significant in any of the age groups. 3.28% of positive biochemical screen referrals were aneuploid, this category was significant in the age groups between 31 and 40. As might be expected, the referral category maternal anxiety despite being the most common referral indication had one of the lowest aneuploid rates detected by NIPT (1.42%), and was found to be significantly lower in the age groups 20 to 34, which is also the groups which were most likely to be referred for this reason (Figure 5.5). Although the rate of aneuploid results significantly increases with maternal age, advanced maternal age as a referral category without any other referral indication had significantly lower aneuploid results in patients over 35. For example, when a patient >35 is referred in combination with another indication such as abnormal ultrasound, they are significantly more likely to have an aneuploid result than if there were no other confounding indication.



1: Abnormal ultrasound; 2: Multiple indications (incl. biochemical screen); 3: Multiple indications; 4: Positive biochemical screen; 5: Advanced maternal age; 6: Unknown; 7: Maternal anxiety; 8: History of affected pregnancy. * statistically significant.

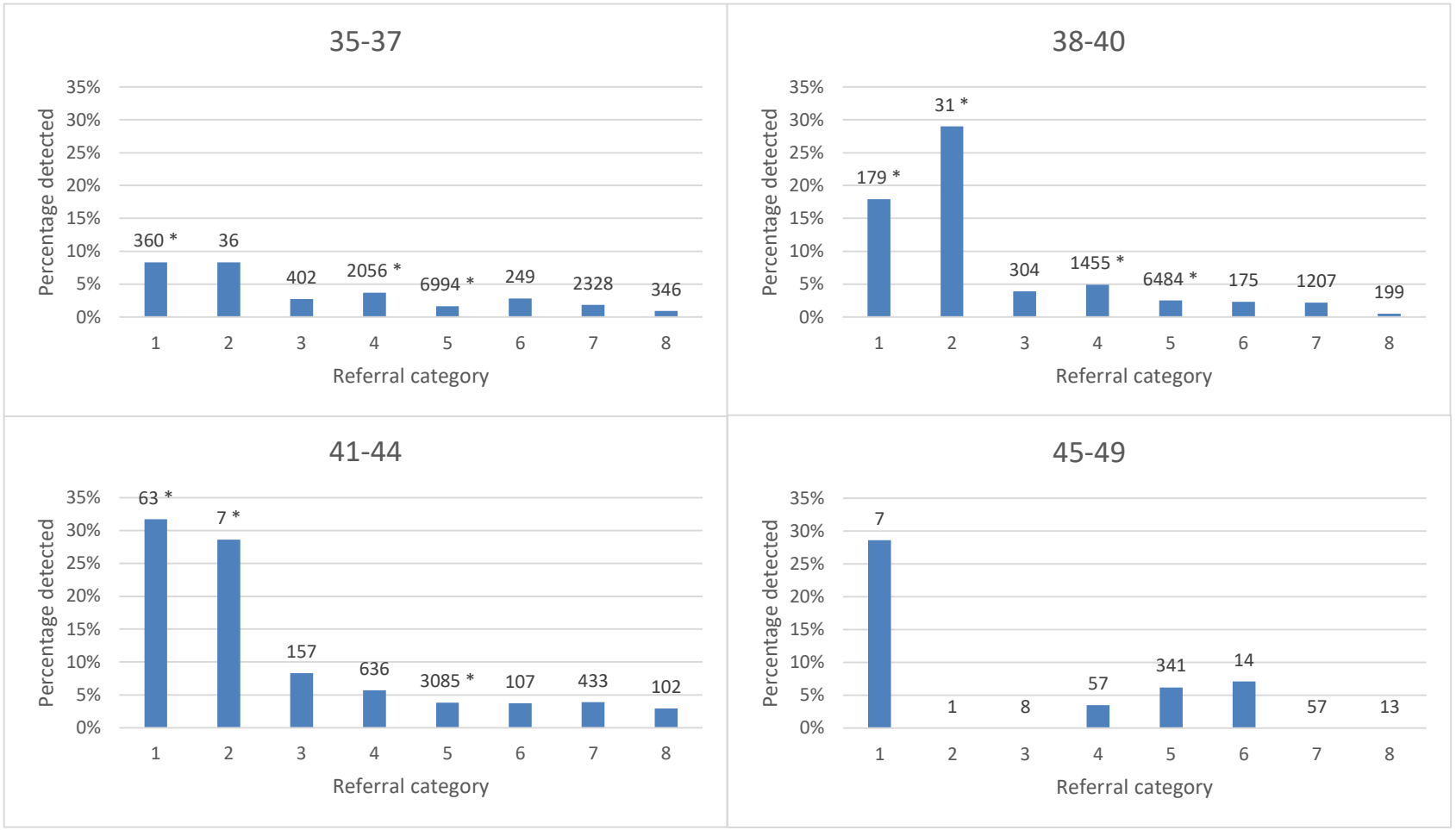
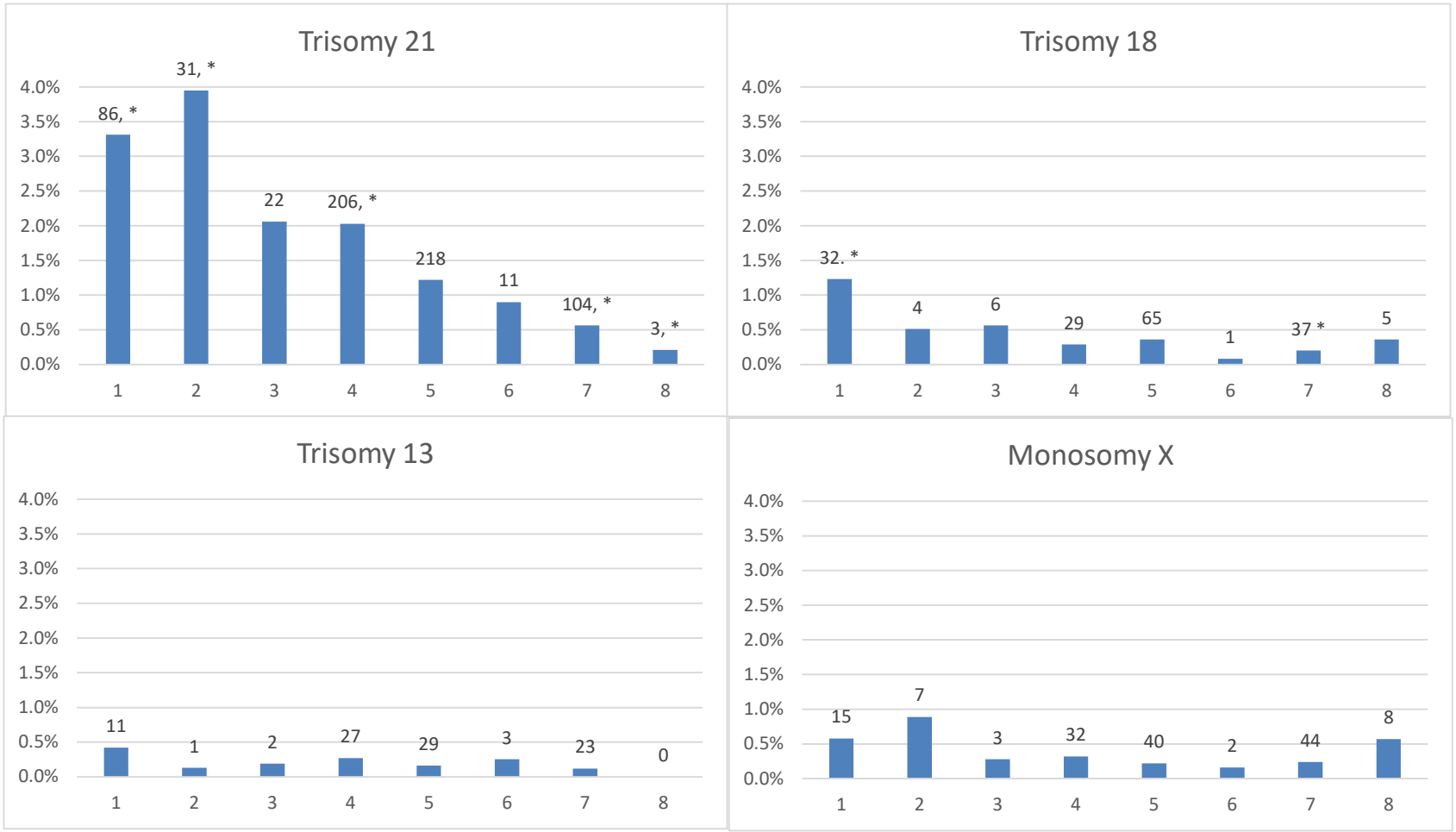


Figure 5.6: Percentage of aneuploidies detected by referral category for each age group. 1: Abnormal ultrasound; 2: Multiple indications (incl. biochemical screen); 3: Multiple indications; 4: Positive biochemical screen; 5: Advanced maternal age; 6: Unknown; 7: Maternal anxiety; 8: History of affected pregnancy. Ch2 test* statistically significant at P<0.05.

		Abnormal ultrasound	Multiple indication (Incl. bio s)	Multiple indications	Positive biochemical screen	Advanced maternal age	Unknown	Maternal anxiety	History of affected pregnancy	Overall P values for age group
<20	**Euploid (N, %)**	55 (98.2%)	19 (100%)	3 (100%)	107 (95.5%)	3 (60%)	7 (87.5%)	130 (98.5%)	5 (83.3%)	
	Aneuploid (N, %)	1 (1.8%)	0 (0%)	0 (0%)	3 (2.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	P value	0.660 NS	0.624 NS	0.849 NS	0.072 NS	0.849 NS	0.772 NS	0.107 NS	0.803 NS	0.311 NS a
20-25	**Euploid (N, %)**	331 (94.8%)	118 (95.2%)	24 (92.3%)	783 (95.7%)	17 (100%)	50 (90.9%)	1277 (98.2%)	72 (91.1%)	
	Aneuploid (N, %)	14 (4.0%)	5 (4.0%)	2 (7.7%)	21 (2.6%)	0 (0%)	1 (1.8%)	13 (1.0%)	1 (1.3%)	
	P value	0.006 NS	0.116 NS	0.444 NS	0.215 NS	0.549 NS	0.952 NS	<0.001 *	0.660 NS	0.0032 * a
26-30	**Euploid (N, %)**	753 (93.9%)	265 (94.0%)	65 (97.0%)	2023 (95.4%)	143 (96.0%)	204 (94.9%)	4844 (96.9%)	245 (98.8%)	
	Aneuploid (N, %)	28 (3.5%)	14 (5.0%)	0 (0.0%)	50 (2.4%)	2 (1.3%)	3 (1.4%)	56 (1.1%)	2 (0.8%)	
	P value	<0.001 *	<0.001 *	0.276 NS	0.013 NS	0.711 NS	0.711 NS	<0.001 *	0.242 NS	<0.001 * a
31-34	**Euploid (N, %)**	741 (94.5%)	271 (95.1%)	93 (96.9%)	2746 (95.5%)	863 (95.7%)	375 (95.2%)	7731 (97.0%)	384 (94.8%)	
	Aneuploid (N, %)	27 (3.4%)	12 (4.2%)	2 (2.1%)	73 (2.5%)	12 (1.3%)	3 (0.8%)	109 (1.4%)	9 (2.2%)	
	P value	<0.001 *	0.002 *	0.841 NS	<0.001 *	0.289 NS	0.126 NS	<0.001 *	0.497 NS	<0.001 *
35-37	**Euploid (N, %)**	314 (87.2%)	33 (91.7%)	382 (95.0%)	1938 (94.3%)	6744 (96.4%)	232 (93.2%)	2243 (96.4%)	334 (96.5%)	
	Aneuploid (N, %)	30 (8.3%)	3 (8.3%)	11 (2.7%)	76 (3.7%)	109 (1.6%)	7 (2.8%)	41 (1.8%)	3 (0.9%)	
	P value	<0.001 *	0.014 NS	0.447 NS	<0.001 *	<0.001 *	0.465 NS	0.112 NS	0.089 NS	<0.001 *
38-40	**Euploid (N, %)**	146 (81.6%)	20 (64.5%)	291 (95.7%)	1349 (92.7%)	6199 (95.6%)	165 (94.3%)	1153 (95.5%)	196 (98.5%)	
	Aneuploid (N, %)	32 (17.9%)	9 (29.0%)	12 (4.0%)	71 (4.9%)	161 (2.5%)	4 (2.3%)	26 (2.2%)	1 (0.5%)	
	P value	<0.001 *	<0.001 *	0.453 NS	<0.001 *	<0.001 *	0.529 NS	0.037 NS	0.029 NS	<0.001 *
41-44	**Euploid (N, %)**	43 (68.3%)	5 (71.4%)	142 (90.5%)	586 (92.1%)	2889 (93.7%)	101 (94.4%)	413 (95.4%)	98 (96.1%)	
	Aneuploid (N, %)	20 (31.8%)	2 (28.6%)	13 (8.3%)	36 (5.7%)	116 (3.8%)	4 (3.7%)	17 (3.9%)	3 (2.9%)	
	P value	<0.001 *	0.0028 *	0.027 NS	0.168 NS	<0.001 *	0.660 NS	0.441 NS	0.407 NS	<0.001 * a
45-49	**Euploid (N, %)**	5 (71.4%)	1 (100%)	7 (87.5%)	53 (93.0%)	312 (91.5%)	12 (85.7%)	57 (100%)	12 (92.3%)	
	Aneuploid (N, %)	2 (28.6%)	0 (0%)	0 (0%)	2 (3.5%)	21 (6.2%)	1 (7.1%)	0 (0%)	0 (0%)	
	P value	0.006 NS	0.810 NS	0.529 NS	0.549 NS	0.171 NS	0.704 NS	0.056 NS	0.407 NS	0.063 NS

Table 5.3: Number and percentage of euploid and aneuploid NIPT cases by referral category and age group. Chi2 P values for each and overall P value for age group given. Adjusted Bonferroni P value calculated at sig <0.003125. NS: Not significant, * statistically significant, a: likelihood ratio, bio s: positive biochemical screen.

Type of aneuploidy detected in each category is shown in Figure 5.7, statistical significance was only observed in trisomy 21 and trisomy 18. For trisomy 21, there were significantly more cases detected in the abnormal ultrasound, multiple indications (incl. positive biochemical screen) and positive biochemical screen, with history of affected pregnancy and maternal anxiety both having a significantly lower incidence of trisomy 21. Trisomy 18 was significantly higher in the abnormal ultrasound referral category and significantly lower in the maternal anxiety category. Abnormal ultrasound had the highest percentage of trisomy 13 cases; this was not statistically significant following the Bonferroni correction at p=0.00253, however had there been more cases overall this is likely trending towards statistical significance. It is hard to draw conclusions for the other types of aneuploidy due to the small numbers observed.



1: Abnormal ultrasound; 2: Multiple indications (incl. biochemical screen); 3: Multiple indications; 4: Positive biochemical screen; 5: Advanced maternal age; 6: Unknown; 7: Maternal anxiety; 8: History of affected pregnancy. * statistically significant.

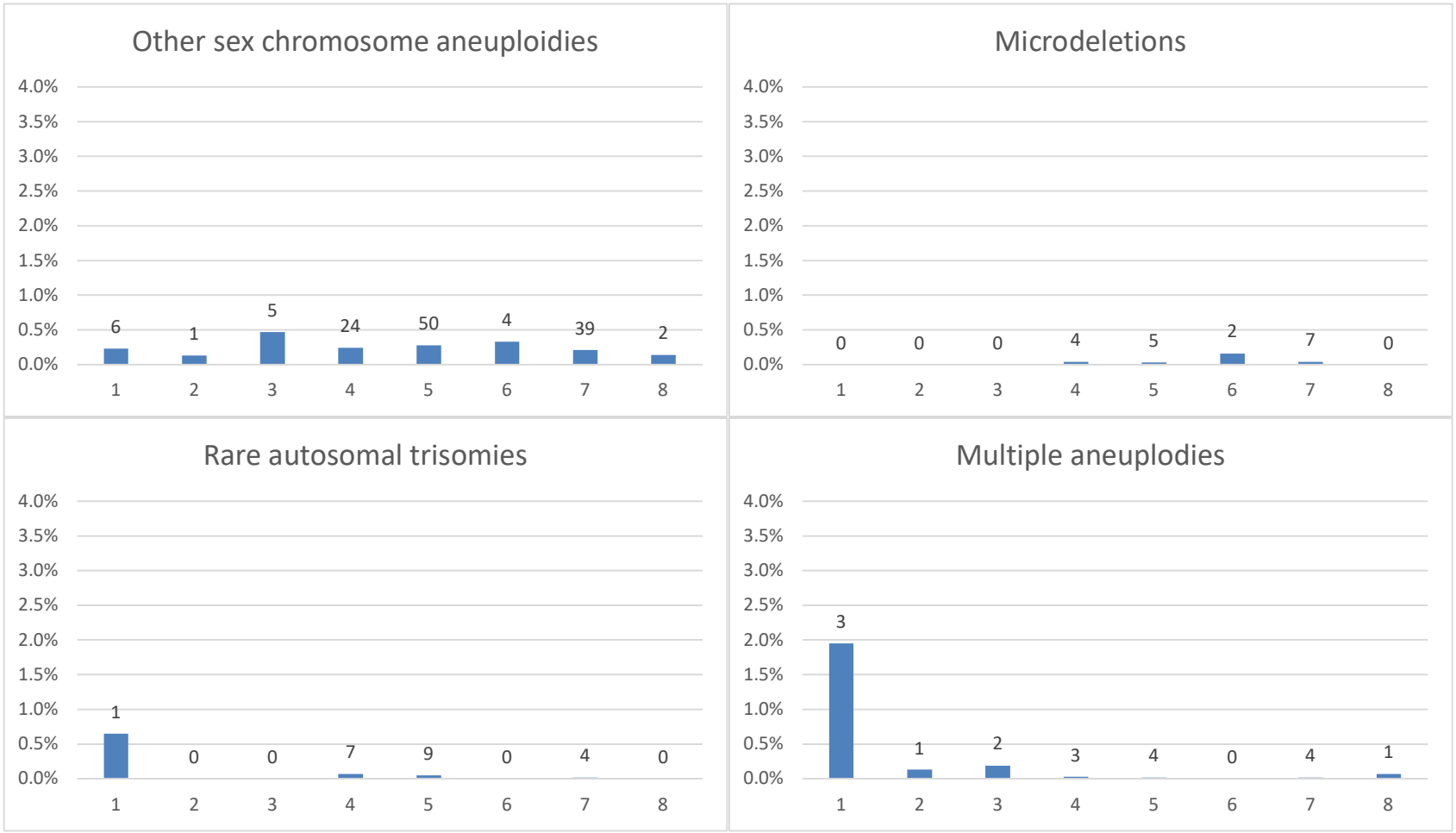


Figure 5.7: Percentage of aneuploidies detected for each referral category by type of aneuploidy.
1: Abnormal ultrasound; 2: Multiple indications (incl. biochemical screen); 3: Multiple indications; 4: Positive biochemical screen; 5: Advanced maternal age; 6: Unknown; 7: Maternal anxiety; 8: History of affected pregnancy. Chi2 test, * statistically significant, $p<0.05$.

5.4 Discussion

In this study, we found as expected statistical significance of increased rates of aneuploidy detected following NIPT with advancing maternal age. We also found that even when maternal age was controlled for, abnormal ultrasound was the most likely referral category to return an aneuploid result following NIPT. The rate of aneuploidy associated with abnormal ultrasound seen in our study is comparable to that reported in an early study of NIPT clinical cases (McCullough *et al.*, 2014). Trisomy 21 and trisomy 18 were both statistically higher in the abnormal ultrasound referral category, although as trisomy 21 and trisomy 18 are the most common autosomal aneuploidy detected through ultrasound, this would be expected (Hume *et al.*, 1995). Multiple indications (incl. positive biochemical screen) was also statistically higher than the other referral categories and almost at the same rate as that seen in the abnormal ultrasound referral category (5.9% and 5.7%, respectively). Although, 84% of those that were reported with an aneuploidy in the multiple indications (incl. positive biochemical screen) were a combination of abnormal ultrasound and positive biochemical screen, therefore it could be argued that it was the ultrasound indication contributing to the higher rate seen in this referral category. Maternal anxiety had a statistically lower rate of aneuploidies detected than all other categories (1.4%), this group would be a good representation of low risk patients within our study cohort. Interestingly, patients with a history of affected pregnancy who are often categorised as high-risk patients had the lowest rate of aneuploidies (1.4%) of all the referral categories, although not statistically significant. This group was also the least affected referral category in a previous publication (McCullough *et al.*, 2014).

It is difficult to state the expected prevalence expected in a general population such as this study. It is estimated that trisomy will affect the pregnancies of 2-3% in younger women and around 30% of pregnancies by a maternal age of 40 (Hassold & Hunt, 2009). Therefore, the average age of motherhood of a population will impact the rate of aneuploidy detected. With this in mind, the percentage of aneuploidies detected in our low -risk patients such as those referred due to maternal anxiety are not far from the expected range. In this study positive biochemical screening had 3.3% reported as aneuploid, whilst this was statistically higher than most referral categories, we would have expected it to be higher than the general mean of all NIPT referrals at 2.4%. It also didn't increase as dramatically along with maternal age at the same rate seen with abnormal ultrasound, which reached 31.8% of abnormal ultrasound referrals in the 41-44 age group, conversely only 5.7% of positive biochemical referrals were aneuploid for the same age group.

Whilst we have confirmed the link between advancing maternal age and increased rates of aneuploidy in this study. When looking at the age groups over 35, advanced maternal age as a referral category on its own had statistically lower rates of aneuploidy than most of the other referral categories. It could be argued that the patients in the age groups over 35 that had been referred for a single reason other than advanced maternal age, in fact had multiple indications (e.g. abnormal ultrasound and advanced maternal age). However, it is likely that advanced maternal age as a referral category on its own becomes the most appropriate category for patient referrals over maternal anxiety once patients are over 35.

Therefore, like maternal anxiety in the younger age groups, these patients are low risk by comparison to those with other referral indications.

Advanced maternal age is widely accepted as a maternal age ≥35, however, in this study 1073 patients under the age of 35 have been referred as advanced maternal age. These are most likely due to an admin error either at the time of referral form completion or at data entry in the genetics laboratory. This highlights a limitation of this study, as this is a retrospective analysis it is not possible to address these potential errors, had it been a prospective study, we would have been able to stress the importance of the accuracy of the referral categories selected, although even in prospective studies human error cannot always be ruled out. It also suggests that there could be some degree of error regarding the other referral categories, it is possible that some patients would have had multiple indication but instead the most relevant was selected. Although, given this study has a considerable data size we believe that these errors are largely mitigated. Other limitations of this study are that we don't have further details available about specific referrals, for example it would be beneficial to assess the risk scores from positive biochemical screens and what impact this has on rates of aneuploidy detected, some of the cases referred for positive biochemical screens could be lower than cut-offs used by national screening programmes. Therefore, it would have been useful to further interrogate this referral category regarding the risk scores observed. It would also be interesting to look at the different types of abnormalities detected at ultrasound, a study with smaller numbers has been able to do this, they found that the highest rates of aneuploidy are observed with,

increased nuchal translucency, multiple congenital abnormalities, anterior abdominal wall defects and gastrointestinal defects (Togneri *et al.*, 2019).

Currently in the UK, NIPT is not offered by the National Health Service (NHS). It is being assessed for use in high-risk patient groups with a screen result ≥1:150 following first trimester combined screen or second trimester quadruple screen, as a contingent screening method, with the intention to reduce the number of invasive procedures being carried out. There is an obvious benefit to preforming less invasive tests and avoiding the associated risk of miscarriage. It is understandable why national programmes would wish to implement NIPT as a contingent screening method prior to offering an invasive option. The hesitance of some programmes to offer NIPT to all pregnant women irrespective of risk level, is most likely related to increased cost of implementation (Bayón *et al.*, 2019). By using NIPT as a contingent screen the focus is on the false positive rates from conventional screening. The false negative rates associated are therefore overlooked by taking this approach, and potentially affected pregnancies would be wrongly deemed low-risk and not receive the NIPT option. False negatives from combined testing have been reported in the literature at rates between 10% for trisomy 21 to 22% for other autosomal aneuploidies (Spencer, 2001; Breathnach *et al.*, 2007; Alldred *et al.*, 2017). The false negative rate was not the focus of this study; however, 6 false negatives were reported during this time making this a rate of 0.5% in our experience. Low frequency of false negatives from NIPT has been reported in many studies (Oepkes *et al.*, 2016; Pescia *et al.*, 2016; Beulen *et al.*, 2017; Meij *et al.*, 2019). In our study, 765 (1.9%) aneuploidies were

detected from 40,081 pregnancies referred for reason which would not be included in many national programmes including the UK. This amounts to 59% of all the aneuploidies detected through NIPT in this study. Having this information can be valuable for the patients and the ongoing management of these pregnancies.

It should be remembered that NIPT is not diagnostic and in fact a screening test, however, for the detection of the common aneuploidies it has superior detection rates (lower false positives and negatives) than traditional first-tier screening methods (Song *et al.*, 2013; Bianchi *et al.*, 2014). We believe that NIPT extended to screen for all chromosomes and microdeletions, along with conventional ultrasound screening would be the most appropriate first stage of screening for patients in early pregnancy. This would lead to lower numbers of patients undertaking invasive tests, improve on the current rates of false negative results with conventional biochemical screening (Okmen *et al.*, 2020). Ultrasound screening is vital to aid in detection of structural fetal abnormalities and pregnancies that may be true mosaics and not detected through NIPT (Nicolaides *et al.*, 1992; Bardi *et al.*, 2019; Grossman *et al.*, 2019). In addition, where NIPT is only used to screen for common aneuploidies, ultrasound screening can aid in the detection of up to 16.6% of RATs (Berger *et al.*, 2020). If it is not financially feasible to offer NIPT to all patients we would suggest that positive biochemical screen threshold be lowered to ≥1:500 or further (Bayón *et al.*, 2019).

5.5 Conclusion

This study presents that abnormal ultrasound is the referral reason with significantly higher rates of aneuploidy detected following NIPT. Referrals due to maternal anxiety had the lowest rates of aneuploidy detected. The rate of aneuploidy detected increases with maternal age as expected. We conclude that by offering NIPT to all pregnant women as a first-tier screening method along with abnormal ultrasound, will provide lower rates of false negatives and reduce the overall number of invasive procedures. Programmes that adopt NIPT targeted to high-risk patients alone will miss a large proportion of affected pregnancies due to high rates of false negatives associated with conventional screening methods.

6 Specific Aim 4: Reanalysis of suspected common aneuploidy NIPT results, for the presence of RATs

6.1 Introduction

NIPT was first successfully introduced clinically in 2011 to detect only common fetal aneuploidies, i.e. those of chromosomes 13, 18, 21, X and Y. A consistently high level of sensitivity and specificity has been reported through NIPT for chromosomes 13, 18 and 21 (Oepkes *et al.*, 2016; Mackie *et al.*, 2017). The incidence of false negatives and positives along with test failures occur at very low rates (<0.1%). In many ways NIPT has some of the drawbacks of direct CVS preparations (as described in chapter 1.2.2.1), as the DNA for analysis for both testing methods are derived from the syncytiotrophoblast. Due to this origin of DNA for NIPT analysis it is therefore a screening test and false-positives and false negative results can occur. Amniocentesis is considered the gold standard for follow up confirmation of a positive NIPT result, however, some patients will opt to continue the pregnancy without further testing and the live birth outcome will be the point where the NIPT is confirmed or a false positive is identified. CVS is not generally advised as this will detect the same DNA as NIPT, where CPM is present this will result in further false positive or false negative results.

Here we break down the possible outcomes and interpretation following NIPT, and potential causes for false positives and negatives:

1. The NIPT diagnosis is normal and this represents the karyotype of the fetus (also normal) accurately (this is by far the most common outcome)
2. The NIPT diagnosis is aneuploid, the fetus is confirmed through additional testing such as amniocentesis to be aneuploid and the diagnosis is accurate, in this case:
    a. If it is of a common trisomy (21, 18, 13, XY) the family may be given the option to have a therapeutic abortion
    b. If it is of a rare autosomal trisomy (RAT) this could indicate that a spontaneous abortion is imminent and/or the pregnancy should be closely monitored for an adverse outcome such as IUGR
3. The NIPT result is normal but the fetus is in fact aneuploid (false negative result), although these events are rarer than false positives, they can be due to:
    a. Insufficient fetal fraction
    b. Aneuploidy not detected because of sensitivity issues
    c. Confined placental mosaicism (CPM) (Grati *et al.*, 2014)
4. The NIPT result is aneuploid but the fetus is in fact normal (false positive result). This may be because of:
    a. A previously unknown “vanishing” twin (aneuploid) pregnancy not otherwise detected
    b. Confined placental mosaicism, possibly of a RAT
    c. The algorithm has incorrectly called the trisomy for technical reasons
    d. Maternal chromosome abnormality
    e. Maternal undiagnosed cancer

5. Additionally, both false positive and false negative results can occur when true fetal mosaicism is present (Wang *et al.*, 2013)
6. Test failures can also occur following NIPT. This may be because of:
    a. Insufficient fetal fraction
    b. Confined placental mosaicism, possibly of a RAT

It has been suggested that both false positives and test failures might be due to an imbalance on a chromosome not originally tested (i.e. other than 13, 18, 21, X and Y). This could occur because the original algorithms for detecting common trisomies were calibrated on the assumption that all the other chromosomes were present in two copies. Rare autosomal trisomies (RATs) may cause the NIPT algorithm to report test failures or false positives on test chromosomes. Up to 80% of test failures lead to abnormal outcomes such as miscarriage (Bianchi, 2017; Pertile *et al.*, 2017).

6.1.1 Rare Autosomal Trisomies (RATs)

RAT is a term used collectively to refer to trisomies that affect chromosomes other than 21, 18, 13, X and Y and have largely come to prominence since the advent of NIPT. RATs, as the name implies, are rare and do not occur as often as the common trisomies. Prevalence studies in Europe have indicated that RATs can occur in around 0.07% of pregnancies (Wellesley *et al.*, 2012). Higher rates of RATs have been noted in more recent NIPT studies which have extended to all chromosomes. In a relatively recent study, the authors reported a rate of 0.78% (50 RATs out of 6388 NIPT cases) (Pescia *et al.*, 2016). In

another study, which performed NIPT on high risk Dutch patients, 1.1% were reported with rare autosomal trisomies (15 RATs out of 1386 cases) and Pertile *et al*, who assessed two cohorts, reported a combined result of 0.3% with rare aneuploidies (306 RATs out of 89817 cases) (Oepkes *et al.*, 2016; Pertile *et al.*, 2017). By comparison, in the prevalence study mentioned above, the common aneuploidies accounted for 0.34% of pregnancies in Europe, and 2.7% of the NIPT cases reported in the Pescia *et al* study mentioned previously (Wellesley *et al.*, 2012; Pescia *et al.*, 2016). Although fewer pregnancies are affected by a RAT, it became apparent that by extending screening to all chromosomes, a further 7.4% of clinically significant aneuploidies could be detected (Fiorentino *et al.*, 2017). For this reason, conventional NIPT, originally only detecting the common trisomies was extended to all chromosomes. A non-mosaic RAT will invariably lead to a first trimester spontaneous abortion; indeed, trisomy is the leading cause of pregnancy loss in humans, with trisomy 16 being the most common. An NIPT diagnosis at 9 weeks therefore may mean that a spontaneous abortion is imminent. A RAT detected by NIPT is however more likely to be mosaic, it has been reported that between 72% and 87% of RATs detected are mosaic and confined to the placenta (Malvestiti *et al.*, 2015; Opstal *et al.*, 2016).

The extension of NIPT to encompass all chromosomes is certain to detect more mosaic RATs. In certain circumstances this information could be useful to clinicians and the patient for identifying pregnancies at high risk. For example, in the case of RATs confined to the placenta, detecting these could identify high risk pregnancies, such as those

affected by preeclampsia and fetal growth restrictions (Bianchi, 2017; Benn & Grati, 2018). Where a pregnancy leads to miscarriage following the detection of a RAT this information can be helpful to those patients, giving them an explanation why the pregnancy has failed (Clark-Ganheart *et al.*, 2015). Live births affected by RATs are extremely rare although they do happen and are likely to result in infant mortality just days after birth. Although there have been some case studies associated with RATs such as Trisomy 19, where the RAT is confined to the blood or bone marrow and associated with Leukemia (Jung *et al.*, 2008). The detection of a RAT in NIPT could have a range of serious outcomes, including birth defects, therefore, appropriate genetic counselling following a positive NIPT result to prepare a patient for a possible adverse outcome along with the likelihood of an uneventful pregnancy could be advantageous. Finally, extending the NIPT detection algorithms to encompass all the chromosomes could be a benefit in reducing the number of test failures and false positives caused by imbalances on non-tested chromosomes skewing the normalisation algorithms.

There has however been some criticism about the prospect of expanding NIPT to all chromosomes. Specifically, many of the RATs detected may not be clinically significant and this could lead to unnecessary anxiety during pregnancy (Bianchi, 2017). Extending to all chromosomes would almost certainly lead to an increase in false positives. A higher frequency of aneuploidies is detected in pregnancies under 15 weeks (first trimester), before amniocentesis routinely occurs (Ferreira *et al.*, 2016) and pregnancies affected by a RAT are more likely to miscarry before this time. Therefore, the patient might not benefit

much from the NIPT test, except perhaps an explanation as to why the miscarriage occurred. Many of the RATs detected will be confined to the placenta and not have any impact on the fetus, leading to increased anxiety or an invasive test such as amniocentesis where it is not required, which would present an unnecessary risk to the pregnancy.

The Serenity Basic (CooperSurgical) was initially validated for NIPT for chromosomes 13, 18, 21, X and Y. In the first quarter of 2018 it was extended to analysis of all chromosomes upon request (Serenity 24). This gave the opportunity to re-analyse samples originally only tested using Serenity Basic and, in some cases, review cases where amniocentesis or live birth outcome suggested the original NIPT result was a false positive.

This chapter is specifically concerned with differentiating if false positives following a suspected aneuploidy result are due to RATs or technical causes (2b and 4c, listed as potential causes for false positives). Here, we particularly focus on the suspected aneuploidy calls. The reporting of a suspected aneuploidy, is due to the a potential imbalance on the chromosome in question, the value observed following the processing of NIPT samples, is elevated above that expected for euploidy but not high enough to reach the expected threshold of an aneuploid result. A discordant result for these cases refers to a euploid result being returned at follow up, either through a second NIPT, CVS, amniocentesis, or live birth observations. Therefore, these suspected aneuploidies could be caused by the presence of a RAT or could simply be a technical artifact.

6.1.2 Specific aims

With the above in mind, the purpose of this study is to test the hypothesis that NIPT (Serenity Basic (chromosomes 13, 18, 21, XY)) cases previously reported with a suspected aneuploidy and found to be discordant were due to a RAT disrupting the algorithm (discordance due to biological reasons) or due to technical causes.

6.2 Methods

Under University of Kent ethical regulations, this project did not require further ethical approval. This project was reviewed internally within CooperGenomics, it was determined that no further ethical approval was needed, and that patient confidentiality and General Data Protection Regulations should be observed throughout. Patient samples were re-run as part of the validation of CooperGenomics Serenity 24, and all samples were anonymised before processing.

A total of 21396 NIPT cases reported between September 2015 and June 2017 were reviewed for those with suspected aneuploidy of chromosomes 13, 18, 21, X and Y. Rates of detected and suspected aneuploidies were calculated for this period. Suspected aneuploid results were reviewed and identified for follow up method and result. Where a discordant result was identified these were considered for inclusion in the study. If there was a spare archived plasma sample in storage, these were then reprocessed with the paired end sequencing protocol (see materials and methods section 2.2), then reanalysed

by Serenity Basic (13, 18, 21, XY). Finally, the re-processed samples were reanalysed by Serenity 24, to assess for RATs. Archived plasma samples from these cases were blinded before being reprocessed.

My own personal contribution was that I actively followed up all suspected and detected aneuploidies reported following NIPT at CooperGenomics UK, with the referring centre to identify if any follow up testing had been carried out and if so, what the testing revealed. I identified the samples for this study from the database of NIPT follow up result and pulled the available samples for processing. After the samples had been reprocessed, I analysed the results.

Given the number of samples ultimately processed and the binary nature of the outcome measures, descriptive statistics alone were considered sufficient for this analysis.

6.3 Results

At total of 21396 NIPT cases were reviewed between September 2015 and the end of June 2017, 2 (0.01%) samples failed processing and 420 (1.96%) were cancelled for various reasons (e.g. time elapsed since draw date and date received into the lab was past the required time of 5 days or patient details not on the tube), leaving 20974 samples. A total of 485 (2.3%) aneuploidies were detected, Table 6.1 A shows a breakdown of the aneuploidies detected and their rate of detection. A further 110 (0.5%) suspected

aneuploidies were detected, Table 6.1 B shows a breakdown of the aneuploidies detected and their rate of detection.

A total of 43 of the suspected aneuploidies had follow up information available. 3 were concordant at follow up testing: 1 was concordant following a second NIPT test; 1 was concordant following amniocentesis; and 1 was concordant at live birth. The live birth was mosaic for the suspected sex aneuploidy reported through NIPT. An additional amniocentesis following a suspected trisomy 13 NIPT was found to have a balanced translocation between chromosomes 11 and 22. Table 6.2, shows the breakdown for outcome method and result for all the suspected cases.

A.

Trisomy 21		**Trisomy 18**		**Trisomy 13**		**Monosomy X**		**Other Sex Aneuploidies**	
N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
248	1.18	82	0.39	30	0.14	77	0.37	48	0.23

B.

Susp Trisomy 21		**Susp Trisomy 18**		**Susp Trisomy 13**		**Susp Monosomy X**		**Susp Other Sex Aneuploidies**	
N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
20	0.09	17	0.08	22	0.10	21	0.10	30	0.14

Table 6.1: Aneuploidies and Suspected Aneuploidies Detected Through NIPT. A) Summary of aneuploidies detected through NIPT and percentage of all samples tested. B) Summary of all suspected aneuploidies through NIPT and percentage of all samples tested.

Follow up method		N
Second NIPT	Concordant	1
	Discordant	7
Amniocentesis	Concordant	1
	Discordant	21[a]
CVS	Concordant	0
	Discordant	3
Live birth	Concordant	1[b]
	Discordant	6
Miscarriage		1
Termination of Pregnancy		2
Lost to follow up		67

Table 6.2: Breakdown of follow up information for suspected aneuploidies following NIPT. [a] 1 pregnancy was found to have a translocation following amniocentesis. [b] 1 live birth was mosaic for sex aneuploidy reported as suspected aneuploidy by NIPT.

A total of 14 cases fulfilled the criteria of suspected aneuploidy cases with discordant follow up information were identified, which still had a spare sample in storage available for reprocessing. All cases had originally been run through single-end sequencing for their first result.

Following Serenity Basic analysis of paired end sequencing samples, 13 cases were euploid and concordant with follow up and outcome data, 1 failed due to low fetal fraction. Following processing of paired end sequencing data through the Serenity 24 pipeline, all 14 cases had full concordance with follow up and outcome data. Table 6.3, summarises each sample's original NIPT result, follow up information and results following Serenity Basic and Serenity 24.

	Original Serenity Basic Result	Follow Up Method and Result	Live Birth, Where Available	Serenity Basic Paired-End		Serenity 24	
				Result	FF%	Result	FF%
1	Suspected 47, XX +13	Amnio, no aneuploidy detected	Not given	46, XX	11	46, XX	13
2	Suspected 47, XX +13	No further testing	Normal	46, XX	15	46, XX	11
3	Suspected 47, XX +13	Amnio, no aneuploidy detected	Not given	46, XX	11	46, XX	13
4	Suspected 47, XY +13	No further testing	Normal	46, XY	5	46, XY	4
5	Suspected 47, Presence of Y, +13	Amnio, no aneuploidy detected	Not given	46, XY	6	46, presence of Y	3
6	Suspected 47, XX +18	No further testing	Normal	46, XX	4	46, XX	4
7	Suspected 47, XX +21	No further testing	Normal	46, XX	7	46, XX	10
8	Suspected 47, XY +21	Amnio, no aneuploidy detected	Not given	46, XY	6	46, XY	9
9	Suspected 45, XO	Amnio, no aneuploidy detected	Not given	46, XX	9	46, XX	15
10	Suspected 45, XO	Amnio, no aneuploidy detected	Not given	46, XX	6	46, XX	16
11	Suspected 45, XO	Amnio, no aneuploidy detected	Not given	46, XX	9	46, XX	9
12	Suspected, 47, XXX	No further testing	Normal	46, XX	<1	46, XX	6
13	Suspected 47, XXY	Amnio, no aneuploidy detected	Normal	Failed	n/a	46, XY	0
14	Suspected 47, XYY	Second NIPT, no aneuploidy detected	Normal	46, XY	12	46, XY	8

Table 6.3: Summary of suspected aneuploidy samples following NIPT reprocessing. Original NIPT result, follow up method and outcome, live birth information and new Serenity Basic and Serenity 24 results.

6.4 Discussion

The results lead us to reject the hypothesis that the suspected aneuploidies with discordant follow up results were due to RATs and accept the hypothesis that the suspected aneuploidies arose because of technical reasons alone. This study demonstrated that these technical drawbacks have since been improved upon through changes in calling thresholds and increased sensitivity from paired-end sequencing.

NIPT is not a diagnostic test like amniocentesis but, because of the issues surrounding vanishing twins, confined placental mosaicism (of common aneuploidies or RATs) and maternal factors, a screening test for the ploidy status of the fetus. The cell-free fetal DNA is widely accepted to originate from the cytotrophoblast and syncytio-trophoblast of the placenta, at the maternal-fetal interface (Flori *et al*., 2004). For this reason, it has been suggested that 'cell-free fetal DNA' is misleading and the term should be replaced with 'cell-free placental DNA' (Neofytou, 2020). If there is mosaicism between the placenta and the fetus, false positives will occur from NIPT. Confined placental mosaicism (CPM) is estimated to be the most common form of mosaicism, found in around 72% of cases of mosaicism detected, therefore this is likely to be the cause of most false positives associated with NIPT (Opstal *et al.*, 2016). In this study we aimed to answer the question if false positives for suspected aneuploidies on the test chromosomes were caused by RATs. Amniocentesis is considered a gold standard for assessing the ploidy status of the fetus; however, it is invasive and presents a risk to the pregnancy. Eight of the 14 cases in this study had follow up information provided using amniocentesis, the results from these

were not concordant with the NIPT result and indicated that these pregnancies were Euploid. The other six cases in this study resulted in an unaffected live birth.

In this instance, the suspected aneuploidies that were reprocessed in this study have not been caused by RATs or CPM. Improvements in testing through the addition of paired-end sequencing and reassessing aneuploidy calling thresholds has meant that once these samples were reprocessed the false positive results were no longer produced. This is promising that continued improvements with NIPT are eliminating unnecessary false positives being reported and prevent unnecessary invasive procedures such as amniocentesis being carried out consequently.

The reporting of suspected aneuploidies in the study period was very low in comparison to the full aneuploidies detected over the same time. The types of suspected aneuploidy reported did not match the rates seen in the detected aneuploidies, where trisomy 21 is the most common of the detected aneuploidies. Suspected sex aneuploidies and trisomy 13 were more commonly observed in the suspected group. The fetal fraction associated with trisomy 21 is normally higher than other aneuploidies making this an easier trisomy to detect (Taglauer *et al.*, 2013).

A limitation of this study is that the sample set available for reprocessing was small, we may have found some cases which were affected by CPM or RATs, if follow-up information was available for the remaining 67 suspected aneuploid cases. It was a challenge of this

study and in general to obtain follow-up information for many of the detected or suspected NIPT cases. However, as the suspected aneuploidies were not reported as commonly as the detected aneuploidies, these numbers again were smaller.

At the end of July 2018, CooperGenomics ceased their NIPT business, we were therefore unable to pursue any further research related to this study e.g. to follow up further for the presence of RATs and the incidence of CPM. In addition to the review of discordant detected and failed cases, I was interested in the concept of expanding NIPT testing to the detection of mosaic cell lines, this would have aided in preventing false negative NIPT cases and detecting true fetal mosaicism. NIPT results that had previously been reported as a suspected aneuploidy could be due to a mosaic cell line present in the placenta, in fact one of the live births associated with a suspected aneuploidy NIPT report was mosaic. Mosaicism within the placenta could present levels of cell-free fetal DNA with a low level of the aneuploidy detected in the maternal plasma which would not reach the threshold of a full aneuploidy. The rates of mosaic forms of aneuploidy detected during pregnancy are not easy to establish as we cannot be sure that our sample represents all fetal and placental lineages, however, mosaic forms of trisomy 13, 18 are more common than trisomy 21 (13.3%, 12.8% and 3.2%, respectively of those trisomies detected). The rates of mosaicism associated with RATs are higher for certain chromosomes, such as chromosome 7 which has been reported to be mosaic in 100% of cases (Brison *et al.*, 2018), this is likely due to mosaic forms of RATs being more viable during pregnancy than full aneuploidies. These reported rates of mosaic forms of trisomy echo the trend we have

seen in comparison to the suspected aneuploidies in this study, with trisomy 13 and 18 being more common than trisomy 21, therefore if we had been able to investigate these suspected cases further we might have found that some were mosaic forms confined to the placenta. The recent TRIDENT-2 study identified 101 (0.18%) RATs, of these, 94% were false positives and believed to be most likely CPM (Meij *et al.*, 2019). In another report, 4 out of 6 patients suspected of having trisomy 16 had fetal growth retardation or preeclampsia, therefore where RATs are reported but not confirmed in amniocentesis or karyotype at birth, there can still be clinically significant effects on the pregnancy likely due to CPM (Chatron *et al.*, 2019).

6.5 Conclusion

The studies described herein suggest that it is beneficial to provide patients with genome wide NIPT results (i.e. for all chromosomes). Predictions about the likelihood that the aneuploidy detected will be mosaic (especially in the instance of RATs) provide the basis for improved genetic counselling pertaining to pregnancy management.

The conclusion in this study that there were no chromosomal imbalances on other chromosomes or CPM causing false positives in our original test. This study also provides evidence that there is always a constant need for technology improvement and no cause for complacency. Improvements to the testing procedures through paired-end sequencing, review of thresholds, and advances in our analysis software, suggests that this test would be far less likely to produce false positives for cases such as these in the future.

7 General Discussion

7.1 Achievement summary

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

1. PGT-A cases were reviewed for frequency of cases that only had a mosaic embryo available for transfer and the type and number of chromosomes affected in these mosaic embryos in line with the CoGEN position statement. It was found that between 10 and 11% of cases do not have a euploid embryo for transfer but do have 1 or more mosaic embryos available for consideration for transfer. When the available mosaic embryos were reviewed it was found that only 4 to 5% of all cases would have a mosaic embryo which would be considered for transfer. The findings also indicate a weak link between maternal age and finding a mosaic embryo, however this link is more related to the increase of meiotic errors with increasing maternal age than a reduction in mitotic errors.

2. Live birth outcomes from four clinics that performed PGT-A were reviewed for success rates when transferring euploid embryos following PGT-A vs conventional IVF without genetic screening. The evidence presented supports that the transfer of euploid embryos following PGT-A results in higher live birth rates than conventional IVF for patients over 35. However, no benefit was detected for

patients under 35. It was also found that these rates varied significantly between individual clinics.

3. NIPT cases were reviewed for reasons for referral and the rate of aneuploidies detected for each category following NIPT. Results showed that abnormal ultrasound was the most likely referral category to have aneuploidy detected following NIPT. Maternal anxiety was the least likely referral category to have aneuploidy detected following NIPT.

4. NIPT cases which had originally been reported with a suspected common aneuploidy but at follow up found to be unaffected pregnancies were identified. Results following reprocessing of these samples through paired-end sequencing and 24 chromosome analysis led to the rejection of the hypothesis that these discordant results were due to RATs. As the cases returned results consistent with follow up outcomes, we were able to conclude that the false positives had occurred due to technical drawbacks.

These findings and their significance are discussed in detail within their respective results section and will not be repeated here, however wider implications relating to PGT-A and NIPT will be discussed hereafter.

7.2 PGT-A

The debate between those for and against the use of PGT-A continues despite technological advancements and continued research into the effectiveness of PGT-A. At the end of 2019 the HFEA categorised PGT-A as a red light add on stating that there is 'no evidence that PGT-A improves live birth rates'. However, several publications in recent years have indicated an improvement associated with PGT-A particularly in older patients. A randomised controlled study by Rubio *et al* in 2017, showed a significantly higher live birth rate in patients between 38 and 41, and reported a significantly lower miscarriage rate (Rubio *et al.*, 2017). A more recent randomised controlled study by Munne *et al* in 2019, while it did not find a difference in younger patients, it did report an increased ongoing pregnancy rate associated with PGT-A for patients between 35 and 40 years (Munné *et al.*, 2019). In addition, a retrospective study has also shown improvements associated with the application of PGT-A on a larger scale on both a per embryo transferred and intention to treat basis (Anderson *et al.*, 2019). And despite its opponents, PGT-A has been shown to be growing in popularity, particularly in the USA (Theobald *et al.*, 2020).

While the debate continues regarding the effectiveness of PGT-A, many scientists within the community can agree that it is important to strive to improve embryo selection criteria to optimise outcomes for patients undertaking ART. Conventional morphological assessments have been shown to have a limit to selecting the best embryo for transfer, with euploid embryos of a poor morphological score having a similar implantation

potential to excellent morphologically scoring euploid embryos (Gonzalez *et al.*, 2019). The application of time lapse algorithms does show positive results and may be more appropriate for younger patients (Fishel *et al.*, 2017).

Non-invasive PGT-A is becoming a popular area for research as an alternative to conventional PGT-A, by carrying out NGS on spent embryo culture media or blastocyst fluid, if successful it would be able to indicate the ploidy status of the developing embryo without the potential damage from embryo biopsy (Gianaroli *et al.*, 2014; Shamonki *et al.*, 2016; Rubio *et al.*, 2020). Perhaps with further research, non-invasive PGT-A along with time lapse algorithms, will result in improved outcomes for patients without the potential damage to embryos associated with biopsy.

One question that has yet to be answered is: why do not all euploid embryos which are transferred result in a healthy live birth? Some of the reasons have been addressed in this thesis. Mosaicism within the embryo, resulting in a false negative following PGT-A could in some instances mean that the embryo does not have the potential to implant or go to term, due to the inner cell mass being affected by aneuploidy. As well as older technologies such as aCGH being unable to detect mosaicism within the biopsied samples. Another proposed cause could be the incorrect timing of transfer of euploid embryos, endometrial receptivity, and sufficiency throughout pregnancy (Tan *et al.*, 2018). Biopsy technique could also be impacting on the chance of a euploid embryo resulting in live

birth (Rubino *et al.*, 2020). Further investigation into why euploid embryos fail to result in a live birth will be required in the future.

7.3 NIPT

Continued improvements with the detection levels of NIPT could ensure that NIPT can be practically applied to offer more information. In this thesis the extension to screening all chromosomes was discussed and the fact that by being able to detect aneuploidy on all chromosomes, false positives for the common aneuploidies could potentially be avoided. The further application of algorithms to predict mosaicism levels when reporting positive NIPT results could mean that with appropriate genetic counselling, such pregnancies could be better managed in the future (Brison *et al.*, 2018; Neofytou, 2020).

A recent alternative application for NIPT has been suggested by Yaron *et al*. 2020, they have demonstrated that NIPT could be used whilst a patient is experiencing early pregnancy loss to detect if there was a chromosomal abnormality in the fetus, which ultimately may have caused the loss of the pregnancy (Yaron *et al.*, 2020). This information could be valuable for the patient and the future management of their attempts to conceive, or management of the pregnancy. This could be especially useful for patients who experience recurrent pregnancy loss. They have demonstrated that this method of analysis is more successful at achieving a result than karyotyping products of conception. This knowledge from applying NIPT to early miscarriage, could be applied

within an ART setting, where conventional IVF cycles result in early pregnancy loss, to identify if these pregnancies failed due to aneuploidy or if other avenues need to be investigated.

In mid-August 2020. The American College of Obstetricians and Gynecologists, issued guidelines relating to NIPT, and recommended that all pregnancies should be offered NIPT regardless of their risk level (ACOG, 2020). As discussed previously within this thesis, NIPT has most commonly been offered to high risk patients, however this means that cases of common aneuploidies in low risk patients are missed. It is likely that with continued introduction of NIPT and eventually a reduction in cost, more countries will roll out NIPT for all patients regardless of risk and NIPT will supersede older methods of prenatal screening.

7.4 Mosaicism

A recurring theme for both PGT-A and NIPT is the impact of mosaicism. For PGT-A the improvement in technology to NGS has meant that mosaic embryos are more readily detected, the challenge moving forward is to gain a better understanding of the effects of transferring these embryos and indeed this is where a lot of research is currently being focused. Perhaps the development of a prediction model based on the percentage of cells affected and the chromosomes affected will be possible in the future. NIPT has been shown to be more sensitive than CVS for the detection of mosaicism confined to the

placenta (Opstal *et al.*, 2020). Whilst CPM leading to false positive or in rare cases false negatives is a drawback of NIPT, with appropriate patient counselling this information can prove valuable to the ongoing management of these pregnancies. Perhaps reviewing knowledge gained from NIPT regarding those mosaic pregnancies that result in a healthy live birth will prove beneficial in the future when applied to the consideration of mosaic PGT-A embryos for transfer.

What has not been discussed in this thesis is the mechanism by which mosaic embryos can result in a healthy live birth despite an aneuploid cell line being present at the time of embryo biopsy. It has often perhaps imprecisely been suggested that the cells containing errors correct themselves, for example a trisomy rescue mechanism. While trisomy rescue is likely the mechanism that leads to UPD (Balbeur *et al.*, 2016), a study in mouse models has indicated that the aneuploid cell lines fail to replicate and are outcompeted by the euploid cell lines which replicate at a normal rate (Bolton *et al.*, 2016). Therefore, it is reasonable to expect those embryos with a lower percentage of aneuploid cells to have the best likelihood of developing and resulting in a successful pregnancy. However, unless the euploid and aneuploid cell lines are uniformly distributed throughout the trophectoderm, it is difficult to predict this from the percentage of mosaicism reported following PGT-A. It has also been shown that there is increased apoptosis in both mosaic and aneuploid embryos (Victor *et al.*, 2019). This apoptosis could have implications for non-invasive PGT-A. Non-invasive PGT-A as mentioned previously assesses DNA from spent media or blastocyst fluid. However, the origin of the cell free DNA which is detected

through non-invasive PGT-A is not clear, it is reasonable that as increased apoptosis is detected in aneuploid and mosaic embryos, that this would be the source of DNA for an aneuploid result, this in turn may lead to mosaic embryos being discounted as aneuploid and not considered for transfer. From the research in this thesis, this could leave those 10 to 11% of patients who do not have a euploid embryo but do have a mosaic embryo, without the option to consider a mosaic embryo for transfer.

Perhaps in the future if non-invasive PGT-A were to become widely introduced in ART, if a patient had all embryos reported as aneuploid, it would be possible to subsequently biopsy these embryos for a confirmatory result prior to discarding. In this situation, where a patient had no euploid embryo for transfer, a mosaic embryo could potentially be detected and considered. This approach draws parallels to NIPT, that a positive result should ideally be confirmed with a more invasive procedure, preferably amniocentesis, especially prior to termination of the pregnancy.

7.5 Thoughts on the overall incidence of aneuploidy

The relative incidence of aneuploidy of each individual chromosome, at each stage of development is complex and not easily defined. Indeed, there are still areas in which further studies need to be performed before a complete picture can be made. The stage of ascertainment, the tissue sampled, the initial rate of error of the chromosome, the

selection against specific abnormalities and the incidence of mosaicism (confined or otherwise) all need to be considered.

In this regard, both PGT-A results (see chapters 3 and 4) and NIPT results (see chapters 5 and 6) provide unique, and hitherto unprecedented, insight. The results contained within chapter 4 indicate how data from the study of trophectoderm biopsies informs the bigger picture of how the incidence of chromosome abnormalities may be interpreted and expressed. In addition to its benefit for prenatal medicine, NIPT outcomes also provide insight into the incidence of a tissue type arising from the trophectoderm, i.e. the syncytiotrophoblast of the placenta.

The most common aneuploidies associated with live birth are trisomy 21, 18, 13 and the sex-chromosome aneuploidies, affecting around 0.3% of newborn's (Hassold & Hunt, 2001). Although the exact incidence in newborn's is difficult to ascertain due to many variables in different countries over time. It is known that these live birth aneuploidies occur more frequently with increasing maternal age, but individual and country-wide attitudes towards screening and termination impact on final live birth numbers as well as average age of motherhood in different countries. For example, Iceland has reported only 1 or 2 live births affect by trisomy 21 per year in recent years due in part to the allowance of termination of affected pregnancies past 16 weeks of gestation (Kluznik & Slepian, 2018). It has been estimated that aneuploidy occurs in around 4% of stillborn's (Hassold *et al*, 1996). Although, the frequency of aneuploidies associated with stillbirth is equally

challenging to quantify for the same reasons previously outlined, however, what is clear is that a very similar frequency of aneuploidies is seen within stillbirth's, that is, trisomy 21 is the most common, followed by the sex-chromosomes aneuploidies, trisomy 18 and 13 and in rarer instances trisomy 9 (Hassold *et al*, 1996). The frequency and distribution of chromosomal abnormalities is much higher in miscarriages, with over 35% being aneuploid (Hassold *et al*, 1996). Almost all other chromosomes are involved with miscarriages, except for chromosome 1. The most common aneuploidies detected other than the common live birth trisomies are trisomy 16, followed by 22, 15, 2 and 14, this indicates that such embryos possessing these aneuploidies are capable of implantation but not compatible with life (Hassold *et al*, 1996). It is likely that the incidence of aneuploidy is much higher than that detected from miscarriage, stillbirth and live birth, however, these pregnancies are likely to demise prior to being clinically recognised, or may go unrecognised altogether following natural conception, this is supported by the rates of aneuploidy being much higher in preimplantation embryos than that clinically recognised pregnancies.

From PGT-A studies, it is known that chromosomal errors are associated with every chromosome, and the frequency of aneuploid errors is positively correlated with maternal age (Franasiak *et al,* 2014). It has also been shown that the frequency of chromosome specific aneuploidies is negatively correlated to chromosome length, that is whole chromosome aneuploidy occurs more frequently with smaller chromosomes (McCoy *et al*,

2015). The rate of aneuploidy detected decreases from the cleavage stage to the blastocyst stage, and it is thought that aneuploidy is the main cause for embryo arrest or delayed development between these two stages, particularly embryos possessing multiple aneuploidies (Harton *et al*, 2013; McCoy *et al*, 2015; Coll *et al*, 2018). It has also been demonstrated that single chromosomal aneuploidies compatible with clinical pregnancy and live birth are morphologically indistinguishable from chromosomally normal embryos at the blastocyst stage (Fragouli *et al,* 2014). This demonstrates that these types of aneuploidies although may not always be the most frequently detected with PGT-A embryos, they do have the greatest capacity to implant and result in an affected pregnancy.

In chapter 5, the types of aneuploidy detected for each referral indication were shown, here we can see that the most frequently detected aneuploidies from NIPT were trisomy 21, followed by sex chromosome aneuploidies, trisomy 18 and trisomy 13, this is in line with that previously reported to be associated with live birth and miscarriage. Although the testing of microdeletions and all other chromosomes was not carried out on all cases included in this study and was introduced late in the period reviewed for consideration, 18 microdeletions and 21 RATs were reported, with trisomy 16 and trisomy 7 being the most frequent. Although not discussed within chapter 4, the distribution of chromosomes affected following PGT-A was reviewed and showed a similar pattern to that previously reported. All chromosomes are affected by aneuploidy within the embryo, and that the smaller chromosomes are more commonly affected (except the y chromosome).

Monosomies were detected at a similar rate as trisomies and segmental errors were less common than both trisomies and monosomies. Despite the frequency of monosomies in relation to trisomies, these are almost never detected at early pregnancy and were not detected following NIPT within our data set (except monosomy X). Despite chromosome 22 and 16 being the most affected at the PGT-A analysis stage, these chromosomes are rare to be detected during pregnancy. It is likely that aneuploidies relating to these chromosomes are lethal to the developing embryo, and either result in the embryo arresting prior to implantation, or the demise of the pregnancy prior to testing at 10 weeks for NIPT. And as discussed within chapter 6, mosaic forms of aneuploidy associated with RATs may be more viable than full aneuploidies, therefore the detection of these aneuploidies following NIPT and miscarriage, are likely due to mosaicism.

7.6 Future studies arising from this thesis

The evidence presented within this thesis has contributed towards answering some questions surrounding PGT-A and NIPT. The work carried out has however highlighted other potential avenues for further exploration, for example:

1. Outcomes following the transfer of mosaic embryos. Whilst there are some recent publications addressing this subject, the data available is understandably limited. It is important that these studies continue, to gain valuable information relating to the viability of mosaic embryos especially relating to the chromosomes affected.

Further studies regarding the frequency of mosaic embryos could also be carried out in lieu of improved PGT-A technologies and artificial intelligence for the calling of PGT-A results.

2. Further prospective randomised controlled trials regarding live birth in relation to PGT-A with mosaic embryos considered. Where these are multi centre studies, I feel that it is important to show what degree the results vary between centres.

3. Further investigation regarding why euploid embryos do not make it to live birth. Carrying out a PGT-A study along with endometrial receptivity could highlight some additional factors which are preventing success following ART.

4. Extending the scope of NIPT to identifying mosaicism. As discussed, many RATs are likely to be mosaic for these pregnancies to be viable and not to miscarry at a very early gestational age. This information could prove to be valuable for all aneuploidies in the management of these pregnancies.

5. The application of NIPT to miscarriage. NIPT has the potential to offer mothers who are miscarrying an explanation regarding the cause of the miscarriage, whether those pregnancies were affected by aneuploidy. This information can be applied for the future management of subsequent conceptions, both naturally and alongside ART.

7.7 Personal perspectives and concluding remarks

PGT-A and NIPT continue to both be subject to debate regarding their application. The increase in incidental findings from both technologies, most commonly in the form of mosaicism propel this debate. However, from my perspective the benefit of both screening methods is clear. The more information regarding the viability of an embryo before transfer, will ultimately result in better outcomes for the patients and reduce adverse outcomes such as miscarriage. NIPT offers a screening test which has high levels of accuracy over conventional methods and reduces the need for invasive procedures. I feel that such a screening method should be available for all patients. Despite the drawbacks that have been mentioned, these all can be minimised by appropriate genetic counselling for patients before and after genetic testing.

In the coming years, further research will be required for both NIPT and PGT-A. NIPT will likely benefit from increased research into extending screening to other genetic anomalies, such as RATs and CNVs and the improvement in accuracy of these tests. PGT-A would also benefit from additional randomised clinical trials to assess it effectiveness as the technology improves and more is known about the transfer of mosaic embryo. I will also be interested to observe as more evidence comes to light regarding non-invasive approaches to PGT-A.

8 References

Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology (2011) Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reproductive BioMedicine Online*, **22**, 632–646.

American College of Obstetricians and Gynecologists. (2015) Committee Opinion No. 640: Cell-free DNA screening for fetal aneuploidy. Obstetrics & Gynecology, 126, e31–e37.

American College of Obstetricians and Gynecologists. (2020) Practice Bulletin 226 – Clinical management guidelines for obstetrician gynecologists: Screening for fetal chromosomal abnormalities. [WWW document] https://www.acog.org/clinical/clinical-guidance/practice-bulletin/articles/2020/08/screening-for-fetal-chromosomal-abnormalities

Adler, A., Lee, H.-L., McCulloh, D.H., Ampeloquio, E., Clarke-Williams, M., Wertz, B.H., *et al*. (2014) Blastocyst culture selects for euploid embryos: comparison of blastomere and trophectoderm biopsies. Reproductive BioMedicine Online, 28, 485–491.

Alfarawati, S., Fragouli, E., Colls, P., Stevens, J., Gutiérrez-Mateo, C., Schoolcraft, W.B., *et al*. (2011) The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. Fertility and Sterility, 95, 520–524.

Alfarawati, S., Fragouli, E., Colls, P. & Wells, D. (2012) Embryos of robertsonian translocation carriers exhibit a mitotic interchromosomal effect that enhances genetic instability during early development. PLOS Genetics, 8 (10): e1003025.

Alfirevic, Z., Mujezinovic, F., and Sundberg, K. (2017) Amniocentesis and chorionic villus sampling for prenatal diagnosis. Cochrane Database of Systematic Reviews. 3.

Alldred, S.K., Takwoingi, Y., Guo, B., Pennant, M., Deeks, J.J., Neilson, J.P., *et al*. (2017) First trimester ultrasound tests alone or in combination with first trimester serum tests for Down's syndrome screening. Cochrane Database of Systematic Reviews.

Anderson, R.E., Whitney, J.B. & Schiewe, M.C. (2019) Clinical benefits of preimplantation genetic testing for aneuploidy (PGT-A) for all in vitro fertilization treatment cycles. European Journal of Medical Genetics, 103731.

Balbeur, S., Grisart, B., Parmentier, B., Sartenaer, D., Leonard, P., Ullmann, U., *et al*. (2016) Trisomy rescue mechanism: the case of concomitant mosaic trisomy 14 and maternal uniparental disomy 14 in a 15-year-old girl. Clinical Case Reports, 4, 265–271.

Bardi, F., Bosschieter, P., Verheij, J., Go, A., Haak, M., Bekker, M., *et al*. (2019) Is there still a role for nuchal translucency measurement in the changing paradigm of first trimester screening? Prenatal Diagnosis, 40, 197–205.

Basile, N., Vime, P., Florensa, M., Ruiz, B.A., Velasco, J.A.G., Remohí, J., *et al*. (2015) The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. Human Reproduction, 30, 276–283.

Bayón, J.C., Orruño, E., Portillo, M.I. & Asua, J. (2019) The consequences of implementing non-invasive prenatal testing with cell-free fetal DNA for the detection of Down syndrome in the Spanish National Health Service: a cost-effectiveness analysis. Cost Effectiveness and Resource Allocation, 17, 6.

Bean, C.J., Hassold, T.J., Judis, L. & Hunt, P.A. (2002) Fertilization in vitro increases non-disjunction during early cleavage divisions in a mouse model system. Human Reproduction, 17, 2362–2367.

Beaudet, A.L. (2016) Using Fetal Cells for Prenatal Diagnosis: History and Recent Progress. American Journal of Medical Genetics Part C Semin Med Genet, 172C, 123-127.

Benn, P. & Grati, F. (2018) Genome-wide non-invasive prenatal screening for all cytogenetically visible imbalances. Ultrasound in Obstetrics & Gynecology.

Berger, V.K., Norton, M.E., Sparks, T.N., Flessel, M., Baer, R.J. & Currier, R.J. (2020) The utility of nuchal translucency ultrasound in identifying rare chromosomal abnormalities not detectable by cell-free DNA screening. Prenatal Diagnosis, 40, 185–190.

Bergh, C.M, Reda, C.V., Glujovsky, D. and Bergh, P.A. (2013) Effect of body mass index on aneuploidy. Fertility and Sterility. S151.

Beulen, L., Faas, B., Feenstra, I., Vugt, J. van & Bekker, M. (2017) Clinical utility of non-invasive prenatal testing in pregnancies with ultrasound anomalies. Ultrasound in Obstetrics & Gynecology, 49, 721–728.

Bevilacqua, E., Gil, M.M., Nicolaides, K.H., Ordonez, E., Cirigliano, V., Dierickz, P.J. Willems & Jani, J.C. (2015) Performance of screening for aneuploidies by cell-free DNA analysis of maternal blood in twin pregnancies. Ultrasound in Obstetrics and Gynecology, 45, 61-66.

Bianchi, D.W. (2004) Circulating Fetal DNA: Its Origin and Diagnostic Potential – A review. Placenta, 25 (18), S93-S101.

Bianchi, D.W., Platt, L.D., Goldberg, J.D., Abuhamad, A.Z., Sehnert, A.J. and Rava, R.P. (2012) Genome-Wide Fetal Aneuploidy Detection by Maternal Plasma DNA Sequencing. Obstetrics & Gynecology 119:5, 890-901.

Bianchi, D.W., Parker, L.R., Wentworth, J., Madankumar, R., Saffer, C., Das, A.F., *et al*. (2014) DNA Sequencing versus Standard Prenatal Aneuploidy Screening. The New England Journal of Medicine, 370, 799–808.

Bianchi, D.W. (2017) Should we 'open the kimono' to release the results of rare autosomal aneuploidies following non-invasive prenatal whole genome sequencing? Prenatal Diagnosis, 37, 123–125.

Bolton, H., Graham, S.J., Aa, N. der, Kumar, P., Theunis, K., Gallardo, E., *et al*. (2016) Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential. Nature Communications, 7, 11165.

Boynukalin, F.K., Gultomruk, M., Cavkaytar, S., Turgut, E., Findikli, N., Serdarogullari, M., *et al*. (2020) Parameters impacting the live birth rate per transfer after frozen single euploid blastocyst transfer. PLOS ONE, 15, e0227619.

Breathnach, F.M., Malone, F.D., Lambert-Messerlian, G., Cuckle, H.S., Porter, T.F., Nyberg, D.A., *et al*. (2007) First- and Second-Trimester Screening: Detection of Aneuploidies Other Than Down Syndrome. Obstetrics & Gynecology, 110, 651–657.

Brison, N., Neofytou, M., Dehaspe, L., Bayindir, B., Bogaert, K., Dardour, L., *et al*. (2018) Predicting fetoplacental chromosomal mosaicism during non-invasive prenatal testing. Prenatal Diagnosis.

Bunt, C.W. & Bunt, S.K. (2014) Role of the family physician in the care of children with Down syndrome. American family physician, 90, 851–8.

Buysse, K., Beulen, L., Gomes, I., Gilissen, C., Keesmaat, C., Janssen, I.M., Derks-Willemen, J.J.H.T., de Ligt, J., Feenstra, I., Bekker, M.N., van Vugt, J.M.G., van Kessel, A.G., Vissers, L.E.L.M. and Faas, B.H.W.(2013) Reliable noninvasive prenatal testing by massively parallel sequencing of circulating cell-free DNA from maternal plasma processed up to 24 h after venipuncture. Clinical Biochemistry 46, 1783-1786.

Campbell, A., Fishel, S., Bowman, N., Duffy, S., Sedler, M. & Hickman, C.F.L. (2013) Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. Reproductive BioMedicine Online, 26, 477–485.

Capalbo, A., Wright, G., Elliott, T., Ubaldi, F.M., Rienzi, L. & Nagy, Z.P. (2013) FISH reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocysts shows high accuracy of diagnosis and no major diagnostic impact of mosaicism at the blastocyst stage. Human Reproduction, 28, 2298–2307.

Capalbo, A., Ubaldi, F.M., Cimadomo, D., Maggiulli, R., Patassini, C., Dusi, L., *et al*. (2015) Consistent and reproducible outcomes of blastocyst biopsy and anueploidy screening across different biopsy practitioners: a multicentre study involving 2586 embryo biopsies. Human Reproduction. 31 (1), 199-208.

Chan, K.C.A., Zhang, J., Hui, A.B.Y., Wong, N., Lau, T.K., Leung, T.N., Lo, K.W., Huang, D.W.S. and Lo, Y.M.D. (2004) Size distributions of maternal and fetal DNA in maternal plasma. Clinical Chemistry, 50 (1), 88-92.

Chatron, N., Till, M., Abel, C., Bardel, C., Ramond, F., Sanlaville, D., *et al*. (2019) Detection of rare autosomal trisomies through non-invasive prenatal testing: benefits for pregnancy management. Ultrasound in Obstetrics & Gynecology, 53, 129–130.

Chatzimeletiou, K., Morrison, E.E., Prapas, N., Prapas, Y. & Handyside, A.H. (2005) Spindle abnormalities in normally developing and arrested human preimplantation embryos in vitro identified by confocal laser scanning microscopy. Human Reproduction, 20, 672–682.

Chuang, T.-H., Hsieh, J.-Y., Lee, M.-J., Lai, H.-H., Hsieh, C.-L., Wang, H.-L., *et al*. (2018) Concordance between different trophectoderm biopsy sites and the inner cell mass of chromosomal composition measured with a next-generation sequencing platform. Molecular Human Reproduction, 24, 593–601.

Cimadomo, D., Capalbo, A., Ubaldi, F., Scarica, C., Palagiano, A., Canipari, R., & Rienzi, L. (2016) The impact of biopsy on human embryo developmental potential during preimplantation genetic diagnosis. BioMed Research International, 2016, 1-10.

Clark-Ganheart, C.A., Fries, M.H., Leifheit, K.M., Jensen, T.J., Moreno-Ruiz, N.L., Ye, P.P., *et al*. (2015) Use of Cell-Free DNA in the Investigation of Intrauterine Fetal Demise and Miscarriage. Obstetrics & Gynecology, 125, 1321.

CoGEN (2016) COGEN position statement on chromosomal mosaicism detected in preimplantation blastocyst biopsies. [WWW document], https://ivf-worldwide.com/cogen/general/cogen-statement.html

Coll, L., Parriego, M., Boada, M., Devesa, M., Arroyo, G., Rodríguez, I., *et al*. (2018) Transition from blastomere to trophectoderm biopsy: comparing two preimplantation genetic testing for aneuploidies strategies. Zygote, 1–8.

Cram, D.S., Leigh, D., Handyside, A., Rechitsky, L., Xu, K., Harton, G., *et al*. (2019) PGDIS Position Statement on the Transfer of Mosaic Embryos 2019. Reproductive BioMedicine Online, 39, e1–e4.

Dar, P., Curnow, K.J., Gross, S.J., Hall, M.P., Stosic, M., Demko, Z., *et al*. (2014) Clinical experience and follow-up with large scale single-nucleotide polymorphism–based non-invasive prenatal aneuploidy testing. American Journal of Obstetrics and Gynecology, 211, 527.e1-527.e17.

Delhanty, J., Griffin, D., Handyside, A., Harper, J., Atkinson, G., Pieters, M., *et al*. (1993) Detection of aneuploidy and chromosomal mosaicism in human embryos during preimplantation sex determination by fluorescent in situ hybridisation, (FISH). Human molecular genetics, 2, 1183–5.

Demko, Z.P., Simon, A.L., McCoy, R.C., Petrov, D.A. & Rabinowitz, M. (2016) Effects of maternal age on euploidy rates in a large cohort of embryos analyzed with 24-chromosome single-nucleotide polymorphism–based preimplantation genetic screening. Fertility and Sterility, 105, 1307–1313.

Edwards, R.G., Fishel, S.B., Cohen, J., Fehilly, C.B., Purdy, J.M., Slater, J.M., *et al*. (1984) Factors influencing the success of in vitro fertilization for alleviating human infertility. Journal of in Vitro Fertilization and Embryo Transfer, 1, 3–23.

Esteki, M.Z., Dimitriadou, E., Mateiu, L., Melotte, C., Van der Aa, N., Kumar, P. *et al.* (2015) Concurrent whole-genome haplotyping and copy-number profiling of single cells. American Journal for Human Genetics, 96 (6), 894-912.

Fan, H.C., Blumenfeld, Y. J., Chitkara, U., Hudgins, L. & Quake, S.R. (2010) Analysis of the size distributions of fetal and maternal cell-free DNA by paired-end sequencing. Clinical Chemistry, 56 (8), 1279-1286.

Fan, J., Wang, L., Wang, H., Ma, M, Wang, S., Liu, Z., *et al*. (2015) The clinical utility of next generation sequencing for identifying chromosome disease syndromes in human embryos. Reproductive BioMedicine Online. 31, 62-70.

Ferreira, J.C.P., Grati, F.R., Bajaj, K., Malvestiti, F., Grimi, M.B., Trotta, A., *et al*. (2016) Frequency of fetal karyotype abnormalities in women undergoing invasive testing in the absence of ultrasound and other high-risk indications. Prenatal Diagnosis, 36, 1146–1155.

Fiorentino, F., Bono, S., Pizzuti, F., Duca, S., Polverari, A., Faieta, M., *et al*. (2017) The clinical utility of genome-wide non invasive prenatal screening. Prenatal Diagnosis, 37, 593–601.

Fishel, S., Campbell, A., Montgomery, S., Smith, R., Nice, L., Duffy, S., *et al*. (2017) Live births after embryo selection using morphokinetics versus conventional morphology: a retrospective analysis. Reproductive BioMedicine Online, 35, 407–416.

Fishel, S., Campbell, A., Montgomery, S., Smith, R., Nice, L., Duffy, S., *et al*. (2018) Time-lapse imaging algorithms rank human preimplantation embryos according to the probability of live birth. Reproductive biomedicine online, 37, 304–313.

Flori, E., Doray, B., Gautier, E., Kohler, M., Ernault, P., Flori, J., *et al*. (2004) Circulating cell-free fetal DNA in maternal serum appears to originate from cyto- and syncytio-trophoblastic cells. Case report. Human Reproduction, 19, 723–724.

Fragouli, E., Alfarawati, S., Spath, K. & Wells, D. (2014) Morphological and cytogenetic assessment of cleavage and blastocyst stage embryos. Molecular Human Reproduction, 20, 117-126.

Fragouli, E., Alfarawati, S., Spath, K., Babariya, D., Tarozzi, N., Borini, A., *et al*. (2017) Analysis of implantation and ongoing pregnancy rates following the transfer of mosaic diploid–aneuploid blastocysts. Human Genetics, 136, 805–819.

Franasiak, J.M., Forman, E.J., Hong, K.H., Werner, M.D., Upham, K.M., Treff, N.R., *et al*. (2014a) Aneuploidy across individual chromosomes at the embryonic level in trophectoderm biopsies: changes with patient age and chromosome structure. Journal of Assisted Reproduction and Genetics, 31, 1501–1509.

Franasiak, J.M., Forman, E.J., Hong, K.H., Werner, M.D., Upham, K.M., Treff, N.R., *et al*. (2014b) The nature of aneuploidy with increasing age of the female partner: a review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. Fertility and Sterility, 101, 656-663.e1.

Friedenthal, J., Maxwell, S.M., Munné, S., Kramer, Y., McCulloh, D.H., McCaffrey, C., *et al*. (2018) Next generation sequencing for preimplantation genetic screening improves pregnancy outcomes compared with array comparative genomic hybridisation in single thawed euploid embryo transfer cycles. Fertility and Sterility.

Friedenthal, J., Maxwell, S.M., Tiegs, A.W., Besser, A.G., McCaffrey, C., Munné, S., *et al*. (2020) Clinical error rates of next generation sequencing and array comparative genomic hybridisation with single thawed euploid embryo transfer. European Journal of Medical Genetics, 63, 103852.

Futch, T. (2013) Initial clinical laboratory experience in non-invasive prenatal testing for fetal aneuploidy from maternal plasma DNA samples. Prenatal Diagnosis, 33.

Gabriel, A.S., Thornhill, A.R., Ottolini, C.S., Gordon, A., Brown, A.P.C., Taylor, J., Bennett, K., Handyside, A., & Griffin, D.K. (2017) Array comparative genomic hybridisation on first polar bodies suggests that non-disjunction is not the predominant mechanism leading to aneuploidy in humans. Mutational mechanisms, 48, 433-437.

Gadsbøll, K., Petersen, O.B., Gatinois, V., Strange, H., Jacobsson, B., Wapner, R., *et al*. (2020) Current use of non-invasive prenatal testing in Europe, Australia and the USA: A graphical presentation. Acta Obstetricia et Gynecologica Scandinavica, 99, 722–730.

Gardner, R.L. and Edwards, R.G. (1968) Control of the sex ratio at full term in the rabbit by transferring sexed blastocysts. Nature, 218, 346-348.

Gardner, D.K., Lane, M., Stevens, J., Schlenker, T. & Schoolcraft, W.B. (2000) Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertility and Sterility, 73, 1155–1158.

Gianaroli, L., Magli, C.M., Pomante, A., Crivello, A.M., Cafueri, G., Valerio, M., *et al*. (2014) Blastocentesis: a source of DNA for preimplantation genetic testing. Results from a pilot study. Fertility and Sterility, 102, 1692-1699.e6.

Gianaroli, L., Magli, C.M., Ferraretti, A.P., Tabanelli, C., Trengia, V., Farfalli, V., *et al*. (2005) The beneficial effects of preimplantation genetic diagnosis for aneuploidy support extensive clinical application. Reproductive biomedicine online, 10, 633–40.

Girardi, L., Serdarogullari, M., Patassini, C., Poli, M., Fabiani, M., Caroselli, S., *et al*. (2020) Incidence, Origin, and Predictive Model for the Detection and Clinical Management of Segmental Aneuploidies in Human Embryos. The American Journal of Human Genetics.

Gleicher, N., Kushnir, V. A., & Barad, D.H. (2014) Preimplantation genetic screening (PGS) still in search of clinical application: a systemic review. Reproductive Biology and Endocrinology. 12, 22.

Gonzalez, X.V., Odia, R., Naja, R., Serhal, P., Saab, W., Seshadri, S., *et al*. (2019) Euploid blastocysts implant irrespective of their morphology after NGS-(PGT-A) testing in advanced maternal age patients. Journal of Assisted Reproduction and Genetics, 36, 1623–1629.

Go, A.T.J.I., van Vugt, J.M.G. & Oudejans C.B.M. (2011) Non-invasive aneuploidy detection using free fetal DNA and RNA in maternal plasma: recent progress and future possibilities. Human Reproduction Update, 17 (3), 372-382.

Grati, F.R., Malvestiti, F., Ferreira, J.C.P.B., Bajaj, K., Gaetani, E., Agrati, C., *et al*. (2014) Fetoplacental mosaicism: potential implications for false-positive and false-negative non-invasive prenatal screening results. Genetics in Medicine, 16, 620–624.

Grati, F.R., Gallazzi, G., Branca, L., Maggi, F., Simoni, G. & Yaron, Y. (2018a) An evidence-based scoring system for prioritizing mosaic aneuploid embryos following preimplantation genetic screening. Reproductive BioMedicine Online.

Grati, F., Gallazzi, G., Branca, L., Maggi, F., Simoni, G. & Yaron, Y. (2018b) Response: scoring of mosaic embryos after preimplantation genetic testing - the rollercoaster ride between fear, hope and embryo wastage. Reproductive BioMedicine Online.

Grati, F.R., Ferreira, J., Benn, P., Izzi, C., Verdi, F., Vercellotti, E., Dalpiaz, C., D'Ajello, P. *et al*. (2019) Outcomes in pregnancies with a confined placental mosaicism and implications for prenatal screening using cell-free DNA. Genetics in Medicine. 22 (2), 309-316.

Greco, E., Minasi, M.G. & Fiorentino, F. (2015) Healthy Babies after Intrauterine Transfer of Mosaic Aneuploid Blastocysts. New England Journal of Medicine, 373, 2089–2090.

Gregg, A.R., Skotko, B.G., Benkendorf, J.L., Monaghan, K.G., Bajaj, K., Best, R.G., *et al*. (2016) Non-invasive prenatal screening for fetal aneuploidy, 2016 update: a position statement of the American College of Medical Genetics and Genomics. Genetics in Medicine, 18, 1056–1065.

Griffin, D.K., Wilton, L.J., Handyside, A.H., Atkinson, G.H.G., Winston, R.M.L. & Delhanty, J.D.A. (1993) Diagnosis of sex in preimplantation embryos by fluorescent in situ hybridisation. British Medical Journal, 306 (6889), 1382.

Griffin, D.K., Millie, E.A., Redline, R.W., Hassold, T.J. & Zaragoza, M.V. (1997) Cytogenetic analysis of spontaneous abortions: Comparison of techniques and assessment of the incidence of confined placental mosaicism. American Journal of Medical Genetics, 72, 297–301.

Griffin, D. K. & Ogur, C. (2018) Chromosomal analysis in IVF: just how useful is it? Reproduction, 156 (1), F29-F50.

Grossman, T.B., Bodenlos, K.L. & Chasen, S.T. (2019) Abnormal nuchal translucency: residual risk with normal cell-free DNA screening. The Journal of Maternal-Fetal & Neonatal Medicine, 1–6.

Guy, C., Haji-Sheikhi, F., Rowland, C.M., Anderson, B., Owen, R., Lacbawan, F.L., *et al*. (2019) Prenatal cell-free DNA screening for fetal aneuploidy in pregnant women at average or high risk: Results from a large US clinical laboratory. Molecular Genetics & Genomic Medicine, 7, e545.

Handyside, A.H., Kontogianni, E.H., Hardy, K. & Winston, R.M.L. (1990) Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature. 344, 768-770.

Handyside, A. H., Lesko, J. G., Tarín, J. J., Winston, R. M. & Hughes, M. R. (1992) Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. N. Engl. J. Med, 327, 905–9.

Handyside, A.H., Robinson, M.D., Simpson, R.J., Omar, M.B., Shaw, M.A., Grudzinskas, J.G. *et al*. (2004) Isothermal whole genome amplification from single and small numbers of cell: a new era for preimplantation genetic diagnosis of inherited disease. Molecular Human Reproduction, 10 (10), 767-772.

Handyside, A.H., Harton, G.L., Mariani, B., Thornhill, A. R., Affara, N., Shaw, M-A. *et al*. (2010) Karyomapping: a universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes. Journal of Medical Genetics. 47, 651-658.

Harton, G.L., Munné, S., Surrey, M., Grifo, J., Kaplan, B., McCulloh, D.H., *et al*. (2013) Diminished effect of maternal age on implantation after preimplantation genetic diagnosis with array comparative genomic hybridisation. Fertility and Sterility, 100, 1695–1703.

Hassold, T., Abruzzo, M., Adkins, K., Griffin, D., Merrill, M., Millie, E., Saker, D., Shen, J. & Zaragoza, M. (1996) Human aneuploidy: incidence, origin and etiology. Environmental and Molecular MutagenesisI, 26, 167-175.

Hassold, T. & Hunt, P. (2001) To err (meiotically) is human: the genesis of human aneuploidy. Nature Reviews Genetics, 2, 35066065.

Hassold, T. & Hunt, P. (2009) Maternal age and chromosomally abnormal pregnancies: what we know and what we wish we knew. Current Opinion in Pediatrics, 21, 703.

Human Fertilisation & Embryology Authority. (2017) PGD conditions licensed by the HFEA. [WWW document] https://www.hfea.gov.uk/pgd-conditions/

Hume, R.F., Kilmer-Ernst, P., Wolfe, H.M., Ebrahim, S., Treadwell, M.C., Johnson, M.P., *et al*. (1995) Prenatal cytogenetic abnormalities: Correlations of structural rearrangements and ultrasonographically detected fetal anomalies. American Journal of Obstetrics and Gynecology, 173, 1334–1336.

Johnson, D.S., Cinnioglu, C., Ross, R., Filby, A., Gemelos, G., Hill, M., *et al*. (2010) Comprehensive analysis of karyotypic mosaicism between trophectoderm and inner cell mass. MHR: Basic science of reproductive medicine, 16, 944–949.

Jung, S.I., Cho, H., Lee, C., Kim, K., Ha, J., Kim, M.-K., *et al*. (2008) Two Cases of Trisomy 19 as a Sole Chromosomal Abnormality in Myeloid Disorders. The Korean Journal of Laboratory Medicine, 28, 174–178.

Kahraman, S., Cetinkaya, M., Yuksel, B., Yesil, M. & Cetinkaya, C.P. (2020) The birth of a baby with mosaicism resulting from a known mosaic embryo transfer: a case report. Human Reproduction, 35, 727–733.

Kalousek, D.K, & Vekemans, M. (1996) Confined placental mosaicism. Journal of Medical Genetics, 33, 529-533.

Kluznik, S. & Slepan, H. (2018) Iceland's abortion policy concerning children with down syndrome: An ethical analysis. Journal of Healthcare and Ethics, 4, 45-48.

Kokkali, G., Traeger-Synodinos, J., Vrettou, C., Stavrou, D., Jones, G.M., Cram, D.S. *et al*. (2007) Blastocyst biopsy versus cleavage stage biopsy and blastocyst transfer for preimplantation genetic diagnosis of β-thalassaemia: a pilot study. Human Reproduction, 22 (5), 1443-1449.

Kort, J.D., McCoy, R.C., Demko, Z. & Lathi, R.B. (2018) Are blastocyst aneuploidy rates different between fertile and infertile populations? Journal of Assisted Reproduction and Genetics, 35, 403–408.

Kotsopoulou, I., Tsoplou, P., Mavrommatis, K. And Kroupis. C. (2015) Non-invasive prenatal testing (NIPT): limitations on the way to become diagnosis. Diagnosis. 2 (3), 141-158.

Kramer, Y.G., Kofinas, J.D., Melzer, K., Noyes, N., McCaffrey, C., Buldo-Licciardi, J., *et al*. (2014) Assessing morphokinetic parameters via time lapse microscopy (TLM) to predict euploidy: are aneuploidy risk classification models universal? Journal of Assisted Reproduction and Genetics, 31, 1231–1242.

Kung, A., Munné, S., Bankowski, B., Coates, A. & Wells, D. (2015) Validation of next-generation sequencing for comprehensive chromosome screening of embryos. Reproductive Biomedicine Online, 31, 760–9.

Kushnir, V.A., Darmon, S.K., Barad, D.H. & Gleicher, N. (2018) Degree of mosaicism in trophectoderm does not predict pregnancy potential: a corrected analysis of pregnancy

outcomes following transfer of mosaic embryos. Reproductive Biology and Endocrinology, 16, 6.

Lee, E., Illingworth, P., Wilton, L. & Chambers, G.M. (2015) The clinical effectiveness of preimplantation genetic diagnosis for aneuploidy in all 24 chromosomes (PGD-A): systematic review. Human Reproduction. 30, 473-483.

Li, Y., Zimmerman, B., Rusterholz, C., Kang, A., Holzgreve, W. and Hahn, S. (2004) Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. Clinical Chemistry, 50 (6), 1002-1011.

Lin, P.-Y., Lee, C.-I., Cheng, E.-H., Huang, C.-C., Lee, T.-H., Shih, H.-H., *et al*. (2020) Clinical Outcomes of Single Mosaic Embryo Transfer: High-Level or Low-Level Mosaic Embryo, Does It Matter? Journal of Clinical Medicine, 9, 1695.

Liu, L., Li, K., Fu, X., Chung, C. And Zhang, K. (2016) A forward look at non-invasive prenatal testing. Trends in Molecular Medicine, 22 (11), 958-968.

Lledó, B., Morales, R., Ortiz, J.A., Blanca, H., Ten, J., Llácer, J., *et al*. (2017) Implantation potential of mosaic embryos. Systems Biology in Reproductive Medicine, 63, 1–3.

Lo, Y.M.D., Lo, E.S.F., Watson, N., Noakes, L., Sargent, I.L., Thilaganathan, B. & Wainscoat, J.S. (1996) Two-way cell traffic between mother and fetus: biologic and clinical implications. Blood, 88 (11), 4390-4395.

Lo, Y.M.D., Corbetta, N., Chamberlain, P.F., Rai, V., Sargent, I.L., Redman, C.W.G. & Wainscoat, J.S. (1997) Presence of fetal DNA in maternal plasma and serum. The Lancet, 350, 485-487.

Mackie, F., Hemming, K., Allen, S., Morris, R. & Kilby. (2017) The accuracy of cell-free fetal DNA-based non-invasive prenatal testing in singleton pregnancies: a systematic review and bivariate meta-analysis. BJOG: An International Journal of Obstetrics & Gynaecology, 124, 32–46.

Magli, M.C., Jones, G.M., Gras, L., Gianaroli, L., Korman, I. & Trounson, A.O. (2000) Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro. Human Reproduction, 15, 1781–1786.

Malvestiti, F., Agrati, C., Grimi, B., Pompilii, E., Izzi, C., Martinoni, L., *et al*. (2015) Interpreting mosaicism in chorionic villi: results of a monocentric series of 1001 mosaics in chorionic villi with follow-up amniocentesis. Prenatal Diagnosis, 35, 1117–1127.

Masuzaki, H., Miura, K., Yoshiura, S., Niikawa, N. & Ishimaru, T. (2004). Detection of cell free placental DNA in maternal plasma: direct evidence from three cases of confined placental mosaicism. J Med Genet, 41, 289-292.

Meij, K.R.M. van der, Sistermans, E.A., Macville, M.V.E., Stevens, S.J.C., Bax, C.J., Bekker, M.N., *et al*. (2019) TRIDENT-2: National Implementation of Genome-wide Non-invasive Prenatal

Testing as a First-Tier Screening Test in the Netherlands. American journal of human genetics, 105, 1091–1101.

Mastenbroek, S., Twisk, M., Echten-Arends, J. van, Sikkema-Raddatz, B., Korevaar, J.C., Verhoeve, H.R., *et al*. (2007) In Vitro Fertilization with Preimplantation Genetic Screening. New England Journal of Medicine, 357, 9–17.

Maxwell, S.M., Colls, P., Hodes-Wertz, B., McCulloh, D.H., McCaffrey, C., Wells, D., *et al*. (2016) Why do euploid embryos miscarry? A case-control study comparing the rate of aneuploidy within presumed euploid embryos that resulted in miscarriage or live birth using next-generation sequencing. Fertility and Sterility, 106, 1414-1419.e5.

McCoy, R.C., Demko, Z.P., Ryan, A., Banjevic, M., Hill, M., Sigurjonsson, S., *et al*. (2015) Evidence of Selection against Complex Mitotic-Origin Aneuploidy during Preimplantation Development. PLOS Genetics, 11, e1005601.

McCoy, R. C. (2017) Mosaicism in preimplantation human embryos: when chromosomal abnormalities are the norm. Trends in Genetics, 33 (7), 448-463.

McCullough, R.M., Almasri, E.A., Guan, X., Geis, J.A., Hicks, S.C., Mazloom, A.R., *et al*. (2014) Non-Invasive Prenatal Chromosomal Aneuploidy Testing - Clinical Experience: 100,000 Clinical Samples. PLoS ONE, 9, e109173.

McFadden, D.E. & Friedman, J.M. (1997) Chromosome abnormalities in human beings. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 396, 129–140.

McLennan, A., Palma-Dias, R., Costa, F.S., Meagher, S., Nisbet, D.L. & Scott, F. (2016) Non-invasive prenatal testing in routine clinical practice – An audit of NIPT and combined first-trimester screening in an unselected Australian population. Australian and New Zealand Journal of Obstetrics and Gynaecology, 56, 22–28.

Minasi, M.G., Colasante, A., Riccio, T., Ruberti, A., Casciani, V., Scarselli, F., *et al*. (2016) Correlation between aneuploidy, standard morphology evaluation and morphokinetic development in 1730 biopsied blastocysts: a consecutive case series study. Human Reproduction, 31, 2245–2254.

Morris JK, Mutton DE, Alberman E. (2002) Revised estimates of the maternal age specific live birth prevalence of Down's syndrome. Journal of Medical Screening, 9 (1), 2-6.

Munne, S., Lee, A., Rosenwaks, Z., Grifo, J. and Cohen, J. (1993) Diagnosis of major chromosome aneuploidies in human preimplantation embryos. Human Reproduction, 8 (12), 2185-2191.

Munné, S., Tang, Y., Grifo, J., Rosenwaks, Z. & Cohen, J. (1994a) Diagnosis of Major chromosome aneuploidies in human preimplantation embryos. Fertility and Sterility, 61.

Munné, S., Weier, H.U.G., Grifo, J. & Cohen, J. (1994b) Chromosome Mosaicism in Human Embryos. Biology of Reproduction, 51, 373–379.

Munné, S. & Wells, D. (2017) Detection of mosaicism at blastocyst stage with the use of high-resolution next-generation sequencing. Fertility and Sterility, 107, 1085–1091.

Munné, S., Blazek, J., Large, M., Martinez-Ortiz, P.A., Nisson, H., Liu, E., *et al*. (2017) Detailed investigation into the cytogenetic constitution and pregnancy outcome of replacing mosaic blastocysts detected with the use of high-resolution next-generation sequencing. Fertility and Sterility, 108, 62-71.e8.

Munné, S., Kaplan, B., Frattarelli, J.L., Child, T., Nakhuda, G., Shamma, F.N., *et al*. (2019) Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. Fertility and Sterility.

Munné, S., Spinella, F., Grifo, J., Zhang, J., Beltran, M.P., Fragouli, E., *et al*. (2020) Clinical outcomes after the transfer of blastocysts characterized as mosaic by high resolution Next Generation Sequencing- further insights. European Journal of Medical Genetics, 63, 103741.

Murtinger, M., Wirleitner, B. & Schuff, M. (2018) Scoring of mosaic embryos after preimplantation genetic testing: a rollercoaster ride between fear, hope and embryo wastage. Reproductive BioMedicine Online.

Nakhuda, G., Jing, C., Butler, R., Guimond, C., Hitkari, J., Taylor, E., *et al*. (2018) Frequencies of chromosome-specific mosaicisms in trophoectoderm biopsies detected by next-generation sequencing. Fertility and Sterility, 109, 857–865.

Natesan, S. A., Handyside, A.H., Thornhill, A.R., Ottolini, C.S., Sage, K., Summers, M.C. *et al*. (2014) Live birth after PGD with confirmation by a comprehensive approach (karyomapping) for simultaneous detection of monogenic and chromosomal disorders. Reproductive BioMedicine Online, 29, 600-605.

Navratil, R., Horak, J., Hornak, M., Kubicek, D., Balcova, M., Tauwinklova, G., *et al*. (2020) Concordance of various chromosomal errors among different parts of the embryo and the value of re-biopsy in embryos with segmental aneuploidies. Molecular Human Reproduction, 26, 269–276.

Neal, S.A., Morin, S.J., Franasiak, J.M., Goodman, L.R., Juneau, C.R., Forman, E.J., *et al*. (2018) Preimplantation genetic testing for aneuploidy is cost-effective, shortens treatment time, and reduces the risk of failed embryo transfer and clinical miscarriage. Fertility and Sterility, 110, 896–904.

Neilson, J. Assessment of fetal nuchal translucency test for Down's syndrome. The Lancet, 350, 754-755.

Neofytou, M. (2020) Predicting fetoplacental mosaicism during cfDNA-based NIPT. Current Opinion in Obstetrics and Gynecology, 32, 152–158.

Newberger, D.S. (2000) Down syndrome: prenatal risk assessment and diagnosis. American family physician, 62(4), 825-832.

Nicolaides, K.H., Snijders, R.J.M., Campbell, S., Gosden, C.M. & Berry, C. (1992) Ultrasonographically detectable markers of fetal chromosomal abnormalities. The Lancet, 340, 704–707.

Niles, K.M., Murji, A. and Chitayat, D. (2018) Prolonged duration of persistent cell free fetal DNA from a vanishing twin. Ultrasound in Obstetrics & Gynecology, 52 (4), 547-548.

Oepkes, D., Page-Christiaens, G.C. (Lieve), Bax, C.J., Bekker, M.N., Bilardo, C.M., Boon, E.M.J., *et al*. (2016) Trial by Dutch laboratories for evaluation of non-invasive prenatal testing. Part I—clinical impact. Prenatal Diagnosis, 36, 1083–1090.

Okmen, F., Ekici, H., Hortu, I., Imamoglu, M., Arican, D., Akın, H., *et al*. (2020) Comparison of indications and results of prenatal invasive diagnostic tests before and after the implementation of the use of cell-free fetal DNA: a tertiary referral center experience. Journal of Assisted Reproduction and Genetics, 1–6.

Opstal, D., Srebniak, M.I., Polak, J., Vries, F. de, Govaerts, L.C., Joosten, M., *et al*. (2016) False Negative NIPT Results: Risk Figures for Chromosomes 13, 18 and 21 Based on Chorionic Villi Results in 5967 Cases and Literature Review. PLOS ONE, 11, e0146794.

Opstal, D.V., Eggenhuizen, G.M., Joosten, M., Diderich, K., Govaerts, L., Galjaard, R., *et al*. (2020) Non-invasive prenatal testing as compared to chorionic villus sampling is more sensitive for the detection of confined placental mosaicism involving the cytotrophoblast. Prenatal Diagnosis.

Ottolini, C.S., Kitchen, J., Xanthopoulou, L., Gordon, T., Summers, M.C. & Handyside, A.H. (2017) Tripolar mitosis and partitioning of the genome arrests human preimplantation development in vitro. Scientific Reports, 7, 9744.

Palmerola, K.L., Vitez, S.F., Amrane, S., Fischer, C.P. & Forman, E.J. (2019) Minimizing mosaicism: assessing the impact of fertilization method on rate of mosaicism after next-generation sequencing (NGS) preimplantation genetic testing for aneuploidy (PGT-A). Journal of Assisted Reproduction and Genetics, 36, 153–157.

Pertile, M.D., Halks-Miller, M., Flowers, N., Barbacioru, C., Kinnings, S.L., Vavrek, D., *et al*. (2017) Rare autosomal trisomies, revealed by maternal plasma DNA sequencing, suggest increased risk of feto-placental disease. Science Translational Medicine, 9, eaan1240.

Pescia, G., Guex, N., Iseli, C., Brennan, L., Osteras, M., Xenarios, I., *et al*. (2016) Cell-free DNA testing of an extended range of chromosomal anomalies: clinical experience with 6,388 consecutive cases. Genetics in Medicine, 19, 169–175.

Pettit, K.E., Hull, A.D., Korty, L., Jones, M.C. and Pretorius, D.H. (2014) The utilization of circulating cell-free fetal DNA testing and decrease in invasive diagnostic procedures: an institutional experience. Journal of Perinatology, 34, 750-753.

Popovic, M., Dheedene, A., Christodoulou, C., Taelman, J., Dhaenens, L., Nieuwerburgh, V.F., *et al*. (2018) Chromosomal mosaicism in human blastocysts: the ultimate challenge of preimplantation genetic testing? Human Reproduction, 33, 1342–1354.

Practice Committee of the American Society for Reproductive Medicine; Practice Committee of the Society for Assisted Reproductive Technology (2018) Blastocyst culture and transfer in clinically assisted reproduction: a committee opinion. Fertility and Sterility, 110, 1246–1252.

Preimplantation Genetic Diagnosis International Society (2016) PGDIS position statement on chromosome mosaicism and preimplantation aneuploidy testing at the blastocyst stage. [WWW document], https://www.pgdis.org/docs/newsletter_071816.html

Qi, S.-T., Liang, L.-F., Xian, Y.-X., Liu, J.-Q. & Wang, W. (2014) Arrested human embryos are more likely to have abnormal chromosomes than developing embryos from women of advanced maternal age. Journal of ovarian research, 7, 65.

Rechitsky, S., Kuliev, A., Sharapova, T., Laziuk, K., Ozen, S., Barsky, I., *et al*. (2006) Preimplantation HLA typing with aneuploidy testing. Reproductive BioMedicine Online, 12 (1), 89-100.

Rienzi, L., Capalbo, A., Stoppa, M., Romano, S., Maggiulli, R., Albricci, L., *et al*. (2015) No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor-prognosis patients: a longitudinal cohort study. Reproductive BioMedicine Online, 30, 57–66.

Robberecht, C., Voet, T., Utine, G.E., Schinzel, A., Leeuw, N. de, Fryns, J.-P., *et al*. (2012) Meiotic errors followed by two parallel postzygotic trisomy rescue events are a frequent cause of constitutional segmental mosaicism. Molecular Cytogenetics, 5, 19.

Roque, M., Lattes, K., Serra, S., Psych, I.S.B., Geber, S., Carreras, R. *et al*. (2013) Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: a systematic review and meta-analysis. Fertility and Sterility, 99, 156-162.

Rosenwaks, Z., Handyside, A.H., Fiorentino, F., Gleicher, N., Paulson, R.J., Schattman, G.L. *et al* (2018) The pros and cons of preimplantation genetic testing for aneuploidy: clinical and laboratory perspectives. Fertility and Sterility, 110 (3), 353-361.

Rubino, P., Tapia, L., Alonso, R.R. de A., Mazmanian, K., Guan, L., Dearden, L., *et al*. (2020) Trophectoderm biopsy protocols can affect clinical outcomes: time to focus on the blastocyst biopsy technique. Fertility and Sterility, 113, 981–989.

Rubio, C., Rodrigo, L., Mercader, A., Mateu, E., Buendía, P., Pehlivan, T., *et al*. (2007) Impact of chromosomal abnormalities on preimplantation embryo development. Prenatal Diagnosis, 27, 748–756.

Rubio, C., Rodrigo, L., Mir, P., Mateu, E., Peinado, V., Milán, M., *et al*. (2013) Use of array comparative genomic hybridisation (array-CGH) for embryo assessment: clinical results. Fertility and Sterility, 99, 1044–1048.

Rubio, C., Bellver, J., Rodrigo, L., Castillón, G., Guillén, A., Vidal, C., *et al*. (2017) In vitro fertilization with preimplantation genetic diagnosis for aneuploidies in advanced maternal age: a randomized, controlled study. Fertility and Sterility, 107, 1122–1129.

Rubio, C., Navarro-Sánchez, L., García-Pascual, C.M., Ocali, O., Cimadomo, D., Venier, W., *et al*. (2020) Multicenter prospective study of concordance between embryo cell-free DNA and trophectoderm biopsies from 1,301 human blastocysts. American Journal of Obstetrics and Gynecology.

Sachdev, N.M., McCulloh, D.H., Kramer, Y., Keefe, D. & Grifo, J.A. (2020) The reproducibility of trophectoderm biopsies in euploid, aneuploid, and mosaic embryos using independently verified next-generation sequencing (NGS): a pilot study. Journal of Assisted Reproduction and Genetics, 37, 559–571.

Shajpal, A. & Siddiqui, F. (2020) Prental screening for common aneuploidies. Obstetrics, gynaecology and reproductive medicine, 30 (11), 337-341.

Shamonki, M.I., Jin, H., Haimowitz, Z. & Liu, L. (2016) Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. Fertility and Sterility, 106, 1312–1318.

Schoolcraft, W.B., Gardner, D.K., Lane, M., Schlenker, T., Hamilton, F. & Meldrum, D.R. (1999) Blastocyst culture and transfer: analysis of results and parameters affecting outcome in two in vitro fertilization programs. Fertility and Sterility, 72, 604–609.

Schoolcraft, W.B., Katz-Jaffe, M.G., Stevens, J., Rawlins, M. & Munne, S. (2009) Preimplantation aneuploidy testing for infertile patients of advanced maternal age: a randomized prospective trial. Fertility and Sterility, 92, 157–162.

Schrurs, B.M., Winston, R.M.L and Handyside, A.H. (1993) Preimplantantion diagnosis of aneuploidy using fluorescent in-situ hydridization: evaluation using a chromosome 18-specific probe. Human Reproduction, 8 (2), 296-301.

Scott, R.T., Ferry, K., Su, J., Tao, X., Scott, K., Treff, N.R. (2012) Comprehensive chromosome screening is highly predictive of the reproductive potential of human embryos. A prospective, blinded, nonselection study. Fertility & Sterility, 97, 870-875.

Scott, R.T., Upham, K.M., Forman, E.J., Hong, K.H., Scott, K.L., Taylor, D., *et al*. (2013) Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly

increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. Fertility and Sterility, 100, 697–703.

Simon, C. (2017) Introduction To transfer or not transfer…a mosaic embryo, that is the question. Fertility and Sterility, 107, 1083–1084.

Snyder, H.L., Curnow, K.J., Bhatt, S. And Bianchi, D.W. (2016) Follow-up of multiple aneuploidies and single monosomies detected by non-invasive testing: implications for management and counseling. Prenatal Diagnosis, 36, 203-209.

Song, Y., Liu, C., Qi, H., Zhang, Y., Bian, X. & Liu, J. (2013) Non-invasive prenatal testing of fetal aneuploidies by massively parallel sequencing in a prospective Chinese population. Prenatal Diagnosis, 33, 700–706.

Spencer, K. (2001) Age related detection and false positive rates when screening for Down's Syndrome in the first trimester using fetal nuchal translucency and maternal serum free βhCG and PAPP-A. BJOG: An International Journal of Obstetrics & Gynaecology, 108, 1043–1046.

Spinella, F., Biricik, A., Minasi, M.G., Varrichhio, M.T., Corti, L., Viganò, P., *et al*. (2019) Impact of chromosomal mosaicism in IVF outcomes: Experience from two hundred mosaic embryos transferred prospectively. Reproductive BioMedicine Online, 39, e17–e18.

Spinella, F., Fiorentino, F., Biricik, A., Bono, S., Ruberti, A., Cotroneo, E., *et al*. (2018) Extent of chromosomal mosaicism influences the clinical outcome of in vitro fertilization treatments. Fertility and Sterility, 109, 77–83.

Steptoe, P.C. and Edwards, R.G. (1978) Birth after the reimplantation of a human embryo. Lancet, 2 (8085), 366.

Taglauer, E.S., Wilkins-Haug, L. & Bianchi, D.W. (2014) Review: Cell-free fetal DNA in the maternal circulation as an indication of placental health and disease. Placenta, 28, S64-S68.

Tang, W., Wu, Y., Liu, J. and Ren, W. (2017) Research Letter: Prenatal diagnosis of low-level trisomy 3 mosaicism. Taiwanese Journal of Obstetrics and Gynecology, 56, 114-115.

Tan, J., Kan, A., Hitkari, J., Taylor, B., Tallon, N., Warraich, G., *et al*. (2018) The role of the endometrial receptivity array (ERA) in patients who have failed euploid embryo transfers. Journal of Assisted Reproduction and Genetics, 35, 683–692.

Taylor, T.H., Stankewicz, T., Katz, S.L., Patrick, J.L., Johnson, L. & Griffin, D.K. (2020) Preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm. Zygote, 1-4.

Theobald, R., SenGupta, S. & Harper, J. (2020) The status of preimplantation genetic testing in the UK and USA. Human Reproduction.

Togneri, F.S., Kilby, M.D., Young, E., Court, S., Williams, D., Griffiths, M.J., *et al*. (2019) Implementation of cell-free DNA-based non-invasive prenatal testing in a National Health Service Regional Genetics Laboratory. Genetics Research, 101, e11.

Van Opstal, D., Srebniak, M.I.,Polak, J., de Vries, F., Govaerts, L.C.P., Joosten, M., Go, A.T.J.I., Knapen, M.F.C.M., van den Berg, C., Diderich, K.E.M. & Gajaard, R.J.H. (2016) False negative NIPT results: Risk figures for chromosome 13, 18 and 21 based on chorionic villi results in 5967 cases and literature review. PLoS One, 11 (1), 1-10.

Vázquez-Diez, C., Yamagata, K., Trivedi, S., Haverfield, J. & FitzHarris, G. (2016) Micronucleus formation causes perpetual unilateral chromosome inheritance in mouse embryos. Proceedings of the National Academy of Sciences, 113, 626–631.

Vera-Rodriguez, M. & Rubio, C. (2017) Assessing the true incidence of mosaicism in preimplantation embryos. Fertility and Sterility, 107, 1107–1112.

Verlinsky, Y., Rechitsky, S., Schoolcraft, W., Strom, C. & Kuliev, A. (2001) Preimplantation diagnosis for fanconi anemia combined with HLA matching. JAMA. 285, 3130-3133.

Victor, A., Viottia, M., McCoy, R., Brake, A., Tyndall, J., Murphy, A., *et al*. (2019a) Aneuploidy concordance between trophectoderm and inner cell mass by next-generation sequencingin 100 blastocysts. Reproductive BioMedicine Online, 38, e15–e16.

Victor, A.R., Tyndall, J.C., Brake, A.J., Lepkowsky, L.T., Murphy, A.E., Griffin, D.K., *et al*. (2019b) One hundred mosaic embryos transferred prospectively in a single clinic: exploring when and why they result in healthy pregnancies. Fertility and Sterility, 111, 280–293.

Walknowska, J., Conte, F.A. & Grumbach, M.M. (1969) Practical and theoretical implications of fetal/maternal lymphocyte transfer. The Lancet, 1, 1119-1122.

Wang, Y., Zhu, J., Chen, Y., Lu, S., Chen, B., Zhao, X., *et al*. (2013) Two cases of placental T21 mosaicism: challenging the detection limits of non-invasive prenatal testing. Prenatal Diagnosis, 33, 1207–1210.

Wang, X., Wu, H., He, X., Jiang, H., Wu, L., Xu, Y., Zhou, P., Wei, Z. & Cao, Y. (2018) Retrospective study to compare frozen-thawed embryo transfer with fresh embryo transfer on pregnancy outcome following intracytoplasmic sperm injection for male infertility. Medical Science Monitor, 24, 2668-2674.

Wellesley, D., Dolk, H., Boyd, P.A., Greenlees, R., Haeusler, M., Nelen, V., *et al*. (2012) Rare chromosome abnormalities, prevalence and prenatal diagnosis rates from population-based congenital anomaly registers in Europe. European Journal of Human Genetics, 20, ejhg2011246.

Wells, D., Sherlock, J.K., Handyside, A.H. & Delhanty, J.D.A. (1999) Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridisation. Nucleic Acids Research, 27 (4), 1214-1218.

Yaron, Y., Pauta, M., Badenas, C., Soler, A., Borobio, V., Illanes, C., *et al*. (2020) Maternal plasma genome-wide cell-free DNA can detect fetal aneuploidy in early and recurrent pregnancy loss and can be used to direct further workup. Human Reproduction, deaa073-.

Zelig, C.M., Knutzen, D.M., Ennen, C.S., Dolinsky, B.M. & Napolitano, P.G. (2016) Chorionic Villus Sampling, early amniocentesis, and termination of pregnancy without diagnostic testing: Comparison of fetal risk following positive non-invasive prenatal testing. Journal of Obstetrics and Gynaecology Canada, 38 (5), 441-445.

Zhang, L., Wei, D., Zhu, Y., Gao, Y., Yan, J. & Chen, Z.-J. (2019) Rates of live birth after mosaic embryo transfer compared with euploid embryo transfer. Journal of Assisted Reproduction and Genetics, 36, 165–172.

9 Publications and activities arising from this thesis

Publications

Sanders, K.D & Griffin, D.K. (2017) Chromosomal Preimplantation Genetic Diagnosis: 25 Years and Counting. *Journal of Fetal Medicine*, **4 (2):** 51-56.

Sanders, K.D & Gordon, A.T. (2019) "Non-invasive prenatal testing" in Woodward, B & Mehta, J. *Female infertility – Core principles and clinical management*. London. New Dehli: JP Medical Publishers, 123-127.

Presentations

Oral presentation, Graduate Induction Week Symposium, University of Kent, Canterbury, UK, September 2017, When should NIPT be carried out after the demise of a twin? Could discordant follow-up NIPT results be an indication for undetected vanishing twin?

Poster presentation, International Society for Prenatal Diagnosis, San Diego, USA, July 2017, **Sanders, K.D.**, Xanthopoulou, L., Kotzadamis, A., Wyatt, M., Pissaridou, S., Golubeva, A., Bakosi, E., Griffin, D.K.& Gordon, A.T. When should NIPT be carried out after the demise of a twin and could discordant follow up NIPT results be an indication for undetected vanishing twin?

Poster presentation, Fertility Conference, Liverpool, UK, January 2018, **Sanders, K.D.**, Xanthopoulou, L., Kotzadamis, A., Wyatt, M., Pissaridou, S., Golubeva, A., Bakosi, E., Griffin, D.K.& Gordon, A.T. Is undetected vanishing twin syndrome causing false positives in NIPT?

Poster presentation, Preimplantation Genetic Diagnosis International Society, Bangkok, Thailand, May 2018, **Sanders, K.D.,** Griffin, D.K., Blazek, J., Large, M. & Gordon, A.T. Mosaic embryos are not the only option for transfer in 90% of PGT-A cases.

Poster presentation, Internation Society for Prenatal Diagnosis, Antwerp, Belgium, July 2018, **Sanders, K.D.**, Xanthopoulou, L., Kotzadamis, A., Wyatt, M., Billington, S., Pissaridou, S., Golubeva, A., Bakosi, E., Ianniello, C., Ali, A., Griffin, D.K.& Gordon, A.T. Re-analysis of non-concordant suspected aneuploid NIPT cases using 24 chromosome software.

Poster presentation, CoGEN congress, Paris, France, November 2018, **Sanders, K.D**., Griffin, D.K., Blazek, J., Large, M. & Gordon, A.T. What proportion of mosaic embryos are suitable for transfer when reviewed using the CoGEN position statement?

Poster presentation, Fertility Conference, Birmingham, UK, January 2019, **Sanders, K.D**., Griffin, D.K., Blazek, J., Large, M. & Gordon, A.T. What proportion of mosaic embryos are suitable for transfer when reviewed using the CoGEN position statement?