Molecular cytogenetic investigation of the origin of chromosomal abnormalities arising in human preimplantation embryos and oocytes

By

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Declaration

I, Anna Mantzouratou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

I dedicate this work to my lovely daughter Eleni

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Abstract

Introduction: Advances in diagnosis and screening of preimplantation embryos or oocytes for chromosomal abnormalities have helped many couples achieve a normal pregnancy. They also pointed to the fact that numerical and structural chromosomal abnormalities are frequent in human preimplantation embryos and can arise at any point during gametogenesis and meiosis through to early embryonic development and mitotic division. However, information coming from studies in this area is far from complete and uniform.

Aim: To investigate aneuploidy and its mechanisms in human preimplantation embryos and oocytes. To develop protocols and improve on existing molecular cytogenetic techniques for the advance of preimplantation genetic diagnosis or screening (PGD/PGS) in routine clinical analysis. To evaluate the impact of PGD and PGS on the treatment of various types of infertility.

Methods: Fluorescent In situ Hybridisation (FISH) and Comparative genomic hybridisation (CGH) were the main methods used. I) Protocols were developed and implemented for the clinical PGD and PGS program. The PGD protocols included 2 couples with rare structural chromosomal abnormalities II) All untransferred embryos were studied and information was obtained for 101 PGS cycles (77 couples-935 embryos) and 18 PGD cycles for structural chromosomal abnormalities. III) Immature and undivided oocytes were studied using CGH from PGS, PGD and routine IVF couples.

Results and discussion: Specific and highly efficient methods and their clinical application to detect a variety of rare and common chromosomal abnormalities in PGD and PGS embryos were achieved. This study adds to the accumulating evidence showing the extent and mechanisms of genetic abnormalities in human oocytes and preimplantation embryos. It is one of the first studies to identify significant differences in the types of chromosomal abnormalities in embryos from couples with different

reproductive history suggesting susceptibility to particular types of aneuploidy in these couples. The problems and effectiveness of PGS and PGD are also discussed.

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List of abbreviations

ADO	Allele dropout
CCD	Charged coupled devise
CGH	Comparative genomic hybridisation
CPM	Confined placental mosaicism
DAPI	4',6'-diamidino-2-phenylindole
Der	Derivative chromosome
DOP-PCR	Degenerate oligonucleotide primed-polymerase chain reaction
ESHRE	European Society for Human Reproduction and Embryology
ET	Embryo transfer
FISH	Fluorescent *in situ* Hybridisation
HFEA	Human Fertilisation and Embryology Authority
ICSI	Intracytoplasmic sperm injection
IVF	*In vitro* fertilisation
MI	First meiotic division
MII	Second meiotic division
PB	Polar body
PCR	Polymerase chain reaction
PGD	Preimplantation genetic diagnosis
PGS	Preimplantation genetic screening
PN	Pronucleus
RCT	Reciprocal translocation
UPD	Uniparental disomy
WGA	Whole genome amplification

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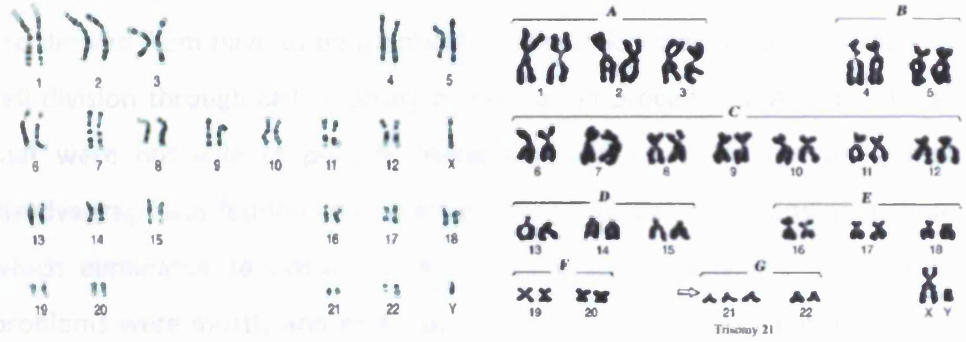
Chapter 1. Introduction

Section 1.1 Studying human chromosomes.

The origins of human cytogenetics, the study of chromosomes, can be traced back to the 19th century in Austria. In 1923, the definitive diploid number of human chromosomes was determined to be 48 but it was not until 1956 that the human chromosome number was correctly identified as 2n=46 by Tjio and Levan (Tjio & Levan, 1956). Once the correct number of human chromosomes was determined various chromosomal abnormalities could be identified by finding the karyotype of affected individuals. In 1959 Down's syndrome was attributed to an extra chromosome 21 by Lejeune, Gautier and Turpin and almost immediately other autosomal trisomies and sex chromosome abnormalities were identified (reviewed in Harper, 2006).

Chromosomes or "coloured bodies" as their name suggests are now widely studied with a variety of methods. Karyotyping is done on chromosomes from cells that have entered the metaphase stage of cell division. Each individual chromosome can be identified as well as various structural chromosomal abnormalities. Following pre-treatment, Giemsa stain is widely used in routine cytogenetics to darkly stain the AT-rich areas of chromosomes producing an individual banding pattern for each chromosome. With this type of banding, called G-banding, on average 300 chromosome bands can be seen on metaphases with normal resolution while high resolution banding can provide 1000 to 2000 bands. It can also provide information about structural chromosomal abnormalities as small as 5Mb in length. There are other types of banding techniques depending on the areas of chromosomes one would like to study and a number of dyes which can be fluorescent or non-fluorescent. These techniques were vital in deciphering the structure of human chromosomes (some examples of this can be found in Schweizer, 1981). Figure 1.1 shows the some examples of Giemsa karyotyping on normal and abnormal samples.

Figure 1.1. Giemsa stain on chromosomes. Left: Karyotype of a normal male. Right: the first karyotype of a male trisomy 21 individual in 1959 (from Smeets 2004).



Currently, there are numerous methods of studying human chromosomes in a variety of cell types ranging from traditional cytogenetic staining of chromosomes to molecular cytogenetic techniques like Fluorescent *In Situ* Hybridisation (FISH) and Comparative Genomic Hybridisation (CGH). In this section a brief introduction to some of those methods and their applications will be provided.

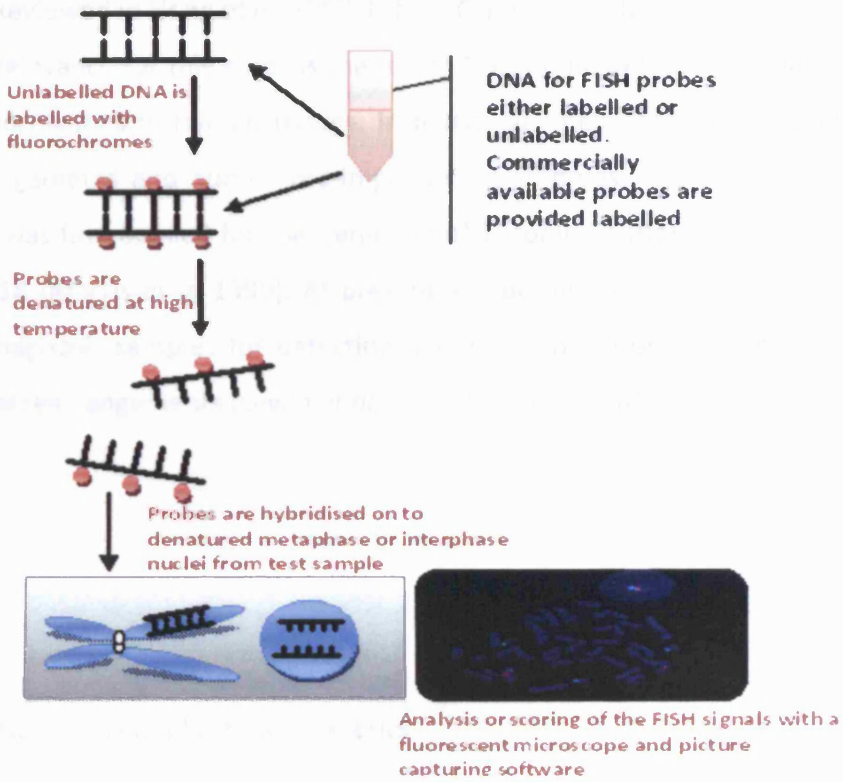
1.1.1. Fluorescent *In Situ* Hybridisation (FISH).

One major disadvantage of the above cytogenetic banding methods is that they require metaphase chromosomes. In order to have these, the cells that the chromosomes are derived from have to be mitotically or meiotically active or to be stimulated to enter cell division through cell culturing or cell fusion procedures. As a result cell populations that were not able to provide metaphase spreads could not be examined. Another disadvantage was found in the relatively low resolution such banding techniques provided which eliminated to possibility of detecting smaller changes in the genome. These problems were mostly addressed by the evolution of Fluorescent In Situ Hybridisation or FISH techniques.

Fluorescent *in situ* hybridisation (FISH) utilizes the complementarily of DNA or RNA strands and the tagging of target DNA with fluorescent probes. The basis of the FISH technique is the detection of specific nucleic acid sequences in cells fixed on a microscope slide. *In Situ* Hybridisation was mainly developed during the 70's and 80's with the use of radioactive RNA and DNA probes. With the utilisation of fluorescent probes it evolved into a powerful technique for studying chromosomes and how they change under certain conditions.

The availability of new fluorochromes and sensitive detection systems have led to the wider use of FISH in order to analyse metaphase or interphase cells and with the advances of molecular techniques and the completion of the human genome project, FISH probes can be made for almost all human DNA sequences. There are mainly three types of DNA probes used for FISH (i) whole chromosome paints that can be used to analyse metaphase chromosomes (ii) repetitive DNA sequences and (iii) locus specific probes that are unique to particular sequences in a chromosome (Kearney & Buckle, 2001). The resolution of FISH based techniques is also much greater since DNA targets from 1kb of DNA in size can be detected. Figure 1.2 illustrates the basic principles of FISH.

Figure 1.2. Basic principle of interphase and metaphase FISH



The creation of whole chromosome probes also lead to the introduction of two fluorescent based karyotyping methods (i) fluorochrome-specific optical filters used for M-FISH; and (ii) interferometer-based spectral imaging (spectral karyotyping or SKY). They are useful for detecting small translocations which are cytogenetically similar in appearance, to classify marker chromosomes and complex chromosomal aberrations (Ried et al, 1998). However, these techniques are not very sensitive in detecting intrachromosomal effects such as deletions and inversions so they are usually combined with standard banding methods.

The remaining two FISH probe types can be used in both interphase and metaphase chromosomes thus allowing analysis of cells that could not be studied in the past. For human cells there are specific FISH probes for each centromere and telomere of each chromosome as well as various locus specific probes commercially available. This availability has lead to the wide use of FISH and FISH based techniques in clinical and

research settings like prenatal diagnosis, cancer genetics, gene mapping and genome structure studies (Reviewed in Heng *et al*, 1997, Lehr & Claussen, 2002).

Of greater relevance for this study is the use of FISH in the detection of numerical and structural abnormalities in human tissues. In particular, their detection in prenatal diagnosis samples, gametes and human pre-implantation embryos. The use of FISH for prenatal diagnosis was first applied for the detection of Trisomy 21 (Romana *et al*, 1993) and later trisomy 18 (Morris *et al* 1999). At present a wide number of FISH probes are used in prenatal diagnosis samples for detecting numerical chromosomal abnormalities and some structural rearrangements (Lewin *et al*, 2000, Pettenati *et al*, 2002).

1.1.1.1 The use of FISH in preimplantation genetics

FISH is used in preimplantation genetic diagnosis (PGD) to detect various chromosomal abnormalities and allows analysis of a single embryonic cell in a limited amount of time (Griffin *et al*, 1991, Harper *et al*, 1994). FISH was initially used for selecting the sex of embryos, to avoid severe X-linked disease (Griffin *et al*, 1994). FISH is also used to detect structural chromosomal abnormalities such as translocations and deletions in preimplantation embryos and for aneuploidy screening of embryos (PGS) from individuals undergoing IVF with poor reproductive history (reviewed in Wells & Delhanty, 2001).

However, there are various limitations for FISH in these clinical settings. The limiting factor for FISH is that at least in interphase cells 3 to 5 chromosomes only can be analysed in each experiment depending on the availability of fluorochromes. Since all the chromosomes cannot be screened with one or two FISH experiments, only those that present an elevated risk in the population are targeted. Rare types of aneuploidy will be

missed if FISH alone is used. Additionally, de novo structural chromosomal abnormalities cannot be detected with FISH only.

Chromosomal polymorphisms can present another source of error. The polymorphisms in various sites of chromosomes can result in a decrease or complete absence of the FISH signal while the chromosome is in fact present, giving false positive results especially when interphase cells are investigated (Tsuchiya et al, 2001, Liehr *et al*, 2002a). In a review by Stumm *et al* 2006, 20 false positive and false negative results were examined since 1998 in prenatal diagnosis samples. Twelve out of the twenty errors were due to heteromorphisms mainly of chromosome X and 18 and three due to mosaicism. The solution in prenatal diagnosis is to combine FISH with G-banding where possible. At the preimplantation stage though where only one or two cells can be tested this is not possible so good preliminary research is needed before the onset of a PGD/PGS cycle (discussed in 1.3).

There are other limitations for FISH diagnosis on single cells. Errors can occur due to the increased levels of chromosomal mosaicism detected in preimplantation embryos (Delhanty *et al*, 1997, Munne, 2002). This means that because only 1 or 2 cells are analysed from each embryo and are diagnosed as normal, if the embryo is mosaic, with chromosomally different cell lines, the diagnosis will not be accurate. Overlapping signals of DNA probes in interphase cells might also lead to similar errors. The probability of misdiagnosis however can be reduced by using 2 cells per embryo for the diagnosis (Kuo *et al*, 1998, Simopoulou *et al*, 2003).

Mosaicism presents a major problem for PGD. A misdiagnosis after FISH PGD for trisomy 21 was attributed to mosaicism following in a trisomy 21 conception (Munne et al, 1999). In some cases, the misdiagnosis rate was estimated to be 7.2% of which 5.6% was attributed to mosaicism (Munne, 2002). PGD for translocation carriers is also complicated by mosaicism since it has been observed that chaotic and mosaic embryos are very frequent in the carriers of translocations that present for PGD (Conn *et al*, 1998, 1999, Iwarsson *et al*, 2000, Simopoulou *et al*, 2000).

Another problem is that the efficiency of hybridisation of probes in interphase FISH is reduced in comparison to that of metaphase FISH (Ruangvutilert *et al,* 2000). Additionally, the hybridisation efficiency is also reduced with each additional probe added to the diagnostic procedure (Harper & Wells, 1999). For these reasons the efficiency of each probe, alone and in combinations, used in each PGD cycle must be calculated on patient and control lymphocyte metaphases and interphases and the stringency conditions of the FISH protocols are adjusted to obtain the best possible results. Informative and efficient probes and probe combinations are then used in embryos.

1.1.1.2. The use of FISH in basic research

Apart from its clinical use, FISH is also a very useful in research. Cancer cells were being studied with FISH from the late 80's (Hopman *et al*, 1988). At present, cancer cells are studied on tissue micro-arrays (TMA) using FISH with computerized signal scoring (Brown & Huntsman, 2007). More relevantly, human gametes and the processes of meiosis (Eckel et al, 2003, Oliver-Bonet *et al*, 2006) were studied with FISH being the preferred method. Untransferred embryos after IVF or PGD have been extensively analysed using various protocols for FISH (Delhanty *et al*, 1997, Daphnis *et al*, 2005, Mantzouratou *et al*, 2007) as well as samples from spontaneous abortions (Jobanputra *et al*, 2002, Lescoat *et al*, 2005).

Interestingly, three dimensional maps of all chromosomes in prometaphase human nuclei have been studied using 3D-FISH (Bolzer et al, 2005). This involved making

chromosome specific paints for all chromosomes by combining different fluorochromes, hybridizing them to prometaphase nuclei and analyzing the results with the help of appropriate computer software. This FISH application has help identify non-random arrangements of gene-dense chromosome territories towards the centre of the nucleus. Three dimensional FISH can possibly allow the identification of all chromosomes in interphase nuclei (Walter et al, 2006) thus allowing maximum information to be obtained even from a single interphase nucleus. Figure 1.3 shows an example of 3D FISH on human fibroblast nuclei.

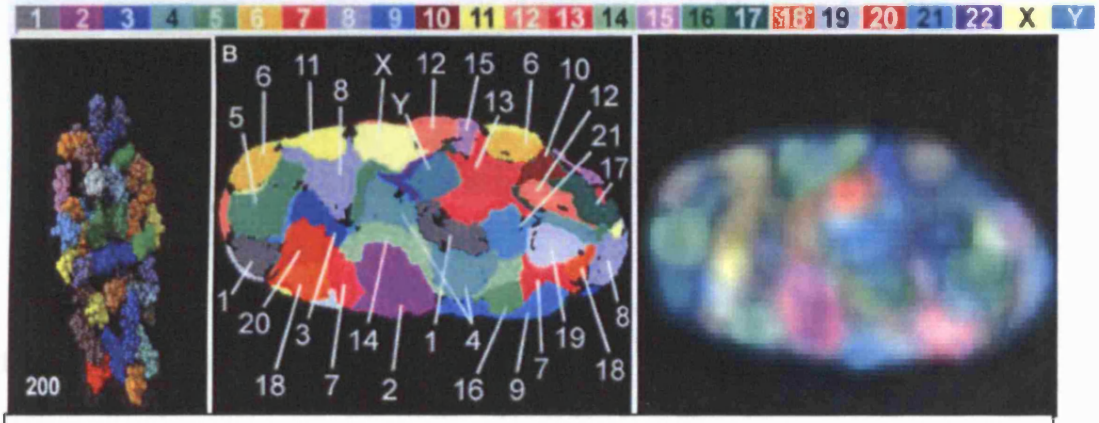
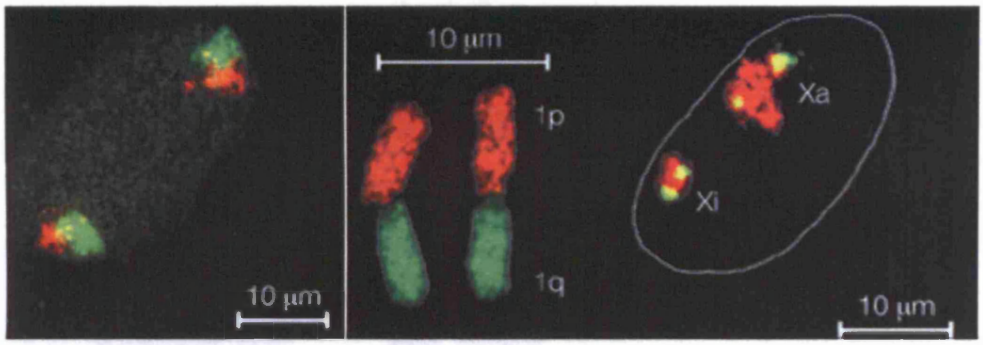


Figure 1.3. Images generated from 3D-FISH on fibroblast prometaphase nuclei *(From Bolzer et al, 2005)*

With the view that each chromosome occupies a distinct chromosome territory in an interphase nucleus, methods were developed to visualize whole chromosome arms or bands in interphase nuclei as well as metaphase ones in combination with telomeric and centromeric probes in human cells (Dietzel *et al*, 1998, Iourov *et al*, 2006). Figure 1.4 shows an example on such experiments in human fibroblast interphase nuclei with localization of the p- and q-arm of the X-chromosome. These techniques provide insight into genome architecture and organization as well as providing information about the mechanisms that produce structural chromosomal abnormalities (reviewed in Cremer & Cremer, 2001). More in the context of this study, in the future, these techniques can be used in embryonic nuclei from preimplantation embryos to allow maximum information

to be drawn about the genetic content of the embryos and the identification of genome organization in this early stage of development.

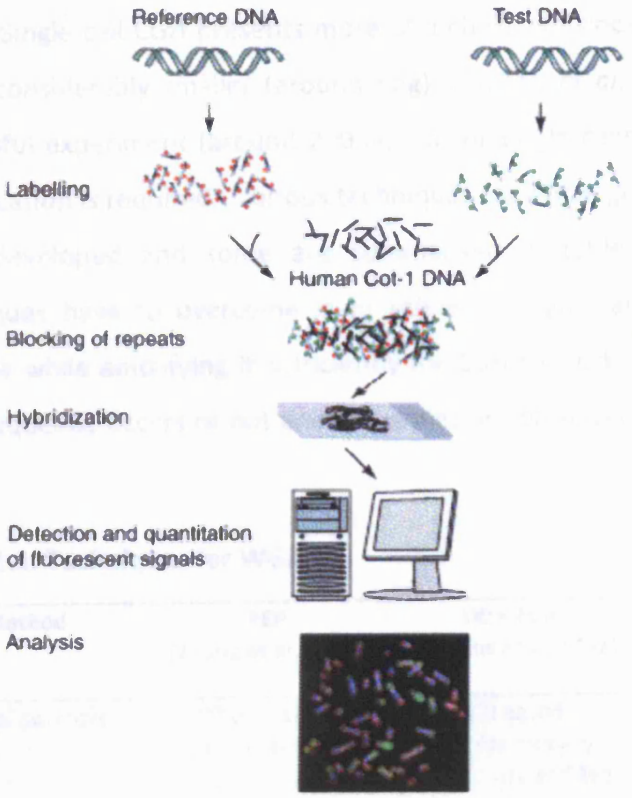
Figure 1.4. FISH with whole chromosome arm microdissection probes in human female fibroblast interphase nuclei identifying the X chromosomes territories and differentiating the inactive from the active X (*From Cremer & Cremer, 2001*).



1.1.2. Comparative Genomic Hybridisation (CGH)

CGH is a technique that allows an overview of the whole genome in a single hybridisation step. It was developed originally for the analysis of solid tumours by Kalioniemi *et al* (1992). It involves differentially labelled test and normal reference DNA which are hybridised simultaneously to normal metaphase spreads. Changes in ratio between the test and reference DNA along a specific chromosome site would be interpreted as deletions or duplications which could represent a monosomy or a trisomy in the tested DNA sample. An overview of the CGH techniques is illustrated in figure 1.5.

Figure 1.5. Basic principles of CGH (*adapted from Mantripragada et al, 2004)*



The advantage of CGH is that it is a DNA based method that can be used in any type of cell whether it is dividing or not, so there is no need for culturing. Its resolution is also greater than standard banding techniques at around 3Mbp (Kirchhoff *et al*, 2000). Also it can all be achieved in a single hybridisation. CGH has been applied to various tissues apart from cancer cells; it was used in cytogenetic analysis to detect unfamiliar or very small chromosomal imbalances (Kirchhoff *et al,* 2000); in spontaneous abortions and prenatal diagnosis samples that could not be analysed with standard cytogenetic methods (Lestou *et al*, 2000, Tabet *et al* 2001). It has also been applied to single cells (Wells *et al* 1999, Klein *et al,* 1999), to single blastomeres from preimplantation human embryos for numerical and structural chromosomal abnormalities (Wells & Delhanty 2001, Voulaire *et al,* 2002, Wells *et al*, 2002, Malmgren *et al*, 2002, Wilton *et al*, 2003) as well as single human oocytes and polar bodies (Guitierrez-Mateo *et al*, 2004, Fragouli *et al*, 2006a).

1.1.2.1 CGH analysis of single cells

Single cell CGH presents more of a challenge since the amount of DNA in a single cell is considerably smaller (around 6pg) (Morton *et al*, 1991) than that required for a successful experiment (around 200ng). So for single cells the additional step of genome amplification is required. Various techniques for whole genome amplification (WGA) have been developed and some are summarised in table 1.1. The problem that these techniques have to overcome is to achieve a significant representation of the whole genome while amplifying it sufficiently for CGH to work. Otherwise, if a bias in a specific DNA sequence occurs or not enough copies are produced then the CGH result will not be valid.

Table 1.1. Techniques for WGA

Method	PEP (Zhang et al, 1992)	DOP-PCR (Telenius et al, 1992)	Linker Adaptor PCR (Klein et al, 1999 for CGH)	MDA (Dean et al, 2002)
General principles	PCR based 15bp random primers Taq polymerase	PCR based Primers partially degenerate and taq polymerase	PCR based Restriction endonuclease, adapter oligonucleotide and ligation to restriction sites. Primer complementary to the adapter.	Isothermal reaction with ϕ29 DNA polymerase, random exonuclease resistant primers at 30°C.
Fragment size produced	450bp	500bp-2kb (Av. 1300bp)	100-1500bp (with MseI)	>10Kb up to 70kb
Amount of DNA produced from a single cell	200pg	Around 1µg	1µg	20-30µg
Single cell genome representation	78-91%	90%	≈92%	80-225%
Applications	PGD, PD for single gene disorders	PGD and PD. CGH and genetic studies on cancer, polar bodies, blastomeres	On single cell analysis and paraffin embedded tissues	PGD for fragile X syndrome, Marfan syndrome. Haplotyping of single cells.
Drawbacks	Amplification bias, ADO, low yield cannot be used for CGH based methods.	Repeat sequences amplification bias	Average fragment size may be too small	Sequence bias, primer dimers are indistinguishable from true product, representation not ideal.

DOP-PCR is the method of choice for CGH since it produces a relatively high amount of DNA from a single cell and offers a good representation of the whole genome. However, amplification bias due to the polymerase slippage in repetitive sequences is observed with DOP-PCR (Wells *et al*, 1999). This produces various artefacts in the heterochromatic and telomeric regions of the amplification products. Multiple Displacement Amplification or MDA is more recently developed and was tried for single cell analysis (Handyside *et al*, 2004). MDA offers a much higher DNA yield and the proofreading properties of the preferred enzyme, ϕ29 polymerase. Additionally, repeat sequences are not prone to biased amplification so MDA can be used in DNA fingerprinting and haplotyping.

However, various problems have been reported with this method (Spits *et al*, 2006). Primer artefacts produced in samples as well as in the negative controls denote that the high yield of MDA is not all true amplified DNA but primer dimers. Allele dropout (ADO), the preferential amplification of one allele over the other and underrepresentation of certain loci are also frequent. Additionally, MDA products are too big to be used for CGH and require digestion prior to DNA labelling. A study comparing MDA and DOP for CGH concluded that DOP-PCR was the most appropriate method for CGH analysis of single cells (Nq *et al*, 2005).

As single cell CGH protocol is time consuming and the hybridisation time is very long (around 72hrs). In a clinical PGD context, with day 3 embryonic nuclei as the starting cells, freezing of the embryo would be needed until the results are obtained. Some PGD groups tried to overcome this obstacle by optimising protocols to work in 30 hours (Wells *et al*, 2002). However, this is not possible in most cases. Testing of the polar bodies would give enough time to avoid the embryo freezing process but polar bodies can only give information on the maternal DNA and any paternal or postzygotic errors would not be detected. Additionally, contamination could be a source of error since only one cell is examined at a time.

However, CGH in a research context is very useful as it can give information on all the chromosomes of certain cells. It has provided a valuable source of information from preimplantation embryos and oocytes by detecting various numerical and structural abnormalities in these cells. One drawback is that it cannot detect balanced chromosomal rearrangements or the ploidy status of the test sample as it is a DNA based method relying on fluorescent ratios.

Another recent advance that is based on CGH is called array-CGH. It is derived from CGH, but instead of metaphase spreads is using human DNA targets constructed on a microarray slide. The resolution of array-CGH is higher at the 1-2Mbp level. This will gradually enable fine chromosomal mapping and identification of new genes as arrays are constructed to provide full genome coverage (Ishkanian *et al,* 2004). Array-CGH has been used to detect minor chromosomal changes in lymphocytes (Veltman *et al,* 2004, Schoumans *et al,* 2005). Also recently, array CGH was used for aneuploidy detection in single cells, like lymphocytes (Gui Hu *et al,* 2004), single fibroblasts and blastomeres (Le Caignec *et al,* 2006).

Section 1.2 Mechanisms of aneuploidy and numerical chromosomal abnormalities

1.2.1. The cell cycle and embryo development

Abnormality in the chromosome number of a cell or aneuploidy can be found in all stages of the human life cycle from gametogenesis and early embryonic development to cancer. Hassold *et al* (1996) estimated that 5% of all human conceptions are aneuploid. This figure is probably an underestimate since data obtained from human oocytes show that the aneuploidy rate at conception can be as high as 20% (Pellestor *et al,* 2002). In addition Hassold *et al* (1996) found that aneuploidy can occur in 0.3% of livebirths and 35% of spontaneous abortions. The specific chromosomes affected were also different in the various stages of development while in liveborns trisomy 21 was most commonly found; in spontaneous abortions (6-20weeks) trisomies of most chromosomes were found with most frequently trisomy 16 to account for one third abnormalities seen. In preimplantation embryos the aneuploidy rates reported are much higher compared to any

other stage of embryonic development and are usually in the range of 50 to 85% (reviewed in, Donoso *et al* 2007). The mechanisms of formation of this level of aneuploidy in humans are being thoroughly investigated and most of the causes of aneuploidy can be located in cell division processes, mitosis and meiosis.

1.2.1.1 Cell division

From any stage in a progenitor cell to the same stage in a daughter cell is called one cell division cycle. It has four stages: S-phase, where DNA synthesis takes place, M-phase, where cell division occurs and in between S and M there are two gaps or intermediate stages called G1 and G2. There are two kinds of cell division: mitosis, which takes place in all dividing cells and meiosis which takes place during gametogenesis.

In normal mitosis the genetic material of a cell is divided equally between two daughter cells. There are five stages for a mitotic cycle to be completed; these are interphase, prophase, metaphase, anaphase and telophase. In interphase the chromosomes are decondenced and form domains. During DNA replication sister chromatid cohesion is established so that the old and new sister chromatids are held together for the onset of mitosis. A multi-subunit complex containing cohesin and numerous other proteins are thought to be involved in this process (examined in detail in Lee & Orr-Weaver, 2001).

During prophase the chromosomes become condensed leading to the prometaphase stage where the nuclear membrane dissolves and the chromosomes start to collect on the equator of the spindle (the metaphase plate).This process was thought to be random but evidence from experiments on mice point to a non-random distribution of newly synthesised and older chromatids at least for some chromosomes in some tissues (Armakolas and Klar, 2006). Before the onset of metaphase, the centrosome, an organelle outside the nucleus, divides and moves to each of the poles of the nucleus. The mitotic spindle forms, sister chromatid cohesion breaks down and the cell enters anaphase where the centromeres via the kinetochores of each chromosome divide and the spindle fibres "drag" the chromatids to opposite poles. Just before sister chromatids separate, the

spindle checkpoint proteins monitor attachment and anaphase does not progress if any of the kinetochores are misaligned (detailed in Craig & Choo, 2005). In telophase, nuclear membranes start to appear around each pole and this is followed by cytoplasmic division to result in two daughter cells.

In normal meiosis, a reduction division takes place that gives rise to haploid daughter nuclei and is restricted to one cell type, the gametes. Meiosis consists of two nuclear divisions meiosis I and meiosis II. Each meiotic division is divided into prophase, metaphase, anaphase and telophase of which the lengthiest is prophase I. Prophase I in also divided into five stages: leptotene, zygotene, pachytene, diplotene and diakinesis.

During zygotene the homologous chromosomes pair through the formation of the synaptonemal complex, a protein structure formed along the length of the pairing region similar to that of the cohesin molecules and can also be found between sister chromatids. In yeast, a molecule called Sgo1 protects the cohesin molecules formed between the centromeres of sister chromatids throughout meiosis I thus keeping them together after the cohesin along the chromosome arms has broken down (Kitajima *et al*, 2004). Deleting this gene in mice results in normal chromosome segregation in meiosis I but random segregation of sister chromatids in meiosis II due to premature loss of cohesion at anaphase I. Whether this is true for human meiosis is not yet known.

In pachytene the pairs condense and thicken. In diplotene the synaptonemal complex disappears and the homologs are now kept together by their crossovers. The crossovers, which occurred during zygotene, are formed from breakage, exchange and reunion between two non-sister chromatids and form structures called chiasmata via a process called recombination. Finally the cells enter metaphase I where each bivalent (pair of chromosomes) takes a position on the equatorial plane like a mitotic division. By the end of meiosis the result is from one diploid parental cell, up to four haploid daughter nuclei are produced.

A checkpoint system is believed to be in place during meiosis in order to avoid genome and chromosomal errors. These molecules guard against abnormal

recombination, DNA damage and chromosomal misalignment on the meiotic spindle (detailed in Borner, 2006). Although checkpoint genes have been found in oocytes (Zhang *et al*, 2005), the observation that most of the autosomal trisomies originate from maternal meiosis has led to the theory that mutations in checkpoint genes may lead to meiotic arrest and infertility in males, whereas in females, the outcome may be a chromosomally abnormal gamete after completing the meiotic divisions (Hassold and Hunt, 2002).

In humans, the germ cells, the precursors of gametes, arise outside the gonads and migrate there during early embryonic development. They start to increase in number by mitosis and they finally become haploid via meiosis. The production of oocytes in the female is called oogenesis and the production of sperm in the male is called spermatogenesis. The female is born with a finite number of oocytes which undergo meiosis very slowly. They first arrest during prophase I were the necessary preparations occur for the potential future embryo. The primary oocytes as these are called remain arrested until puberty where small numbers of them start to progress further with each menstrual cycle. The other oocytes can remain arrested for as long as 50 years. The completion of the first meiotic division happens just before ovulation which results in two unequal daughter cells, the secondary oocyte and the first polar body which degenerates. It is thought that a heterodimer protein called maturation (M-phase) promoting factor (MPF) plays a pivotal role in oocyte maturation and in their exit from prophase I (Jones, 2004). The secondary oocyte enters the second meiotic division and is arrested again at metaphase II. Upon fertilisation the oocyte resumes meiosis and the second meiotic division is completed with the extrusion of the second polar body.

In contrast, meiosis in the males does not begin until puberty and the production of sperm is infinite. When sperm complete meiosis I the result is two equal sized secondary spermatocytes which enter meiosis II immediately and result in 4 haploid spermatids. The total length of human spermatogenesis is 64 days (Carlson, 1999).

1.2.1.2. Fertilisation and embryogenesis

Once the spermatozoon has fused with the oocyte, the entry of other sperm is prevented through a rapid electrical depolarisation of the plasma membrane of the egg and through "hardening" of the zona pellucida caused by a wave of Ca++ that prevents other sperm adhering (Wilding M., 1996). Then the oocyte resumes meiosis and the sperm nucleus starts to decondensate and the female and male haploid pronuclei are formed. DNA replication occurs at this point as the pronuclei move closer to each other and finally come together. The zygote is formed and the first mitotic divisions occur as it travels through the fallopian tube.

Blastomeres, the embryonic cells, following the third cleavage division start to compact and become tightly connected during a process called compaction. Three to 4 days after fertilisation the embryo reaches the 8-16 cell stage, and it is called a morula. By day 5 it reaches the blastocyst stage where the embryo is composed of an inner cavity called the blastocele, an inner cell mass and an outer cell mass called the trophectderm.

Paternal imprinting, the selective activation and silencing of genes according to the parent of origin, is thought to occur during gametogenesis through to the pronuclear stage (Balrlow, 1995). However, it has been found at least for the mouse embryos that the two parental genomes demonstrate a topological separation which was preserved up to the 4 cell stage (Mayer et al, 2000). The authors suggest that this may be associated with epigenetic programming during the early preimplantation stages. Additionally, it has been found that all chromosomes in diploid, triploid and trisomy 21 human cells are incorporated into a single rosette (radial array) throughout mitosis and arranged into tandemly positioned haploid sets in which chromosome spatial order was preserved (Nagele *et al*, 1998). The authors suggest that this arrangement is a remnant of fertilisation and separates the maternal and paternal genomes during mitosis.

Normal development however is not always possible and errors have been found in all stages during the cell cycle, fertilisation, gametogenesis and embryonic development. In the following paragraphs the nature and the mechanisms of these errors will be discussed.

1.2.2. Mechanisms of aneuploidy

1.2.2.1. Errors in fertilisation

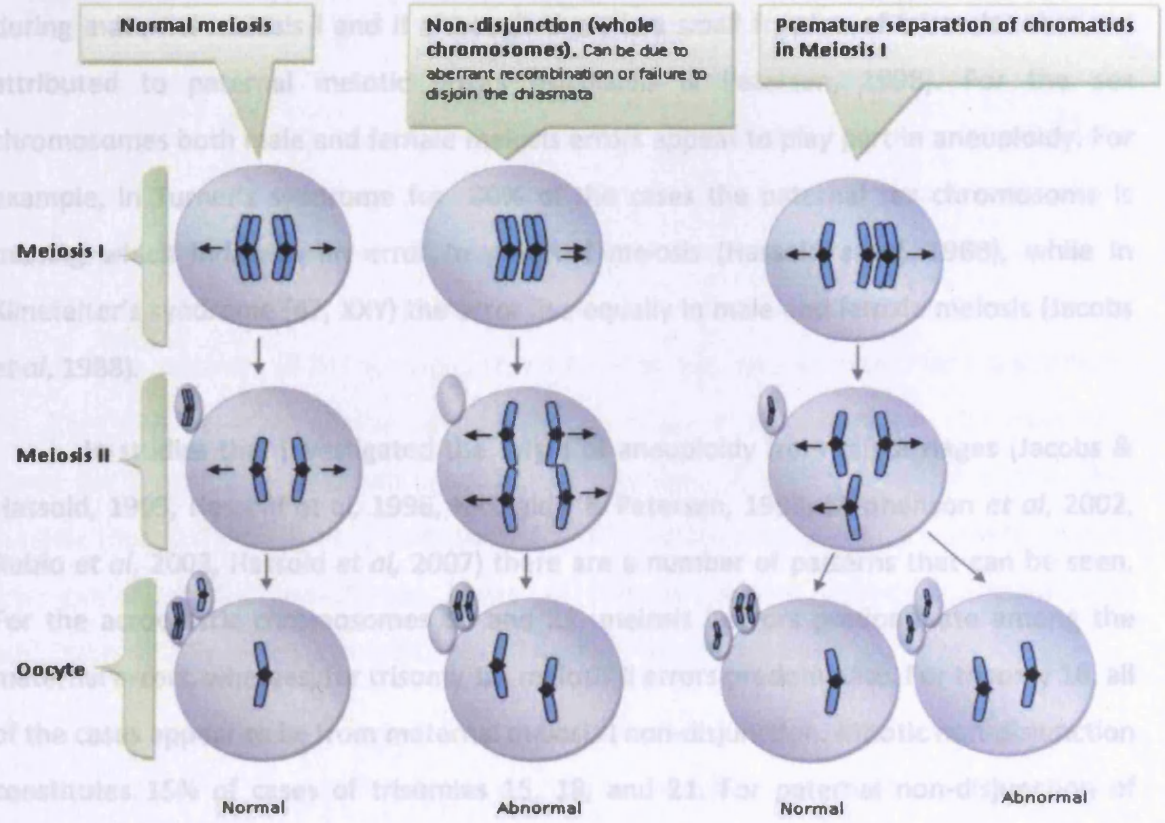
Errors in fertilisation (reviewed in Malan *et al*, 2006) can arise from i) fusion of two different zygotes in a single embryo producing a tetragametic chimera, ii) formation of triploid zygote by dispermy or digyny ether because two sperm fertilised one oocyte, or was fertilised by a diploid sperm or the polar body had not been extruded from the egg, iii) fertilisation of the second polar body and fusion with the oocyte iv) parthenogenetically activated zygotes containing only the maternal genome mitotically divide producing haploid cells. If the sperm pronucleus is also present after the activation of the oocyte, one of the haploid maternal cells can fuse with it producing a diploid cell line while the other maternal cell can undergo endoreduplication and become diploid producing a maternal isodisomic cell line. This mechanism has been proposed to explain the finding of isodisomy in a child with such mosaicism (Strain *et al,* 1995). Androgenetic chimeras have also been reported. Studies from pregnancies with mesenchymal dysplasia of the placenta showed the coexistence of cell lines with complete paternal isodisomy and biparental cells in the placenta (Kaiser-Rogers *et al,* 2006). The authors suggest that the androgenetic cell line arose after failure of division of the female pronucleus followed by endoreduplication and division of the male pronucleus.

1.2.2.2 Errors in meiosis

Errors in meiotic chromosome segregation, generally called non-disjunction, can be formed by a number of mechanisms. The failure to resolve chiasmata or the aberrant or altered recombination between homologous chromosomes during meiosis I will result

in aneuploid gametes (Hassold & Hunt 2001). Additionally, premature division of bivalents during metaphase I will lead to the formation of univalents that can randomly segregate in each pole (Angell *et al,* 1997) and can lead to cells with numerical chromosome abnormalities. Univalents are also predisposed to premature separation of their chromatids, leading to chromatid errors at anaphase I, that may result in aneuploidy gametes after anaphase II. Some examples of the aneuploidy mechanisms in the female meiosis are illustrated in figure 1.6. Anaphase lag is also responsible for the production of abnormalities (Delhanty, 2005). Such errors have usually a devastating effect on the resulting gametes and embryonic development.

Figure 1.6. Female meiosis and mechanisms of aneuploidy via whole chromosome non-disjunction and premature separation of chromatids. Normal gametes can be produced from premature separation of chromatids if the surplus chromatids segregate in the polar body during meiosis II.



Warren *et al* (1987) first noticed reduced levels of recombination in chromosome 21 in trisomy 21 meioses. Further studies in conceptuses with trisomy 21 and other chromosomes showed that absent, reduced or aberrant recombination is involved in non-disjunction of chromosomes by creating univalents susceptible to random segregation (Lamb et al, 1997, Savage et al, 1998). Altogether, it was found that altered recombination patterns are a key feature of most MI division trisomies. More specifically, within homologous chromosomes the susceptibility to abnormal division is associated with the distance between the centromere and the closest exchange (Lamb *et al,* 1997). So the location of certain chiasmata (either proximal or distal to the centromere) makes the bivalent susceptible to non-disjunction. The effect of reduced recombination has also been found in males with abnormal sperm parameters (Rives *et al,* 1999).

Most numerical autosomal anomalies have been found to originate from errors during maternal meiosis I and II although there is a small fraction of trisomies that are attributed to paternal meiotic errors (Nicolaidis & Petersen, 1998). For the sex chromosomes both male and female meiosis errors appear to play part in aneuploidy. For example, in Turner's syndrome for 80% of the cases the paternal sex chromosome is missing which indicates an error in paternal meiosis (Hassold *et al,* 1988), while in Klinefelter's syndrome (47, XXY) the error lies equally in male and female meiosis (Jacobs *et al,* 1988).

In studies that investigated the origin of aneuploidy from miscarriages (Jacobs & Hassold, 1995, Hassold et al, 1996, Nicolaidis & Petersen, 1998, Stephenson *et al,* 2002, Rubio *et al,* 2003, Hassold *et al,* 2007) there are a number of patterns that can be seen. For the acrocentric chromosomes 15 and 21, meiosis I errors predominate among the maternal errors, whereas, for trisomy 18, meiosis II errors predominate. For trisomy 16, all of the cases appear to be from maternal meiosis I non-disjunction. Mitotic non-disjunction constitutes 15% of cases of trisomies 15, 18, and 21. For paternal non-disjunction of chromosomes 18 and 21, meiosis II errors are more frequent. Table 1.2 shows the current knowledge of the origin of errors of individual chromosomes.

Table 1.2. Meiotic origin of human trisomy

Trisomy	Origin (%)				
	Paternal MI	**Paternal MII**	**Maternal MI**	**Maternal MII**	**Postzygotic**
2	28	-	53	13	6
7	-	-	17	26	57
8	-	-	50	50	50
13	3	5	57	34	1
14	-	19	37	37	8
15	-	15	72	9	4
16	-	-	100	-	-
18	-	-	33	59	8
21	2	2	70	24	3
22	2	-	86	10	2
XXY	50	-	25	15	9
XXX	-	-	63	17	20

Adapted from Hassold and Hunt, 2001 and Hassold *et al*, 2007.

More recently the non-disjunction patterns of chromosomes 13 and 22 have been studied. From trisomy 13 conceptuses it was found that the extra chromosomes was maternally derived in 89% of the cases with an equal number of maternal meiosis I and II errors (Bugge *et al*, 2007). The study found that all of the paternal errors originated from meiosis II. They also observed reduced or aberrant (33% of cases) recombination in all cases of chromosome 13 errors. Trisomy 22 was examined in 120 spontaneous abortions by Hall *et al* (2007). In 96% of the cases the extra 22 originated from maternal meiosis and mostly from meiosis I (90%). Reduced recombination was also observed for chromosome 22. They also observed similar patterns in the errors of all acrocentric chromosomes; these are i) over 80% of errors arise during oogenesis, ii) mostly from MI errors. They suggest that there might be chromosome specific factors predisposing to non-disjunction that can affect all chromosomes, groups of chromosomes or individual chromosomes which are directly linked to recombination during meiosis.

The underlying causes of chromosomal non-disjunction in relation to recombination and aneuploidy are not yet fully understood, but certain correlations have been made, for example with advanced maternal age (Eichenlaub-Ritter, 1998). However, the aetiological factors and mechanisms responsible for increases in errors in

chromosome distribution during germ cell formation are still not well understood. In oocytes it is estimated that 20% of all human oocytes, produced by IVF, carry a numerical abnormality (Eichenlaub-Ritter, 1998, Pellestor *et al*, 2005, Fragouli *et al*, 2006a). Additionally, instead of whole chromosome abnormalities in metaphase II oocytes there are also single chromatids, whose presence was elevated with maternal age and which can then segregate randomly in anaphase I (Angell, 1997). This lead to the hypothesis for predisposition to maternal age related aneuploidy via precocious separation of chromosomes during meiosis I. This model requires a susceptible bivalent formed during gametogenesis, a process that is age independent, as gamete formation starts during embryonic development. It also requires another factor that deteriorates with maternal age in order to form aneuploid gametes (e.g. Deterioration of the spindle or of the synaptonemal complex).

Recent studies have found that abnormalities due to predivision of chromatids in the oocytes might exceed those due to non-disjunction (Pellestor *et al*, 2005) and both types of aneuploidy are highly correlated with advanced maternal age. A study using CGH on immature oocytes has shown the same correlation (Guitierrez-Mateo *et al*, 2004). However, other studies on human oocytes using FISH and CGH (Pujol *et al*, 2003, Cupisti *et al*, 2003, Fragouli *et al* 2006a, 2006b) have shown no correlation of aneuploidy with maternal age due to the operation of age independent factors in some younger women in the study group. Additionally, the relationship between recombination and maternal age appears to be a complex one, since it was reported that for chromosome 21, reduced recombination and susceptible chiasmata are observed in trisomy 21 pregnancies from younger women in significantly higher frequency than older ones in meiosis MI non-disjunction while the opposite was seen for meiosis II non-disjunction (Sherman et al, 2006). The authors suggest that multiple mechanisms lead to non-disjunction, some age-dependent and some age-independent.

In Fragouli et al (2006a) CGH was used to detect anomalies in 100 polar bodies and oocytes and found 22% aneuploidy rate. They also found abnormalities were due to whole

chromosome non-disjunction, unbalanced chromatid predivision and chromosome breakage. However, chromatid abnormalities were limited to the smaller chromosomes suggesting either the effect of reduced recombination or that in larger chromosomes increased amounts of cohesions play a role. Steuerwald et al (2007) studied expression profiles of human oocytes and found different expression profiles correlating to changes in maternal age. Mouse studies suggest that failure of histone (DNA binding proteins) deacetylation in older females during meiosis might increase the incidence of errors leading to the maternal age effect of aneuploidy (Akiyama *et al*, 2006). However, the question as to what predisposes older mothers to aneuploidy is still open. Reduced or altered meiotic recombination appears to be involved in aneuploidy but its link to maternal age requires more investigation.

1.2.2.3 Predisposition to aneuploidy

Increasing maternal age has an undisputed link with constitutional embryonic aneuploidy and the risk of various trisomies. This increased aneuploidy is also observed in the preimplantation embryos from couples undergoing IVF but to a much more severe degree than in prenatal studies (Delhanty *et al*, 1997, Munne, 2003, Munne *et al*, 2002,). Although the impact of maternal age is seen in the pregnancy rate in various studies, maternal age is not a major factor affecting the overall frequency of abnormalities seen in preimplantation embryos and oocytes (Bielanska *et al*, 2002, Baart *et al*, 2006, , Fragouli *et al*, 2006a, 2006c, Mantzouratou et al, 2007). Meiotic abnormalities in IVF embryos from older women are not significantly different from those in some of the predisposed younger age groups.

Although older females show higher rates of meiotic aneuploidy in prenatal studies, some younger females going through IVF programs have an almost equally increased chance of chromosomal abnormalities. Conclusively, there might be other parameters either related to IVF processes and/or in these couples genetic makeup that predisposes them to an increased risk of aneuploidy in their gametes or embryos (Warren

& Gorringe, 2006). One factor that increases the risk of aneuploidy in preimplantation embryos and gametes is the existence of gonadal or germinal mosaicism in the parents (Mahmood *et al*, 2000, Somprasit *et al*, 2004).

In preimplantation embryos a wide range of errors has been reported (Ruangvutilert *et al*, 2000, Magli *et al*, 2001, Clouston *et al*, 2002, Mantzouratou *et al*, 2007) where the incidence of chromosomal aneuploidy is thought to be around 50- 80%. Additionally, these studies have shown that a very high proportion of these errors are due to postzygotic events possibly during mitosis in the embryonic cells and to a much lesser degree during meiosis in the gametes. They show that in the preimplantation stage a greater variety of chromosomal abnormalities exist that were not detectable from studies that were done post implantation since most of the abnormal conspectuses would be lost either before the implantation stage or before the stage of recognized pregnancy.

Mantzouratou *et al* (2007 (This study) also found evidence of a genetic predisposition to various aneuploidy mechanisms in couples undergoing PGS that presented with recurrent miscarriage or recurrent implantation failure. Namely, couples with repeated miscarriage (RM) presented with increased meiotic errors in their embryos irrespective of maternal age. Couples presenting with repeated implantation failure (RIF) on the other hand seem to be predisposed to increased postzygotic abnormalities. This is also suggested by another study by Voulairre *et al* (2007) where more complex abnormalities in RIF couples were observed. Another study also found 56.5% aneuploidy rate in preimplantation embryos from RM couples compared with 40% in the control group (X-linked disorders) irrespective of maternal age (Rubio *et al*, 2003). However, this study was without follow up on the untransferred embryos and mosaicism was assessed only after two blastomeres were biopsied. Similarly, meiotic and mitotic errors could not be established. Clearly more detailed studies are needed since it appears that there are some molecular factors that predispose cells to various types of aneuploidy at different stages of development. Although, such factors are not yet elucidated in humans there

many candidate genes that have been found in other organisms which are discussed in the following paragraphs.

Mutations in genes involved in all aspects of cell division can theoretically predispose cells to aneuploidy. Ideal candidates will be the cycle checkpoint genes. In studies looking at male infertility, reduced sperm numbers have been linked to increased chromosomal abnormalities. This might suggest that cell cycle check point proteins might be involved to reduce the number of chromosomally abnormal sperm (Lewis-Jones *et al,* 2003, Mateizel *et al,* 2002). If mutations occur that render these genes inactive, an increase in chromosomally unbalanced sperm will follow.

However, it appears that mammalian female meiosis lacks the metaphase/anaphase checkpoint in experiments done with mice abnormal for chromosome X (LeMaire-Adkins *et al,* 1997). This may explain the high incidence of maternally derived errors in human meiosis but cannot explain the maternal age effect and how normal female meiosis actually works without any fully functioning checkpoints. There is some evidence to suggest that meiotic silencing of unpaired homologues might exist in mammals involving the BRCA1 gene (Turner *et al,* 2005). Additionally, apoptosis analysis of Turner's syndrome ovaries revealed massive apoptosis of oocytes (up to 70% of oocytes) (Modi *et al,* 2003). This means that there is some kind of check in order to detect this abnormality in the gametes, however the study did not include a cytogenetic analysis of the oocytes which would be useful in order to see if the apoptotic oocytes were also chromosomally abnormal.

Additionally, some genes have been identified in mice that interfere with the synaptonemal complex or the meiotic spindle assembly and their loss of function led to aneuploidy. Specifically, in mice lacking the protein SPC3 (synaptonemal complex protein 3) defective meiotic chromosome segregation was observed (Yuan *et al,* 2002). In, female mice mutant for MLH1 (a DNA mismatch repair gene) meiotic recombination was significantly reduced which resulted in unpaired univalents entering anaphase I (Woods *et al,* 1999). Interestingly, mutations in meiosis-specific cohesin protein SMC1β has been

found to exhibit age-dependent defects in mouse meiosis (Hodges *et al*, 2005). If this is true for human oocytes, it would explain some aspects of the maternal age effect. It would provide a good candidate gene to screen for mutations in younger women that produce highly aneuploid gametes and embryos.

Other studies have indicated additional factors that influence gamete formation. Mutations that affect oocyte growth were found to increase the frequency of chromosome misalignment on the meiotic spindle during meiosis II (Hodges *et al,* 2002). Also, in a case report by Schmiady and Neitzel, 2002, they suggest the existence of an autosomal recessive trait that affects the chromatin structure of the oocytes. Additionally, Hodges *et al* (2001), found sexual dimorphism in meiotic chromosome segregation. In the male, remnants of the synaptonemal complex remain associated with the centromeres until anaphase II but in females, all traces of the synaptonemal complex are lost from the chromosomes before the onset of the first meiotic division. This may be relevant to the increased error rates of maternal meiosis.

Interestingly, in an extensive study of recombination events in humans, Cheung *et al* (2007) found large scale individual variation in the number of female and male recombination events. The regions identified as having the largest number of recombination events were at ends of chromosomes but the preferred sites of meiotic recombination events varied greatly among individuals. More recombination events occurred in female than male meiosis with a ratio 1.6:1 but no correlation was found with maternal age. Similar results have been seen in other studies of oocytes and sperm in relation to recombination events (Lenzi *et al,* 2005, Codina-Pascual *et al,* 2006). Since meiotic recombination is so important in the prevention of aneuploid gametes (depending on the number and position of chiasmata) some individuals will produce more favourable recombination patterns while others with fewer recombination events or unfavourable positioning of chiasmata will be at higher risk of producing abnormal gametes.

1.2.2.4 Environmental factors

Environmental factors seem to also affect the production of abnormal gametes and embryos. This was demonstrated when pregnant mice were exposed to synthetic oestrogen Bisphanol A (Susiarjo *et al*, 2007). The oocytes from the exposed female foetuses displayed aberrations in meiotic prophase I thus predisposing them to produce aneuploid gametes even before they were born. However this toxic effect would not have been detected in exposed human foetuses until they reached the reproductive age. Humans are exposed to Bisphenol A (BPA) in their daily lives as it is contained in many everyday products. The questions is does this exposure to BPA put human developing embryos at increased risk of producing abnormal gametes even before their birth. Clearly more research is needed in this area.

Finally, predisposition to aneuploidy in gametes and preimplantation embryos can also relate to IVF procedures which are not part of the natural human reproductive cycle; the conditions that the oocytes, sperm and embryos are subjected to do not resemble a natural cycle. So, IVF conditions play a vital role in this process. There are various problems that were reported relating IVF procedures with genetic abnormalities. Specifically, Intracytoplasmic sperm injection (ICSI) has risen significant concerns regarding the potential for transmission of abnormal genes because many of the natural barriers to conception have been bypassed (Rubio *et al*, 2001, McElreavy & Mitchell, 2002). It has also been suggested that an increased number of chromosomal abnormalities possibly result from the ICSI technique itself (Ludwig *et al,* 2001).

Additionally, frozen thawed embryos present an elevated level of chromosomally chaotic embryos after thawing and in vitro culture indicating a negative impact of cryopreservation of embryos (Salumets *et al,* 2004). These embryos were compared with frozen thawed but not cultured embryos. An earlier study, however, did not show such results (Cobo *et al*, 2001). It could be that the study group of embryos was too small and subject to distortions. Indirect and animal studies (Redding et al, 2006, Carrell *et al*, 2005, Bean *et al*, 2002) also provide some evidence that the IVF process can induce errors in the

early non- human embryos at least when a predisposition to chromosomal instability exists.

Very recently, it was found that milder ovarian stimulation in IVF reduces aneuploidy in human preimplantation embryos while increased stimulation resulted in increased mosaic aneuploidy (Baart *et al*, 2007) which means that hormonal stimulation acting on the ovaries has some effect on the chromosomal complement of the preimplantation embryo and on post-zygotic cell division via a still unknown mechanism. However, Baart *et al* (2007) did not produce any follow up studies of the untransferred embryos and mosaicism was only scored after different results of two biopsied blastomeres of the same embryo were found. Consequently, this study cannot provide any error rates of the PGS protocol that might have contributed to the biopsy results or the existence of true mosaicism in the embryos. Follow up studies of oocytes and embryos after various stimulation protocols will provide more information about the origin of aneuploidy and mosaicism in relation to the IVF setup.

1.2.2.5. Errors in mitosis leading to embryonic mosaicism

Errors in mitosis can occur through mitotic non-disjunction or chromosome loss/ chromosome gain due to anaphase lag. In the preimplantation stage, such errors produce mosaic cell lines with frequency depending on how early in development this error has occurred (Harper *et al*, 1995, Delhanty 2005). In cytogenetic studies done on human preimplantation embryos using interphase FISH with limited probes (Delhanty *et al*, 1997, Ruangvutilert et al, 2000, Bielanska et al, 2002. Baart et al, 2004, Coonen et al, 2004) a high incidence of aneuploidy was detected and about 40% of embryos had mosaic cell lines suggesting one or more postzygotic errors had occurred. Also chaotically dividing embryos were observed where in each of their cells there were different chromosomal defects.

The consequences of chromosomal mosaicism on the normal development of the human preimplantation embryo are unknown. Such a high degree of mosaicism detected in preimplantation embryos has led to the hypothesis that mosaicism is a normal process in early human embryonic development and the mosaic cells might be the precursors of the trophoblastic cells (Benkhalifa *et al*, 1993) although this is not supported by the current evidence. In a mouse tetraploid/diploid mosaic model, the tetraploid cells were segregating to extra-embryonic lineages, with no selective loss of conceptuses (James & West, 1994). It is not known if the tetraploid cells in this mouse model were segregating to the trophectoderm selectively or they were being eliminated from the embryonic lineages. However, in studies of human blastocysts, it appears that no preferential allocation of the aneuploid cells to the trophectoderm exists (Magli *et al*, 2000, Evsikov & Verlinsky, 1998) although this maybe be true for polyploid cells.

Another explanation for such high incidence of mitotic errors in preimplantation embryos could be due to reduced expression of certain cell cycle checkpoint genes (reviewed in Artus *et al*, 2006) that allow the proliferation of aneuploid cells (Handyside & Delhanty, 1995). Some evidence of this comes from cancer cells that also exhibit various mitotic errors due alterations to the function of the mitotic checkpoints (Kops *et al*, 2005). The mutated checkpoint gene loses its function and allows cells with abnormal chromosome alignment to proceed with cell division, thus starting an aneuploid cell line. Additionally, a link has been established between defects in the oocyte and an increased incidence in mitotic segregation errors in a mouse model with an inactivated protein subunit of the meiotic synaptonemal complex (SCP3) (Lightfoot *et al*, 2006). This study revealed an increased level of segregation errors at the first meiotic division but also a substantial increase in mitotic segregation errors during the first embryo cleavage divisions. It was also evident in this study that loss of embryo viability due to mosaicism was caused by the activation of a p53-independent apoptotic mechanism and not from a failure to progress through mitosis.

Additionally, Shi and King (2005), have postulated that chromosome non-disjunction produces tetraploid rather than aneuploid cells in human cancer cell lines. They found that non-disjunction can promote regression of cytokinesis and as a result the production of a tetraploid/ binucleate cell instead of two aneuploid daughter nuclei. Aneuploidy arises after any subsequent divisions. It is thought that cells that become tetraploid after prolonged arrest by the spindle assembly checkpoint but can re-enter G1 as tetraploids, a process called mitotic slippage (Brito & Rieder 2006). In cancer cells the gene that is responsible for the arrest of tetraploid cells (p53) is usually non functional (Fujiwara et al, 2005). The resulting tetraploid cell might present extreme genome instability and erroneous attachments of both sister chromatids to the same poles during any subsequent mitotic divisions, thus producing aneuploid cells (Ganem *et al*, 2007).

The frequent finding of tetraploid cells and diploid binucleated cells in preimplantation embryos and the extreme chromosomal aberrations that are detected do seem to fit in with this tetraploidy first model. This was also suggested for human preimplantation embryos after various spindle abnormalities have been detected (Chatzimeletiou *et al*, 2005). However, definite proof of this would require studies that show tetraploidy as an intermediate of aneuploidy and also the aberrant function of p53 in human preimplantation embryos. The above and also the amplification of centrosomes (as in cancer cells) could also explain the formation of chaotic cell lines in the embryos. The unstable nature of the tetraploid cells and the existence of multipolar nuclei would theoretically produce a highly abnormal set of mitotic divisions.

In conclusion, it is clear there is a variety of mechanisms through which aneuploidy can arise. These can occur at any stage of human development and can affect gametes, preimplantation embryos and somatic cells. The processes that govern cell division, meiosis and mitosis, can be error prone under certain conditions. Additionally, there are a variety of factors either genetic or environmental that produce predispositions to types of aneuploidy in human cells. However, the exact causes of aneuploidy are still not fully understood and clearly more research is needed in this area.

1.2.3. Numerical chromosomal abnormalities

1.2.3.1 Incidence of numerical chromosomal abnormalities in livebirths and spontaneous abortions

Although aneuploidy is affecting 0.3% of livebirths, is one of the main causes of foetal death since around 50% of spontaneous abortions before 15 weeks of gestation are attributed to chromosomal abnormalities with most trisomies of chromosomes 16, 18 and 21 (Nicolaidis & Petersen, 1998). Foetuses with trisomy 13 or trisomy 18 can survive to birth and trisomy 21 is compatible with long term survival accompanied by severe mental retardation and multiple congenital anomalies (Hassold & Jacobs, 1984). Sex chromosome anomalies appear to have a much wider range of viable aneuploidy mostly due the requirement of only one X chromosome in each diploid cell in females and the fact that very few genes are contained on the Y chromosome.

Additionally, aneuploidy can affect all the cells of an individual or particular cell lines resulting in mosaicism which has been documented in various aspects of development. It has been found in the preimplantation stages (Delhanty & Handyside, 1995), in the embryonic stages where the placenta is aneuploid while the foetus is diploid, termed confined placental mosaicism (CPM) (Van Opstal, *et al*, 1998), as well as the adult stages in human development (Cozzi *et al*, 1999, Somprasit *et al*, 2004) in the cases of gonadal mosaicism where the germ cells only appear to be affected by aneuploidy.

Spontaneous abortions have been studied extensively for the understanding of their cause as well as the types and extent of aneuploidy they present. Table 1.3 summarises the main findings of some of these studies. The rate of abnormality ranges from 35% to 72%. The chromosome mostly affected by aneuploidy is 16 as well as X. However in contrast with stillbirths and livebirths, trisomy for all chromosomes have been found and not just 13, 18, 21 and X as complete autosomal monosomy is lethal at earlier stages. A maternal age effect has been observed for some trisomies but not for

monosomy or for chromosome X (Eiben *et al*, 1990). Interestingly, the studies done with interphase FISH revealed a high incidence of mosaicism in spontaneous abortions (Table 1.3).

This had lead to the conclusion that post-fertilisation errors and mosaicism could also contribute to spontaneous abortions to a higher degree than previously thought. Azmanov *et al* (2007) also found that the earliest forms of spontaneous abortions, blighted ovums, also appeared to have higher rates of aneuploidy (50%) compared to more advanced pregnancies. Therefore working backwards from live births, the aneuploidy rate appears to increase exponentially at each step towards the gametogenesis.

Table 1.3. Summary of some cytogenetic studies done on spontaneous abortions.

Study	Method	Results			
		Abnormal	Types of abnormality	Chromosomes affected	Significant findings
Hassold & Jacobs, 1984	Karyotyping Review of 4088 cases	35%	Trisomy 26% Monosomy X 9%	16 most common followed by 22 and 21	Abnormalities of almost all chromosome were been identified
Eiben *et al*, 1990	Karyotyping of 750 cases before 12th week	50.1%	Trisomy 62% Triploidy 12% Monosomy X 11% Tetraploidy 9%	16 (21.8%) 22 (17.9) 21 (10%)	Maternal age effect for trisomies 16, 18, 20, 21, 22
Fritz et al, 2001	CGH in 60 cases with failed culture	72%	Trisomy 68% Triploidy 17% Monosomy X 10%	16 (32%) 7 & 22 (11%) 4, 13, 15, 21 (7%)	Suggest that aneuploidy rate for spontaneous abortions 70%
Lebedev *et al*, 2004	FISH in 60 spontaneous abortions. Studied two tissues from each sample. All chromosomes tested.	53%	Trisomy 50% Sex chromosome aneuploides 13% Triploidy and tetraploidy 9%	No information on individual chromosomes. Found diploid/ aneuploid mosaicism with monosomies 7, 15, 21 & 22.	High frequency of intra tissue and confined placental mosaicism attributed to mitotic errors.
Vorsanova *et al*, 2005	FISH in 148 spontaneous abortions. 11	60%	Aneuploidy 83% Mosaicism	X- Most frequent mosaic aneuploidy.	Mitotic errors are more frequent in spontaneous

	chromosomes		48%		abortions than previously thought.
Azmanov *et al*, 2007	CGH of 106 spontaneous abortions from different gestational ages	38%	Trisomy 40% Monosomy X 25%	16 and X were most frequently affected	Blighted ovums have higher frequency of aneuploidy (50%) compared to other gestational ages.

1.2.3.2. Aneuploidy in oocytes

Aneuploidy in oocytes has been found to be around 20-25% (Delhanty, 2001) although some diagnostic studies of polar bodies have found a rate as high as 62% (Kuliev et al, 2005). Table 1.4 shows a summary of some of the studies done in human oocytes. It appears that non-disjunction of whole chromosomes, unbalanced separation of chromatids and chromosome breakage are all involved in the genesis of aneuploidy during female meiosis I and II. Chromatid errors appear to be the most frequent cause of aneuploidy particularly with advancing maternal age. However, the link between maternal age failed to show up with in some of these studies. This can be due to smaller samples allowing the effects of certain younger females that were predisposed to a particular type of aneuploidy. However, the authors also suggest that there might be a more general age-independent factors that can lead to such meiotic errors. This is also demonstrated in a study by Fragouli et al (2006c) where aneuploidy was found in the oocytes of a 18 year old cancer patient. Other studies have established the link of increased aneuploidy in oocytes. The chromosomes most frequently involved in these abnormalities are most of the smaller ones from the autonomies as well as X. Interestingly, chromosome 21 has been found to be much more frequent in errors when oocytes from women over the age of 40 were investigated (Vialard *et al*, 2006).

There are several problems with studies on IVF oocytes however that leave a lot of gaps in the picture of aneuploidy in meiosis. Firstly, only spectral karyotyping and CGH can give accurate information on the whole chromosome set since G-banding cannot be applied. FISH can only provide information for some abnormalities. Additionally, the

oocytes that have matured in vitro might have been altered by the IVF process itself (Emery et al, 2005, Magli et al, 2006). However, fresh oocytes have been studied and showed similar results to other studies (Sandalinas et al, 2002) but the sample sizes are very small. Additionally, a lot of the oocytes studied for IVF cycles have failed to fertilized or progress to the next stage. This can also present deviation from the natural incidence of aneuploidy for the IVF oocytes. Genetic predisposition to aneuploidy e.g. by gonadal mosaicism or variations in recombination among IVF patients has to be further investigated. Some studies from natural pregnancies also suggest a predisposition to recurrent triploidy of maternal origin, clearly referring to recurrent errors in meiosis in the same individual (Brancatti et al, 2003, Huang et al, 2004) due to unknown reasons.

Table 1.4. Aneuploidy studies in human oocytes.

Study	Method	Results		
		Aneuploidy rates	Types of aneuploidy	Mechanisms of aneuploidy and main findings
Pellestor et al, 2002	R-banding in 1397 IVF oocytes	22%	E and G chromosome groups had higher frequency of aneuploidy than expected. A & B groups- lower	Whole chromosome non-disjunction and chromatid predivision was observed for most chromosomes. Predivision was more frequent. No correlation between aneuploidy rate and type of infertility.
Sandalinas et al, 2002	Spectral karyotyping of 131 fresh oocytes.	22/47 complexes	22 had most frequently errors followed by 21 and 19	Maternal age effect in all aneuploidy mechanisms. Increase of balanced predivision of chromatids with decrease in chromosome size.
Pujol et al, 2003	FISH in 89 1st PBs and 54 oocytes (In Vitro matured). 9 chromosomes	47.5%	16 more frequently affected followed by 13 and 22.	56% whole chromosome alterations 44% chromatid alterations No maternal age effect.
Kuliev et al, 2005	FISH in 4584 IVF polar bodies from advanced maternal age females. 5 chromosomes	62%	21 and 22 with higher error rates. 50% of aneuploidy involved complex errors.	16 and 22 errors arising more frequently in MII. The rest in MI. Chromatid errors significantly higher than chromosome errors. Maternal age affect in all types of aneuploidy.
***Fragouli et al*, 2006b**	CGH in 107 IVF oocyte and PBs (MII).	22%	X and 21 most frequent errors.	All mechanisms observed. More abnormalities in the D-G chromosome groups. No maternal age effect. Chromatid errors confirmed to smaller

				chromosomes.
Vialard et al, 2006	FISH in 141 PBs from advanced maternal age couples	30.5%	21 (20%) 16 (5.6%)	80% of aneuploidy was due to chromatid errors.
Fragouli et al, 2006c	CGH on 14 oocytes and PBs from 18 year-old cancer patient	2/14 complexes	X+21	Aneuploidy due to unbalanced predivision of chromatids. Existence of age independent factors for aneuploidy.

1.2.3.3. Aneuploidy in sperm

At least 2% of sperm from normal men have been found to have numerical chromosomal abnormalities (Martin, 2006). Additionally, all chromosomes appear to be involved in non-disjunction but some studies have indicated that chromosomes 21, 22 and sex chromosomes have an increased frequency of errors (Martin *et al* 1991, Williams *et al* 1993, Sun *et al*, 2006). Structural chromosomal aberrations are also widely observed in the sperm of healthy men (Sloter et al, 2000). Interestingly, it appears that some karyotypically normal males present an elevated aneuploidy risk for all chromosomes in their sperm. This is demonstrated in Tomascik-Cheeseman *et al* (2006) where the male that fathered four consecutive trisomic pregnancies had increased aneuploidy in his sperm.

With the advances in assisted reproduction many infertile men managed to father children that naturally would be an impossible task. However, sperm from infertile men appears to be more prone to aneuploidy than the general population, exhibiting a decreased frequency of recombination (Rives *et al,* 1999) and particularly for chromosomes 13, 18, 21 and the sex chromosomes (Rubio *et al*, 2001, Ma *et al*, 2006). Abnormal morphology in sperm is also associated with increased aneuploidy rates (Lewis-Jones et al, 2003, Morel et al, 2004). These studies show that for couples seeking IVF due to male infertility there are a variety of factors that need to be considered. Although sperm is more available for study than human oocytes, there are still many mechanisms that produce aneuploidy in male gametes that are not fully understood.

1.2.3.4. Aneuploidy in embryos

In preimplantation embryos a more complex genetic picture exists since mitotic as well as meiotic errors can exist. Table 1.5 summarizes some studies performed on preimplantation embryos. The overall aneuploidy rate reported ranges between 30% and 80%. Although some of this variation is mostly due to patient selection, there is no uniformity in the data coming from these studies. This variability could be due to different experimental procedures. The recurrent theme in these studies is mosaicism which is found to be the most common abnormality in preimplantation embryos with frequency ranging from 30% to 70%. The most significant finding clinically, is the co-existence of normal and abnormal cell lines in the same embryo. This can cause various problems for preimplantation genetic diagnosis or screening which are discussed in section 1.4.

Maternal age effect in preimplantation embryos is less obvious, although it does exist (Munne *et al*, 2002, Munne *et al*, 2007). However, a high incidence of aneuploidy has been observed in the embryos of younger women with no indication for genetic screening prior to IVF (Baart *et al*, 2006). All chromosomes seem to be affected but aneuploidy rates for individual chromosomes also vary significantly in these studies. The FISH studies mentioned in table 1.5 mostly screen for the chromosomes that present a high aneuploidy rate in prenatal samples. Within these, chromosome 22, 16, 18 and 21 seem to be most frequently affected. The sex chromosomes do not appear to be affected as severely as in the prenatal studies mentioned above.

The existence of chaotic embryos, where each cell of the same embryo is showing a different chromosomal error, is also confirmed in these studies. Although it is thought of as a patient specific anomaly (Delhanty *et al*, 1997, Mantzouratou et al, 2007) there is still no correlation of chaotic embryos with any significant predisposing factor. It is also possible that chaotic embryos are a result of the *In Vitro* process but studies on this are nonexistent. The main problem is that the human preimplantation embryonic stage can only be examined through IVF and not in natural cycles.

It is clear however that some couples are presenting with an increased risk of having a high rate of abnormal embryos. Evidence is beginning to emerge that couples that have repeated implantation failure (RIF) after IVF are showing increased postzygotic and complex abnormalities in their embryos (Mantzouratou *et al,* 2007, Voullaire *et al,* 2007). This would indicate a predisposing factor to postzygotic aneuploidy that exists in embryos from this particular group of IVF couples. Couples that experience unexplained recurrent miscarriage (RM) are also found to have an increased risk of overall aneuploidy at a rate of 71% (Rubio *et al,* 2003). The reasons for this are not yet fully understood since so few studies are designed to distinguish meiotic from mitotic errors.

Table 1.5. Preimplantation embryo studies

Study	Method	Results		
		Aneuploidy rate	Types of abnormality and chromosome errors	Main conclusions
Studies in routine IVF pre-implantation embryos				
Harper et al, 1995	FISH on 69 day 3 embryos. Chromosomes X, Y 1 and 17 were tested	46% for the autosomes 15% mosaicism for the sex chromosomes. (Different sets of embryos).		Frequent mosaicism in morphologically normal embryos. Consequences on PGD.
Wells and Delhanty, 2000	CGH for 12 day 3 embryos	9/12 embryos contained abnormal cells	8/12 embryos were mosaic. 1/12 uniformly abnormal. 2/12 diploid uniformly	CGH accurate for single cell analysis. Frequent mosaicism and chaotic cell divisions in preimplantation embryos.
Voullaire et al, 2000	CGH for 12 day 3 embryos. 63 blastomeres tested.	10/12 embryos abnormal	42% embryos mosaics with normal/abnormal cell lines	Extensive postzygotic errors. Chromosome loss, gain, breakage and mitotic non-disjunction seen. Chaotic embryos were also observed
Ruangvuil ert et al, 2000	FISH with 5 probes (13, 18, 21, X,Y) in 39 embryos	33/39 embryos abnormal (17/19 blastocysts)	All 33 embryos were mosaic	High incidence of mosaicism of normal/abnormal cells, persisting to the blastocyst stage
Bielanska et al, 2002	FISH with 9 probes (2,7, 13, 16, 18, 21, 22, X, Y) on 216 embryos	70%	48% mosaicism, mostly 2N/polyploidy mosaics.	Incidence of mosaicism increased with cell stage and reached 91% at the blastocyst stage (majority was diploid/polyploidy mosaics)
Baart et	FISH with 10	Day 3- 57%	Lack of correlation of	Day 3 results most

al, 2004	chromosomes (1, 7, 13, 15, 16, 18, 21, 22, X, Y) in 17 embryos from day 3 and day 5 blastomeres	mosaic Day 5- 50% mosaic	aneuploidy and morphology. Cytogenetic confirmation of day 3 abnormality 32% due to mosaicism	reliable if two concordant results were obtained. Analysis of two blastomeres on day 3 for clinical diagnosis is recommended
Daphnis et al, 2005	FISH with 6 probes for 3 chromosomes (1, 11, 18) on 42 embryos	39/42 embryos were mosaic	Predominant type of mosaicism was diploid/aneuploid	Mitotic chromosome loss was the most common finding followed by gain and mitotic non-disjunction. FISH artefacts affecting 5% of nuclei
Coonen et al, 2004	FISH with 3 probes for 3 chromosomes (X, Y, 18) on 299	28%	26% simple mosaicism 31% complex mosaicism 11% chaotic mosaics	Anaphase lagging leading to chromosome loss or gain is the major aneuploidy mechanism for mosaicism
	Studies from embryos on PGD/PGS patients with follow up			
Delhanty et al, 1997	FISH with 3 probes (XY1) in 93 IVF embryos	50%	30% mosaics Mostly ploidy mosaics	Frequent mosaicism observed in embryos from fertile patients. Chaotic embryos were a patient specific finding
Magli et al, 2000	FISH with 6 probes (13, 16, 18, 21, X, Y) on 143 embryos (PGS)	51%	40% of blastocysts were abnormal mosaics. One was uniformly abnormal	High degree of mosaicism. Aneuploid cells do not preferentially move to trophectoderm.
Munne et al, 2002	FISH with 6 probes (13, 16, 18, 21, X, Y) on 1235 embryos (PGS/PGD/IVF) *Not all with follow up.	60%	45% mosaicism not affected by maternal age. Chaotics most common form of mosaicism.	Chromosome 16 was most commonly involved in mitotic non disjunction errors which increased with maternal age. Error rate was 5.6%
Munne et al, 2004	FISH with various probes for different embryos on 2058 embryos (PGS/PGD/IVF) *Not all with follow up.	Chromosome susceptibility Monosomy more common than trisomy. Chromosomes mostly affected 22, 16, 21 and 15. Least common X, Y and 14		Aneuploidy rate increased with maternal age in some autosomes but not X, Y, 1 and 14.
Li et al 2005	FISH with 5 probes (13, 18, 21, X, Y) on 660 embryos (PGS).	43%	60% of blastocysts aneuploid.	False positive rate 7.8%
Baart et al, 2006	FISH with 10 probes on 196 embryos from younger women (no indication for PGS).	64%	50% mosaicism 28% normal/abnormal mosaics. 23% meiotic errors.	Best confirmation rate after diagnosis was based on two cells biopsied on day 3 Mosaicism may affect screening
Mantzou-	FISH with 6 probes	82%	58% chaotic mosaics	Couples with repeated

ratou et al, 2007 (this study)	(13, 15, 16, 18, 21 and 22) on 523 embryos (PGS)		**37%** mosaics **5%** uniformly abnormal **16%** meiotic errors	implantation failure are more prone to post zygotic errors. Errors for chromosome 22 was most common overall followed by 21.
		Studies on PGS embryos with no follow up		
Magli et al, 2001	FISH on 1596 day 3 PGS embryos with 10 different probes.	**66%**	Complex abnormalities most common then monosomies.	Abnormal embryos reached blastocyst stage. Morphological criteria alone are not sufficient selection for poor prognosis patients.
Munne et al, 2007	FISH on 6000 PGS embryos with 9 different probes. *Not all with follow up. Results from 10 years	**70%** <35: 60% 35-37.9: 66% 38-40.9: 78% <41: 80%	No individual abnormalities were studied only normal and abnormal. False positive rate in 1132 reanalysed embryos: **9%**	Aneuploidy increased with maternal age. No differences in normal abnormal ratios according to indication.

Not only the number but the parental origin of chromosomes is important for normal embryonic development. A special case of numerical abnormality is uniparental disomy (UPD), which is the presence of a chromosome pair derived from only one parent in a diploid offspring. UPD has been reported for many human chromosomes and is thought to have an effect due to the presence of imprinted genes the expression of which depends on the parental origin (Morison & Reeve, 1998). UPD is thought to arise from trisomic or monosomic rescue of an aneuploid foetus (Ledbetter and Engel, 1995). Therefore, in the presence of such imprinting, UPD can result in various abnormal phenotypic manifestations in foetuses, placentas and live births (Purvis-Smith *et al*, 1992, Van Opstal *et al*, 1998, Salafsky *et al*, 2001). UPD has been reported for several regions of the genome that have resulted in the inheritance of a recessive trait or the manifestation of an imprinting disorder like Prader-Willi and Angleman syndrome (reviewed in Engel, 2006).

Section 1.3 Structural chromosomal abnormalities

Structural chromosomal abnormalities are usually the result of chromosome breakage and abnormal DNA rejoining thus disrupting the normal DNA sequence in one or more chromosomes. They can be grouped in two categories, balanced or unbalanced chromosomal rearrangements. Balanced structural chromosomal abnormalities include rearrangements where there is no visible loss or gain of genetic material, for example inversions and balanced reciprocal translocations; they are found in about 1 in 500 individuals. Unbalanced chromosome abnormalities include rearrangements such as deletions, duplications and translocations where there is loss and/or gain of genetic material and account for about 3% of all recognized chromosomes anomalies (Shaffer & Lupski, 2000). The frequency of some structural abnormalities in humans are shown in table 1.6

Table 1.6. Frequency of structural chromosomal rearrangements (From Shaffer & Lupski, 2000.

Rearrangement	Frequency in the population
Robertsonian translocations	1 in 1000
Reciprocal translocations	1 in 625
Marker chromosomes	1 in 2000
Terminal deletions	1 in 5000
Interstitial deletions	1 in 4000
Interstitial duplications	1 in 4000

Chromosomal rearrangements can be further grouped into inter- or intra-chromosomal. Interchromosomal rearrangements involve different chromosomes and may occur between non-homologous chromosomes. These include reciprocal and Robertsonian translocations and interchromosomal insertions and inversions. For example Robertsonian translocations involve the acrocentric chromosomes and are very common. Although all acrocentrics have been found to be involved it appears that rob(13q14q) and rob(14q21q) constitute around 85% of all Robertsonian translocations (Therman *et al*, 1989).

In reciprocal translocations all chromosomes have been reported to participate and are thought to occur in a unique way for each carrier. However the translocation t(11;22)(q23;q11.2) is a recurrent reciprocal translocation in unrelated families (Hill *et al*, 2000). Additionally, once recognizable syndromes have been excluded, abnormalities that involved the ends of the chromosomes have been found to be the commonest cause of mental retardation in children with unexplained mental retardation (Knight *et al*, 1999, Menten et al, 2006). In addition, complex chromosomal rearrangements involving more than two chromosomes and/or more than three chromosomal breaks have been documented in balanced and unbalanced forms (Madan et al, 1997).

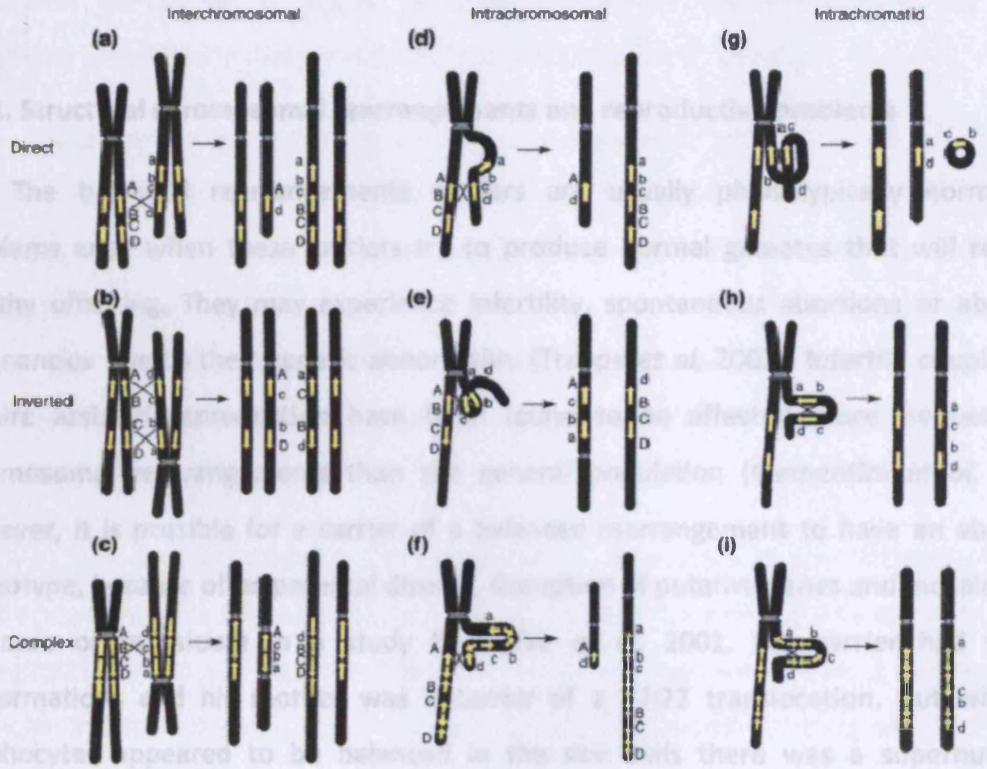
Intrachromosomal rearrangements involve a single chromosome and include duplications, deletions, inversions and marker chromosomes. Some may involve a single homologue and others both homologous chromosomes. It appears that any region of the genome might be subject to rearrangements but certain parts of the genome are more susceptible than others (Brewer *et al*, 1999). Interstitial duplications and deletions usually result from breakage within a chromosome arm and both have been associated with specific genetic syndromes like Di-George syndrome for deletions in chromosome 22 and Charcot-Marie-Tooth disease type 1A for interstitial duplication in chromosome 17 (Shaffer *et al*, 2000).

Inversions have been reported for every human chromosome and the most commonly found is the pericentric inversion of the heterochromatin of chromosome 9 (Shaffer & Lupski, 2000). Balanced chromosomal inversions, are the result of two breaks within a single chromosome and reorientation of the chromatin between the breaks and can be pericentric, which involve the centromere, or paracentric, where only one arm of the chromosome is affected (Therman & Susman, 1993). Although carriers of such inversions are phenotypically normal the inverted chromosome region can cause synaptic and recombinational problems during meiosis and the production of chromosomally unbalanced gametes (Jaarola *et al*, 1998).

Marker chromosomes are usually structurally abnormal chromosomes. The most common marker chromosomes come from chromosomes X, 15 and 22 (Schwartz *et al*, 1997). Isochromosomes are structurally abnormal chromosomes that result from a whole arm duplication within an individual chromosome and their origin is equally divided between paternally derived and maternally derived rearrangements (Shaffer *et al*, 1993). The most common in humans involves the long arm of chromosome X (Wolff *et al*, 1996) and about 15% of individuals with Turners syndrome have an isochromosome of Xq in a population of their cells. Ring chromosomes constitute about 10% of the cases of marker chromosomes and exhibit varying degrees of mitotic instability within individuals thus interfering with any genotype/phenotype correlations (Anderlid et al, 2001, Starke et al, 2003, Jeffries et al, 2005).

The mechanism that produces these structural chromosomal rearrangements is thought to involve i) a number of DNA double strand breaks ii) homology directed sequence repair and interaction of the sequence substrates for recombination iii) resolution of the recombination intermediate with the formation of a novel recombination product. Recombination substrates that are identified so far consist of significant lengths of sequence homology or low copy repeats (LCRs) which would enable abnormal recombination to occur (Stankiewicz & Lupski, 2002) within and between chromosomes or chromatids. Figure 1.7 illustrates the how the LCRs can produce abnormalities during recombination.

Figure 1.7 *Mechanisms of formation of structural chromosomal abnormalities via LCRs. The yellow arrows denote the position of LCRs. Abnormal recombination within these arrears can lead to inversions, duplications, deletions and ring chromosomes among other abnormalities (From Stankiewicz & Lupski, 2002)*



The breakpoints on the common Robertsonian translocations cluster between two repetitive DNA families of satellite III DNA (Page *et al*, 1996).The most common of the Robertsonian translocations have the same breakpoints and are found to arise mainly during oogenesis but can also arise postzygotically (Bandyopadhyay et al, 2002). The authors suggest that the duration and unique nature of oogenesis might predispose to the formation of Robertsonian translocations in the female gametes.

For the reciprocal translocations is more difficult to identify a common factor since most of them are unique to each carrier apart from the 11;22 translocation. Breakpoint studies in unrelated families have shown that this translocation is due to specific Alu repeats on both chromosomes 11 and 22q11.2 (Hill *et al*, 2000, Babcock *et al*, 2007). In another study of this translocation it was detected a non-random asynchronous replication of the 22q11.2 region of the paternal chromosome 22 which, the authors

suggest, increases the probability of an initial mispairing of the parental alleles (Baumer et al, 2004).

1.3.1. Structural chromosomal rearrangements and reproductive problems

The balanced rearrangements carriers are usually phenotypically normal but problems arise when these carriers try to produce normal gametes that will result in healthy offspring. They may experience infertility, spontaneous abortions or abnormal pregnancies due to their genetic abnormality (Trappe *et al,* 2002). Infertile couples that require assisted reproduction have been found to be affected more frequently by chromosomal rearrangements than the general population (Clementini *et al,* 2005). However, it is possible for a carrier of a balanced rearrangement to have an abnormal phenotype, because of uniparental disomy, disruption of putative genes and mosaicism. In the case of mosaicism in a study by Dufke *et al,* 2001, the carrier had various malformations and his mother was a carrier of a 17;22 translocation, but while his lymphocytes appeared to be balanced in the skin cells there was a supernumerary chromosome present.

The chromosomal segregation patterns at meiosis during gametogenesis in a carrier of a structural chromosomal abnormality are thought to determine the formation of a genetically balanced or unbalanced embryo, termed the reproductive risk of the carrier and depends on the size of the rearrangements, the breakpoints and the chromosomes involved (Scriven *et al,* 1998). Sometimes, the sex of the carrier is also included in the reproductive risk since it has been observed that there is an excess in maternal origin abnormalities and maternal age effect (Faraut *et al,* 2000).

The haploid autosomal length (HAL) is a quantitative amount of a particular segmental imbalance and has been used in order to give a more precise risk estimate to carriers of translocations (Neri *et al,* 1983, Davis *et al,* 1985, Cans *et al,* 1993, Cohen et al, 1994, Brewer et al, 1999). In these studies it was found that 96% of the viable imbalances

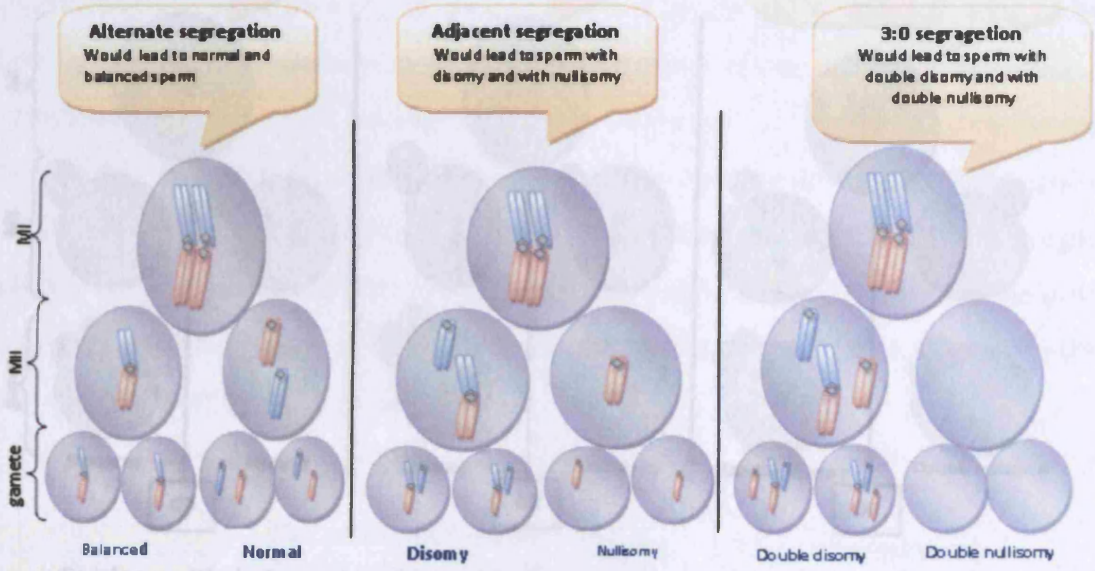
arise for 2-3% of HAL for monosomies and up to 4-5% of HAL for trisomies. However some imbalances demonstrate higher viability thresholds according to their gene content and sometimes the parent of origin. Maternal imbalances are characterized by higher thresholds of viability in monosomy and trisomy (Cohen et al, 1994).

Robertsonian translocation carriers are associated with repeated spontaneous abortions and infertility. For the most common translocation rob(13q14q) there is a 20-25% chance of having a spontaneous abortions because of the translocation (Neri et al, 1983) and for rob(14q21q) there is a 15 % risk of a Down syndrome pregnancy and it is usually due to maternal transmission (Gardner & Sutherland, 1996). In carrier males infertility might arise due to the translocation and can result in spermatogenetic failure (Guichaoua *et al* 1990).

In the case of Robertsonian translocation, at meiosis in the heterozygote the translocated chromosome and the two normal acrocentrics synapse as a trivalent. A 2: 1 segregation can produce up to 6 different types of gametes where only 2 are normal or balanced. Figures 1.8 and 1.9 illustrate the meiotic chromosome pairing and segregation patterns in the male and female carrier of a Robertsonian translocation. Unbalanced conceptuses are essentially trisomic or monosomic for the chromosomes involved referring more to numerical chromosomal abnormalities risk (Gardner & Sutherland, 1996). Sperm studies have shown that at least in males the most common mode of segregation is the one that produced normal or balanced gametes (alternate) and in some carriers other chromosomes were also affected as well as the translocation chromosomes (termed interchromosomal effect)(Ogur et al, 2006).

Figure 1.8. Meiosis in the heterozygote male carrier of a Robertsonian translocation.

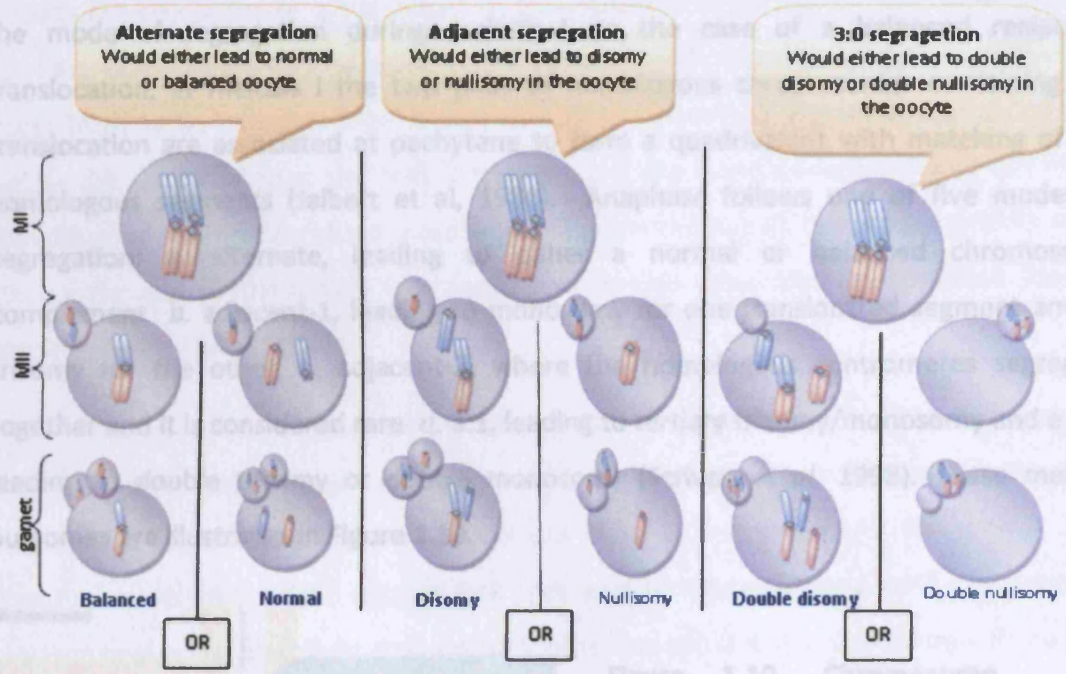
In the alternate segregation 50% of gametes will be normal and 50% carriers. Adjacent and 3:0 segregations will result in unbalanced gametes. Each meiosis will produce four sperm after meiosis II. Errors of meiosis I can also be corrected in meiosis II for some sperm if sister chromatids fail to disjoin (in the case of 2:1 disomy and 3:0 double disomy).



Few studies exist for the segregation patterns in the oocytes due to the difficulty of obtaining such samples. Most data come from preimplantation genetic diagnosis where polar body biopsy is used for the female carriers of Robertsonian translocations. Two such studies showed a variable abnormality rate of around 50% (Durban *et al*, 2001, Munne *et al,* 2000). Figure 1.9 illustrates possible gamete types from meiosis of a female carrier.

Figure 1.9. Meiosis in the heterozygote female carrier of a Robertsonian translocation.

Each meiosis generates one oocyte which can be normal, balanced or unbalanced. Theoretically, errors in meiosis I can be corrected in meiosis II in some cases if the extra products segregate to the polar



Carriers with balanced reciprocal translocations have a higher risk of producing abnormal offspring due to abnormal segregation of chromosomes during meiosis (Jalbert et al, 1980). Reproductive risks for balanced reciprocal translocation carriers are only estimates, and although several risk assessment studies have been done for translocation carriers (Neri et al, 1983, Midro et al, 1992, Barisic et al, 1996) the conclusion is that individual risk estimates have to be performed as the basis of genetic counselling for reciprocal translocation carriers that wish to have offspring.

Carriers of balanced translocations are usually phenotypically normal and thus the translocations are detected when there is the presentation of abnormal offspring due to genetically abnormal gametes, recurrent miscarriage or IVF-implantation failure (Therapel et al, 1985, Stern *et al*, 1999). Couples where one partner is a translocation carrier and that need to resort to PGD maybe predisposed to failure of normal embryo development

and in the formation of abnormal embryos (Conn *et al*, 1998, Iwarsson *et al*, 2000). Additionally, the translocation might be increasing the risk of having offspring with severe congenital malformations and mental retardation if the pregnancy continues.

The inheritance of reciprocal translocations is unpredictable and is determined by the mode of segregation during meiosis I. In the case of a balanced reciprocal translocation, at meiosis I the two pairs of homologous chromosomes containing the translocation are associated at pachytene to form a quadrivalent with matching of the homologous segments (Jalbert et al, 1980). Anaphase follows one of five modes of segregation: a. alternate, leading to either a normal or balanced chromosome complement b. adjacent-1, leading to monosomy for one translocated segment and to trisomy for the other, c. adjacent-2, where the homologous centromeres segregate together and it is considered rare d. 3:1, leading to tertiary trisomy/monosomy and e. 4:0 leading to double trisomy or double monosomy (Scriven *et al*, 1998). These meiotic outcomes are illustrated in Figure 1.10.

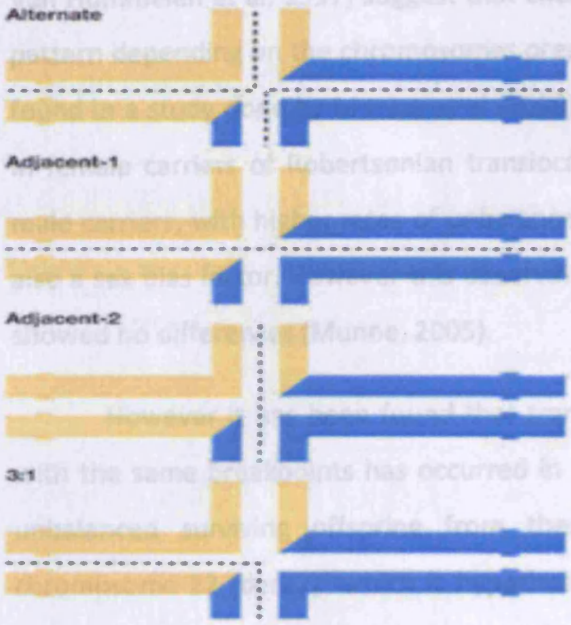


Figure 1.10. Chromosome Segregation outcomes in a reciprocal translocation carrier during meiosis. *The dotted lines denote how the chromosomes can separate during the first meiotic division; 4:0 not shown here will result in all 4 chromosomes in one daughter cell and none in the other.* (from Braude et al 2002)

From studies of individuals with unbalanced chromosomes and spontaneous abortions it is thought that there are some factors that predispose a particular

translocation to a particular segregation pattern. For example although in most cases adjacent 1 is the most common unbalanced segregation that could produce viable pregnancies in some cases adjacent 2 is more frequent, when at least one acrocentric is involved and also chromosome 9 and is nearly always maternal (Jalbert & Sele, 1979). Usually the crucial factor is the length of the translocated fragments, the larger they are the less likely they are to produce viable gametes with adjacent 1 non-disjunction and more likely to be adjacent 2 (Jalbert *et al,* 1980). Chiasmata formation during meiosis I within the translocated or the centric fragments of the translocations can also determine the mode of segregation of the quadrivalent by determining its orientation and its shape during the end of metaphase I. Evidence for this also come from mice models carrying translocations (Tease, 1998).

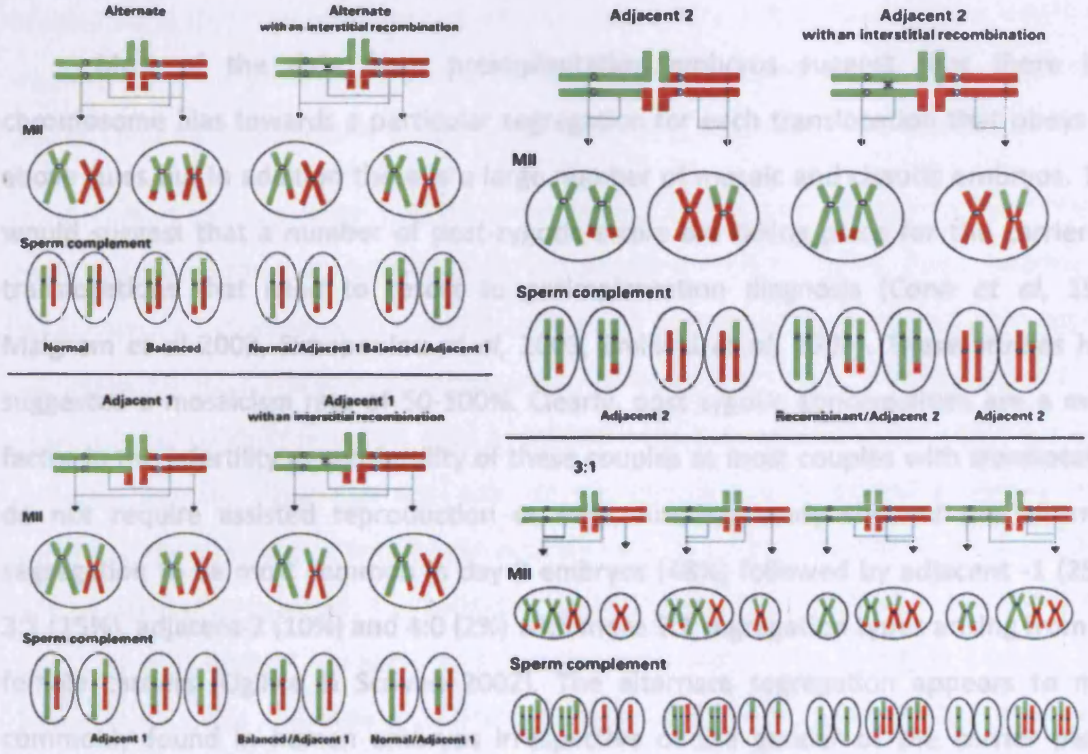
The products of all modes of segregation may be present in gametes but only chromosomally balanced gametes will produce a normal embryo. Studies done on the segregation analysis of various translocations (Zakai & Emanuel, 1980, Estop et al, 1995, Van Hummelen et al, 1997) suggest that each translocation exhibits a distinct segregation pattern depending on the chromosomes present and the size of translocation. It has been found in a study done by Munne et al (2000) that the meiotic segregation patterns found in female carriers of Robertsonian translocations are different from those described in male carriers, with higher rates of unbalanced gametes in females than males, suggesting also a sex bias factor. However this observation has not been confirmed and later studies showed no differences (Munne, 2005).

However it has been found that translocation between chromosomes 11 and 22 with the same breakpoints has occurred in many unrelated families and the majority of unbalanced surviving offspring from these families have an additional derivative chromosome 22 (der22) which is hypothesised to result from a 3:1 segregation in the parental gametes (Zakai & Emanuel, 1980). Although one study performed on human spermatozoa of a t(11;22) carrier supported the above hypothesis for the particular carrier (Estop et al, 1999) another study on male meiosis for the same translocation suggested

that preferential appearance of the extra der22 chromosome constitution is a result of postzygotic selection against other unbalanced karyotypes rather than the 3:1 preferential segregation (Armstrong et al, 2000). That is, of all the unbalanced modes of segregation this one produced the only viable pregnancies.

Studies of human gametes in carriers of reciprocal translocations in have found a varying degree of abnormalities. In sperm the proportion of abnormal gametes ranges widely from 23 to 81%. This figure depends mainly on the size of the imbalance and the individual breakpoints as well as the chiasmata position in meiosis I (Guichaoua et al, 1992, Benet et al, 2005). Figure 1.11 illustrates various 2:2 and 3:1 segregation outcomes in sperm meiosis from a reciprocal translocation carrier. From this it is obvious the even if the alternate segregation occurs, crossing over can still produce unbalanced gametes.

Figure 1.11. Outcomes of the male meiosis from reciprocal translocation carriers *(From Benet et al, 2005)*



In contrast, female meiosis has the added complexity of producing one oocyte form each meiotic division while the polar bodies produced do not play a further role in

reproduction. Hence the random separation of the translocation chromosomes into the oocyte or polar body will also determine the production of a balanced or unbalanced gamete. Oocytes from translocation carriers are more difficult to come by but in a study by Pujol et al (2003) where 1st polar bodies were studied there was a high frequency of unbalanced polar bodies as well as generalized numerical aneuploidy in some cases. The problem with this study however, is that 1st PBs are not always the best indicators of normality or abnormality in the oocyte as meiosis II can change the final outcome. Indeed, chromatid abnormalities of the chromosomes involved in a translocation have been observed in the polar bodies of one translocation carrier as a result of meiotic recombination (Munne et al, 1998). This resulted in metaphase II oocytes having chromosomes with one normal and one derivative chromatid which after meiosis II could result in normal (balanced) or unbalanced oocytes. A further complication is that Escudero *et al* (2000) also found that segregation patterns in oocytes from carriers with similar translocations can vary between individuals even when the breakpoints are similar.

Most of the data from preimplantation embryos suggest that there is a chromosome bias towards a particular segregation for each translocation that obeys the above rules but in addition there is a large number of mosaic and chaotic embryos. That would suggest that a number of post-zygotic errors are taking place for the carriers of translocations that need to resort to preimplantation diagnosis (Conn *et al*, 1998, Malgrem *et al* 2002, Simopoulou *et al*, 2003, Emiliani *et al*, 2003). These studies have suggested a mosaicism rate of 50-100%. Clearly, post zygotic abnormalities are a major factor in the infertility or sub-fertility of these couples as most couples with translocation do not require assisted reproduction or PGD. Another study showed the alternate segregation to be most common in day 3 embryos (48%) followed by adjacent -1 (25%), 3:1 (15%), adjacent-2 (10%) and 4:0 (2%) with more 3:1 segregation types arising from the female carriers (Ogilvie & Scriven 2002). The alternate segregation appears to most commonly found in human embryos irrespective of the gender of the carrier parent (Simopoulou *et al*, 2003) although the high degree of mosaicism makes segregation differentiation at the embryo stage more difficult.

Another interesting suggestion about translocations was that the presence of reciprocal translocations may increase the aneuploidy frequency of chromosomes not involved in the translocation by exerting an interchromosomal effect altering their recombination and segregation pattern (Estop *et al*, 2000). In a study by Pellestor *et al* (2001), it was suggested that this interchromosomal effect appears to be real and should be taken into consideration. In another study by Gianaroli *et al*, 2002, it was concluded that interchromosomal effect seems to play a role in Robertsonian translocations. However, in other studies this interchromosomal effect could not be detected (Oliver-Bonet *et al*, 2004).

Inversions, insertions and duplications carry a reproductive risk that depends again on the size of the fragment and sometimes its gene content. If crossing over occurs within the rearranged regions then it can result in an abnormal chromosomal segment (Gardner & Sutherland, 2004). The reproductive risk for inversions is said to correlate with its size. Studies suggested that inversions of less than 40 or 50% of the chromosome length have significantly less chance of producing unbalanced gametes (Anton *et al*, 2006, Morel *et al*, 2007). This might be due to a lesser chance of crossing over within the inversion.

Insertions can occur interchromosomally, termed insertional translocations and can result in various abnormalities in the gametes (White, 1954). For example, in a heterozygous individual with a small insertional translocation there are two types of pairing in meiosis I. The four chromosomes involved can form two bivalents were they can segregate at random in which case a deleted or a duplicated region may exist in the resulting gamete. They can also pair via the common translocated sequence where recombination can occur. In this case entirely new chromosomes will be produced which will be mostly unbalanced. The incidence of insertional translocation is estimated to be 1:80,000 with 60% of the cases to be of maternal origin (Van Hemel & Eussen, 2000). Interchromosomal insertions specifically, between the chromosome arms, present a high reproductive risk as the recombination during meiosis in a heterozygote carrier will

produce normal/balanced/duplication/deletion outcomes in a 1:1:1:1 ratio (Gardner & Sutherland, 2006).

Ring chromosomes are usually not associated with a normal phenotype due to the high incidence of loss or gain mosaicism that was mentioned earlier. However, some individuals are apparently balanced carriers of stable ring chromosomes and in almost all instances of parent to child transmission this is due to maternal inheritance due to possibly spermatogenic arrest in the male carriers (MacDermot *et al* 1990). A small derivative ring chromosome can also be found either in the balanced or unbalanced and mosaic form in a genome. This can randomly segregate during meiosis thus increasing the risk of unbalanced offspring. The risk for these carriers is very difficult to assess as these are extremely rare events and have to be ascertained according to the individual imbalance.

Finally, complex chromosomal rearrangements provide a particular problem in assessing the reproductive risk of a carrier. When three or more chromosomes are involved a multivalent is formed in meiosis. The size of the imbalance, the position of chiasmata will also determine the segregation mode in this arrangement but the possible combinations in the gametes are not easily predictable as in the simple translocations and very few of the gametes produced will have a balanced chromosomal complement (Siffroi *et al*, 1997). It is usually assumed that most of the unbalanced outcomes would be lethal to the resulting embryos.

1.3.2. Sex chromosome abnormalities

The sex chromosomes need special attention due to the fact that the two homologues are different and that they determine the sex of an individual since females have two X chromosomes, one of which is inactivated, and males have an X and a much smaller Y chromosome.

In terms of numerical abnormalities for the sex chromosomes there are 4 major types. 45, X and 47, XXY carriers are mostly infertile while 47, XXX and 47, XYY appear to have normal fertility but all have variable and usually mild phenotypic effects (Hall et al, 2006). This is because one X in the female is inactivated early in embryogenesis. It is initiated by the X inactivation centre (XIC) on Xq13 and spreads in both directions and inactivates the whole chromosome apart from a few pseudoautosomal regions that help pairing with the Y chromosome (Ballabio et al, 2006). In addition, the Y chromosome carries only a few genes for gender determination.

Turner Syndrome involves a missing X or Y chromosome and it was found that in around 80% of the cases the paternal sex chromosome is lost (Jacobs et al, 1997). Paternal meiotic errors also appear to predominate in the other sex chromosome aneuploides in contrast with the errors in the autosomes which mostly originate from maternal meiosis. This implies that male meiosis is more prone to sex chromosome errors although it is not clear entirely why that is. There is evidence that reduced recombination in spermatogenesis between the terminal regions of the X and Y chromosomes leads to sex chromosome abnormalities (Martin 2005).

Translocations between a sex chromosome and an autosome complicate matters even more than with two autosomes. This is mostly because of the spread of X inactivation into the autosomal segments. Additionally, translocations involving the sex chromosomes can be associated with variable sexual phenotype (Sharp *et al*, 2004). For example in the carrier of an X-autosome translocation the derivative X will carry a portion of the autosomal genes and vice versa. If the derivate chromosome is inactivated there is a danger that it will switch off the autosomal genes or if the X inactivation centre has been moved to the autosome it will inactivate genes there.

The reproductive risks for such carriers is that, even in the case that their offspring is a balanced carrier, if female it might suffer from abnormalities due to inactivation of autosomal genes (Waters *et al*, 2001) or if it is male, he might be infertile due to lack of pairing with the X during spermatogenesis (Ashley, 2002, Lee *et al*, 2003). Some studies

show that the spread of inactivation in the autosomal region is not extensive therefore not severe or that the cell lines with the normal X inactive are usually predominant and most viable but cases have been reported where the derivative X was mainly inactive with very severe consequences (Glaser *et al,* 2004). On the other hand, when a carrier is unbalanced for an X;autosome translocation, a mild phenotype could result due to the inactivation of the extra chromosomal regions (Stankiewicz et al, 2006). However further investigation is needed in order to calculate the reproductive risks for each carrier since the phenotype of the offspring cannot be predicted with any certainty.

Section 1.4. Preimplantation genetic diagnosis and screening (PGD and PGS)

PGD involves the diagnosis of a known genetic condition before an embryo is implanted in the uterus and has been developed as an alternative to prenatal diagnosis in patients at high genetic risk (undergoing IVF treatment), to increase their chance in having a normal pregnancy and avoid termination of pregnancy (Handyside & Delhanty, 1997). The aim is to ensure that only genetically balanced embryos are selected for implantation. PGS is a more general screen of embryos for various abnormalities of the chromosomes most likely to be involved in aneuploidy. PGS is used for patients with normal karyotypes but who are at high risk of producing abnormal gametes and embryos.

The indications for PGS/PGD usually involve a poor reproductive history or affected children (ESHRE PGD consortium, 2000). Patients seeking PGD usually fall into three categories (i) objection to termination of an affected pregnancy, (ii) genetic risk coupled with low fertility, (iii) patients that have undergone previous terminations of pregnancy (Wells & Delhanty, 2001). The advantages of selecting and transferring genetically normal embryos are the avoidance of termination of an affected pregnancy and secondly that for aneuploidy PGD can increase the implantation rate and livebirth rate in routine patients

experiencing implantation failure or recurrent miscarriages (Gianaroli *et al,* 1997, Munne, 2003).

The first successful pregnancies after PGD were reported by Handyside et al, (1990) where PGD was used to select the sex of the preimplantation embryos in order to avoid X-linked inherited diseases. Since then PGD has been applied to various genetic conditions including single gene disorders and chromosomal abnormalities as well as aneuploidy screening. According to the European Society of Human Reproduction and Embryology (ESHRE) PGD consortium (2003) over 9000 cycles of PGD have been performed for various genetic conditions (Sermon *et al*, 2007). The success rate of PGD however is limited firstly by the success rate of IVF procedures, secondly by the technical difficulties encountered in making a diagnosis and thirdly because of the best quality embryos may be affected with the disorder.

However, there are ethical considerations to PGD and PGS since it involves human embryos and their selection. There are several groups of people who disagree with its application for religious reasons or the fear of a eugenic approach to reproduction. Attitudes towards embryo research and PGD vary across Europe (Viville & Pergament, 1998) and the ethical dimension of PGD (Beylveld, 2000) and the moral status of the preimplantation embryo (ESHRE Task Force on ethics and law, 2001) are at the centre of great debate.

1.4.1. Technical aspects of PGD/PGS

There are two main stages to the PGD procedure; embryo biopsy, where a sample is obtained directly from the embryo, and the diagnosis, where the embryonic material is subjected to genetic tests. Embryo biopsy can be undertaken at three main stages: (i) Polar body biopsy by sampling the first and the second polar body of the oocyte/zygote (Verlinsky & Kuliev, 1996), (ii) cleavage stage biopsy by removing 1 or 2 blastomeres from the 6-10 cell stage embryo and this is the most commonly used procedure (Handyside *et*

al, 1990) and (iii) blastocyst biopsy where cells of the trophectoderm are removed (Dokras *et al*, 1990). There are problems and advantages associated with each of the above methods but it is not clear yet which of the above methods is the least detrimental to normal embryo development. Technical advances in embryo biopsy methods will make the procedure safer and more robust.

The diagnosis is performed by two main methods, FISH and Polymerase Chain Reaction (PCR) and sometimes Comparative Genomic Hybridisation (CGH) is used. FISH is used to detect structural and numerical chromosomal abnormalities, such as aneuploidy, translocations, cytogenetically visible deletions and the sex of the embryos. PCR is used to detect mainly single gene disorders (reviewed in Findlay, 2000). Diagnosis from single cells requires extreme sensitivity and both the above procedures require a great degree of accuracy and reliability in order to be used clinically. Time for the diagnosis is also limited to a maximum of 48hrs since the embryos must be transferred by day 5/6 post fertilisation to allow for their successful implantation. Both FISH and PCR protocols used in PGD/PGS must allow for this limitation.

The diagnosis or screening of polar bodies has been applied clinically for aneuploidy using FISH (Munne *et al*, 1995a, Verlinsky and Kuliev, 1996, Magli *et al*, 2004, Montag *et al*,2005) or CGH (Wells *et al*, 2002, Sher *et al*, 2007) and for single gene disorders of maternal origin (Verlinsky and Kuliev, 1996). The removal of polar bodies does not seem to affect embryo viability since it is a by-product of meiosis and plays no further part in reproduction. The genetic information that the polar body can confer directly relates to the oocyte content so an accurate diagnosis can be achieved.

There are several problems however. First, only the maternal genome is being looked at, so paternal and postzygotic abnormalities will not be detected unless subsequent biopsy on the embryo is performed (Magli *et al*, 2004, Cieslak-Janzen *et al*, 2006). Secondly, for a diagnosis to be accurate, both PB1 and PB2 have to be studied so errors in both meiotic divisions can be detected. Finally, the degradation of the polar body after meiosis I can result in loss of chromosomal material or DNA damage that will hinder

the production of a conclusive result. Still, polar bodies provide an alternative to blastomere biopsy in cases were maternal origin errors are suspected e.g. maternal age related aneuploidy or where law restrictions do not allow embryo biopsy.

Blastocyst biopsy has the added advantage of more cells to test but limits the time for the production of the results unless the normal embryos are frozen and used later. However, it has been applied successfully in clinical PGD for all indications (McArthur *et al,* 2005). The biopsy, genetic analysis and embryo transfer have to done on the same day so the implantation window will not be missed. This time limitation leaves no room for errors or reanalysis of the biopsied material. Secondly, mosaicism arising between the trophectoderm and the inner cell mass will not be detected. However, blastocyst biopsy can alleviate the problems of single gene diagnosis using PCR based methods by increasing the genetic material available and decreasing the error rate of mutation detection (Kokkali *et al,* 2007).

Day 3 embryo biopsy (at the 6-8 cell stage) allows for one or two cells of the embryo to be tested and provides information for meiotic or postzygotic errors. Most centres use blastomere biopsy for their PGD or PGS as it is not as time limiting as blastocyst biopsy. The main arguments against day 3 biopsies involve the removal of cells at such an early stage of embryo development which might impair further development. The removal of one or two cells during biopsy is also being debated. Although two cells offer greater information about abnormalities and mosaicism, the removal of two cells may be detrimental to the embryo thus cancelling the beneficial effects of PGD (Emiliani *et al,* 2004, Michiels *et al,* 2006).

For PGD that requires FISH, blastomeres need to be fixed onto a microscope slide (Coonen et al, 1994, Harper et al, 1994) which also presents with different technical difficulties due to the delicate manipulations required in the movement of the single cell. There are three methods for blastomere fixation on slides that mostly use either Tween 20, 3:1 methanol acetic acid or both. Studies comparing these methods (Dozortsev & McGinnis, 2001, Velilla et al, 2002) concluded that using a combination of Tween 20 and

methanol acetic acid is the best option. However, such comparisons done by one person who might be used to a specific fixation method are not entirely objective. In addition, methanol /acetic acid fixation cannot be used in a room where embryo biopsy is taking place. Fixing single blastomeres correctly with any method is a delicate procedure and it requires certain amount of training. As with the biopsy, all fixation methods have their advantages and disadvantages.

PGD with PCR or CGH requires blastomeres to be placed into a tube and subjected to multiple cycles of amplification of certain DNA sequences or of the whole genome of the cell. There are various considerations mainly having to do with contamination. A completely contamination-free environment has to exist from the time of the biopsy to the time of DNA amplification. Since only one cell is placed in each tube a contaminant cell from maternal cumulus cells or sperm or from the PGD team will lead to a false result. These considerations and other aspects of single cell PCR are discussed in the next section

1.4.2. PGD and single gene disorders

PGD based upon PCR is a very versatile technique mainly because certain PCR protocols can amplify the minute quantities of DNA present in one blastomere. It is used to identify single gene disorders in a variety of ways. The first application for PGD using PCR involved sexing of preimplantation embryos by selectively amplifying a Y-chromosome sequence (Handyside et al, 1990). Sexing by this method however proved to be less accurate since it screened for the presence of Y only and failure of amplification of the Y marker would result in a false female result (Kontogianni et al, 1996). Most centres at present use FISH for the sexing of embryos (Griffin *et al* 1991, Griffin *et al,* 1994).

Since then PCR has been used to detect a variety of single gene disorders by using different approaches to mutation detection depending on the nature of the mutation. Therefore PCR based techniques have been used to identify embryos with Huntingdon's disease, Myotonic dystrophy, Fragile-X syndrome (Sermon *et al,* 1998, Sermon *et al* 2001, Jasper et al, 2006) that amplified triplet DNA repeats that are characteristic for these diseases. Also tested was sickle cell anaemia (Xu et al, 1999), thalassemias (Kuliev *et al,*

1998) and inherited cancer predisposition like familial adenomatous polyposis coli and retinoblastoma (Ao *et al*, 1998, Xu *et al*, 2004, Moutou et al, 2007). PCR protocols include heteroduplex analysis (Handyside et al, 1992), analysis of size of the DNA fragment (Sermon *et al*, 1998), single strand conformational polymorphisms (SSCP) (Ao et al, 1998), minisequencing (Fiorentino *et al*, 2006) and haplotyping with MDA whole genome amplification (Renwick *et al*, 2006).

However, there are several problems with single cell PCR for PGD mainly due to the fact that amplification of such minute quantities of DNA present a higher probability of misdiagnosis due to contamination with foreign DNA, amplification failure or allele dropout. Contamination from cumulus cells, sperm or other DNA presents a major problem in single cell analysis. To decrease the probability of parental contamination intracytoplasmic sperm injection (ICSI) is used, and to avoid maternal contamination cumulus cells must be stripped from the zona pellucida before analysis (Harper & Wells, 1999). Allele dropout is another problem, referring to the preferential amplification of one of the two alleles in the cell and can lead to misdiagnosis (Wells & Sherlock, 1998).

Various strategies have been employed in order to avoid some of the above problems. These include the use of multiplex PCR which involves simultaneous amplification of the mutation site and an informative DNA polymorphism that is inherited with the disease and so there are two ways of detecting if a mutation is present (Xu et al, 1999) although this strategy is not always possible due to lack of informative polymorphic markers. Additionally, whole genome amplification discussed previously (Wells *et al,* 1999, Renwick *et al,* 2006) has been used in order to maximise the information taken from a single cell and to reduce the probability of a misdiagnosis. In general, diagnosis based upon PCR offers great potential but has been problematic. Technological advances in this area can increase the sensitivity and accuracy of single cell analysis.

1.4.3. Preimplantation Genetic screening (PGS)

FISH is used to detect various chromosomal abnormalities and has the advantage of allowing interphase cell analysis in a limited amount of time (Harper et al, 1994). FISH was initially used for selecting the sex of embryos, to avoid severe X-linked disease (Griffin *et al*, 1994). It is now also used to investigate aneuploidy in preimplantation embryos for patients going through routine IVF to try to improve pregnancy rates (Munne *et al,* 1995). Originally, this involved FISH probes to detect chromosomes 18, 13, 21, X and Y which account for 95% of all postnatal chromosome abnormalities. Autosomal probe sets to include chromosomes 16 and 22 have also been developed for clinical use (Munne et al, 1999).

The problem with chromosomal screening in preimplantation embryos is that relatively few chromosomes can be detected at any one time due to various technical limitations of FISH mentioned in section 1.1.1. Results are usually based on one or two biopsied cells and follow up of the untransferred embryos is not carried out in most centres. So for screening purposes in PGS only those chromosomes that are deemed at high risk of error are being checked, for example chromosomes 13, 16, 18, 21, 22 and X, Y).

There are two ways of thinking about the number of chromosome to screen for a PGS program. One is that the more chromosomes are screened the better the chances of a subsequent pregnancy since most chromosomal abnormalities will be eliminated. The other is that the efficient screening of the chromosomes mostly affected by aneuploidy is the best approach. With the first method more chromosomes are tested in a single and subsequent hybridizations but the efficiency of the FISH procedure drops while the error rate increases. With the second approach 3 or 4 probes are used in each hybridization which does not compromise the efficiency of FISH but of course some chromosomal abnormalities will be missed.

Different centres use different approaches for PGS and up to 9 chromosomes can being screened for routinely, using FISH; There have been reports of 10, 13 and 15

chromosomes have been screened in single cells (Abdelhadi *et al*, 2003, Baart *et al*, 2004a, Baart *et al*, 2007a). Most centres rely on commercially available probe sets like the PB MultiVysion probe set developed by Vysis (Abbott) that simultaneously tests for five autosomes (13,16,18, 21 & 22) plus additional hybrisidisations with X and Y probes, or alternatively the Vysis Aneuvision probe set which includes chromosomes 13, 18, 12, X and Y.

As mentioned in section 1.1.1, there are problems in using multiple probes in order to achieve accurate diagnosis in a single blastomere, including decreased hybridisation efficiency and overlapping signals. The FISH error rate has also been calculated to be around 5% when three FISH probes are used in the same experiment (Daphnis *et al*, 2005), while the false positive rate for monosomy in embryonic nuclei when using FISH has been found to be around 4% (Cooper *et al*, 2006). For these reasons strict scoring criteria must be used in order to increase the accuracy of the FISH diagnosis (Hopman *et al*, 1988, Munne *et al*, 1998b). Furthermore, polymorphic chromosomal regions can present additional limitations in PGS as was observed in one case of a 16qh-polymorphism by Colls *et al* (2004). Their solution was to use a different probe for chromosome 16 in order to proceed with the PGS cycle.

Another limitation for FISH diagnosis on single cells arises from the increased levels of chromosomal mosaicism detected in preimplantation embryos (Delhanty et al, 1997, Munne *et al*, 2002) as discussed in section 1.2. Mosaic embryos can produce a normal result in the tested cell while the rest of the embryo could be abnormal and vice versa. Munne (2002) calculated that a misdiagnosis rate of around 6% is attributed to mosaicism in embryos. While this percentage is not great combined with the FISH error rate it results in a number of false results. It is clear that the need for highly efficient and specific FISH protocols for embryo aneuploidy screening is crucial to the success of any PGS cycle.

Circumventing the problems and limitations of FISH, CGH can give information for all the chromosomes (Wells and Delhanty, 2000, Wilton *et al* 2001) in one single hybridization. CGH however is more time consuming than FISH and for day-3 biopsy it will

require the freezing of embryos until the results can be obtained due to the 72hrs hybridization time required. The limitations of CGH are also concentrated on how successfully the whole genome of the sample will amplify and the strict conditions needed to avoid contamination.

Whichever method is used for aneuploidy screening, PGS is the biggest category of clinical preimplantation genetics application around the world (Sermon et al, 2007). It is usually performed for couples that present with severe infertility due to i) Advanced maternal age (AMA- usually over 37 years of age), ii) Recurrent miscarriage (RM- with three or more miscarriages), iii) Repeated IVF failure (RIF- with three or more failed IVF attempts) and iv) severe male factor (SMF) that are at increased risk of chromosomal abnormalities. There are various studies of clinical PGS that show benefits but also drawbacks of this method (reviewed in Twisk *et al*, 2006, Donoso *et al*, 2007).

Aneuploidy assessment in embryos is thought to reduce trisomic pregnancies, reduce early pregnancy loss and improve implantation rate in women of advanced maternal age (Munne *et al*, 2006). Thus women that are over 37 years old and undergoing routine IVF treatment might benefit from PGD for aneuploidy screening. It has been estimated PGD for aneuploidy reduces by half the risk of having a trisomic pregnancy in the cases of advanced maternal age (Gianaroli *et al*, 2001).

Furthermore, in a prospective randomised control study (Staessen *et al*, 2004) it was found that in AMA couples undergoing PGS there was no significant difference in implantation rate between the control group and the PGS group when there was no restriction in the number of embryos to be transferred. However there were significantly fewer embryos transferred in the PGS group with a normal embryo rate of 36.8%. In addition it is clear that PGS for AMA will be beneficial only when there will be an adequate number of good quality embryos for testing (Platteau *et al*, 2005).

In the RM/PGS couples up to 70% of embryos tested were found to be abnormal (Rubio *et al*, 2003) however again there are different opinions as to whether PGS is actually improving the prognosis of RM couples (Platteau *et al*, 2005a, Munne *et al*,

2005a). Unexplained recurrent miscarriage involves the treatment of couples that can conceive naturally but are unable to sustain a pregnancy. The debate focuses as to whether these couples have an increased risk of aneuploidy and thus PGS can help. Or they present miscarriages due to other reasons such abnormal immune responses during pregnancy (Yokoo et al, 2006).

For the RIF group a similar picture is also emerging, where around 50% of embryos tested are found to be abnormal and the benefits of PGS for this group are still unclear (Platteau *et al*, 2006, Voullaire *et al*, 2002). Platteau et al (2006), suggested that the couples with RIF must have at least 6 embryos for biopsy in order to benefit from PGS. In addition, an increased incidence of postzygotic embryo abnormalities have been found for this group (Mantzouratou et al, 2007, Wilton et al, 2007). This high incidence of mosaicism might provide an explanation of the failure of implantation. On the other hand, mosaicism could hinder the detection of abnormal embryos through PGS since only a single cell is tested. Clearly more research is needed in order to identify which patients will benefit from PGS.

Results are more encouraging for PGS for SMF. Studies have suggested that implantation and ongoing pregnancy rates were increased after PGS for obstructive, non-obstructive azoospermia and teratozoospermia (Rubio *et al*, 2005, Donoso *et al*, 2006).

The difficulties in assessing the benefits of PGS lie also in the great variation between PGD centres in terms of methodology and patient selection criteria (Shahine et *al*, 2006). Since there is no unified standard procedure in doing and accessing PGS, most studies will provide only part of the information. Additionally, couples referred for PGS have very poor reproductive histories that are difficult to be matched with any control group from routine IVF patients.

The pregnancy rate in the latest ESHRE data for the above PGS groups is as follows:

- PGS general 18% per oocyte retrieval (OR), 24% per embryo transfer (ET);

- AMA 12% per OR 19% per ET;
- RM 22% per OR 29% per ET;
- RIF 20% per OR 24% per ET;
- SMF 28% per OR 32% per ET.

Although these figures are not very high they are comparable with reports from routine IVF patients. The European IVF-monitoring program (EIM-ESHRE) reported the outcome of 324,238 IVF/ICSI cycles done in 2002 from 25 countries (incl. 1563 PGD/PGS cycles) IVF/ICSI cycles (EMI-ESHRE Andersen *et al,* 2006) with pregnancy rates being IVF 26% per OR 29.5% per ET; ICSI 27.2% per OR and 29.4% per ET. The world collaborative report on IVF for the year 2000 reported on data from 49 countries and 460,157 cycles with pregnancy rates 18.6% per OR for IVF and 20.4% per OR for ICSI (Adamson et al, 2006). Although the pregnancy rates on all these studies are not significantly different one has to consider that couples referred for PGS have usually poorer reproductive history than routine IVF and ICSI couples. The pregnancy rate in PGS cycles appear to show that maybe aneuploidy screening can help poor prognosis IVF patients achieve better pregnancy rates.

1.4.4. PGD for structural chromosomal abnormalities

Carriers of structural chromosomal abnormalities are at high risk of producing abnormal gametes and thus have an increased probability of producing chromosomally unbalanced offspring. PGD can help those individuals by selecting normal or balanced embryos thus avoiding or reducing recurrent miscarriage and the birth of chromosomally unbalanced children. FISH is the preferred method of diagnosing structural chromosomal abnormalities in preimplantation embryos using a combination of probes to detect specific abnormalities.

Each case of a structural chromosomal abnormality can require unique probe combinations and additional adjustments in FISH protocols, a procedure that is time-consuming but necessary (Harper & Wells, 1999). Pachytene diagrams are used for the establishment of the probe combination that can be used to help the prediction of the

segregation pattern during meiosis (Scriven *et al*, 1998) and help identify the origin of abnormal FISH signal patterns in preimplantation embryos.

Carriers of balanced structural chromosomal abnormalities present problems due to pairing and separation of chromosomes in meiosis I during gametogenesis that increases the risk of genetically unbalanced gametes. For PGD of these abnormalities in embryos, the difficulty lies in distinguishing between balanced and unbalanced embryos using the limited available probes that must be specific to the structural abnormality. If the carrier is female then preconception diagnosis can be performed by analysing the 1st and or second polar body of the oocyte (Verlinsky *et al*, 1996, Munne et al, 1996), although chromatid errors in the oocyte can cause a false diagnosis. The FISH probes used for such PGD cases must be very specific to each case and highly informative in order to make an accurate diagnosis.

Various strategies have been developed in order to achieve an accurate diagnosis. One involves probes that span the breakpoints of the abnormality and has been used in PGD for reciprocal translocations and inversions (Cassel *et al*, 1997, Munne *et al*, 1998c, Weier *et al*, 1999). This approach required making and labelling the specific DNA probes as well as optimizing the FISH protocol for each individual translocation, a process that usually required around 3-6 months. Since structural abnormalities present a variety of breakpoints this approach is very time consuming and laborious and not used widely in clinical practice. The advantage of the spanning probe strategy is that carrier or non-carrier as well as unbalanced embryos can be differentiated. Similarly, prior to the commercial availability of subtelomeric probes, the use of probes that flank either side of the breakpoints at close proximity have also been used in clinical PGD for reciprocal translocations (Munne *et al*, 1998c, Conn *et al*, 1999) but there were also laborious to prepare and optimize for clinical use.

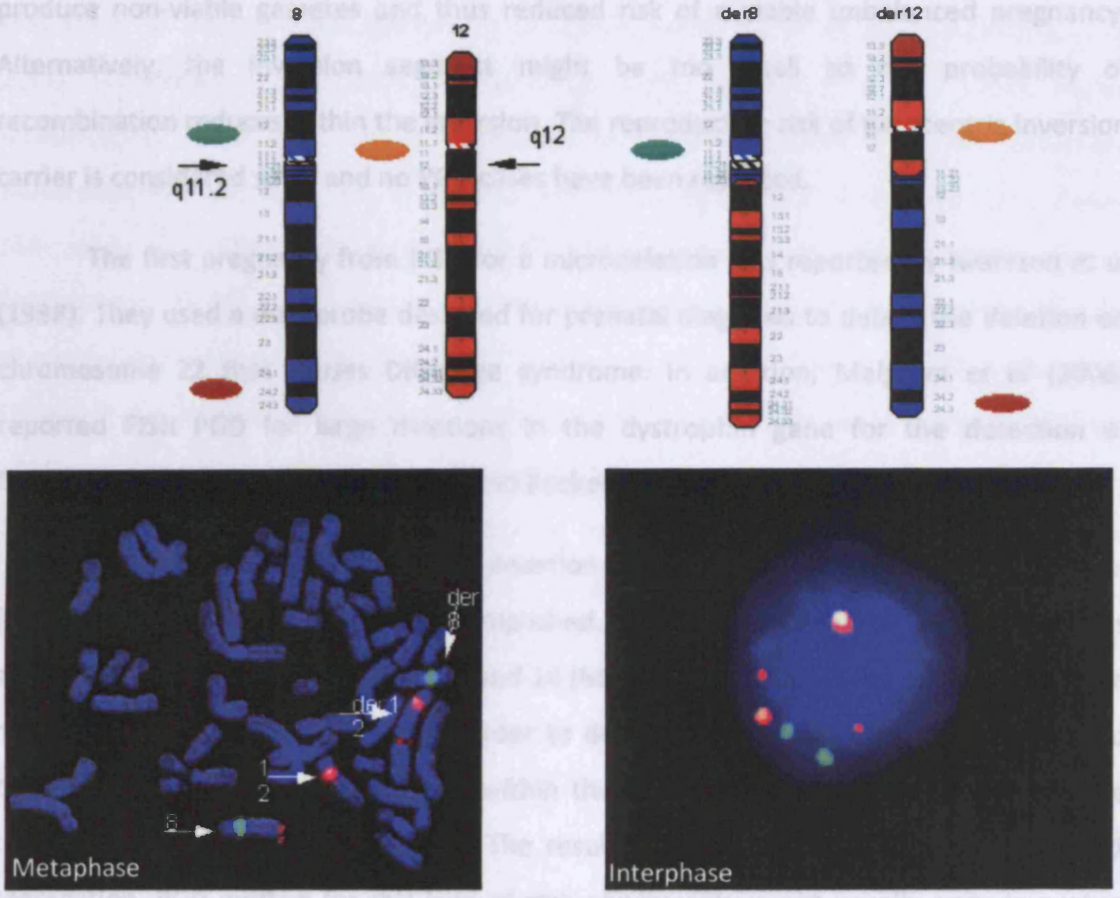
Other approaches used whole chromosome paints on polar body chromosomes. In a study by Munne et al (1998a), it was reported that pregnancy rate was increased and spontaneous abortions were reduced after preconception diagnosis in female carriers of

translocations using whole chromosome paints for the chromosomes involved in the translocations in order to distinguish between genetically balanced and unbalanced polar bodies. However, this approach cannot be used when the male is the carrier and cannot detected postzygotic errors that are usually high in preimplantation embryos.

With the production of a great variety of commercial FISH probes for all chromosomes, the strategy commonly in use now utilizes commercially available subtelomeric, centromeric and locus specific probes. For reciprocal translocations a three or four probe strategy using combinations of telomeric and centromeric are now routinely used in PGD (Scriven *et al*, 1998, Van Assche *et al*, 1999, Munne *et al*, 2000a, Simopoulou *et al*, 2003). Two of the probes are chosen to flank the breakpoints on one chromosome while the third can map to any position in the second chromosome. The decision as to which probes to use will depend on the chromosomes involved, the size and the breakpoints of the translocation.

Usually, three probes are used for simple reciprocal translocations, either two centromeric probes and one telomeric (or locus specific) or two telomeric and one centromeric probe. Figure 1.12 shows an example of this strategy for a reciprocal translocation and how it is translated into FISH signals on metaphase chromosomes and on interphase embryonic nuclei. This approach however cannot differentiate embryos that carry both the derivative chromosomes (balanced carriers) from those that carry the normal chromosomes. This however is not considered as a disadvantage since balanced carriers are phenotypically normal. The only issue concerns the future fertility of carrier individuals not diagnosed during PGD.

Figure 1.12. FISH probe strategy for a carrier of 46XX, t(8;12)(q11.2;q12) for use in PGD. *Two centromeric (green and orange) and one telomeric (red) probes are used in this instance. The arrows denote the breakpoints. Normal diploid cells will produce two signals for each probe on metaphase or interphase nuclei.*



Robertsonian translocation carriers do not present a problem in terms of PGD since whole chromosomes can be missing or gained according to the translocations. One probe can be used for each translocated chromosome which can be either subtelomeric or locus specific (Conn *et al*, 1998, Scriven *et al*, 2001). In addition, the use a third probe unrelated to the translocation would detect any ploidy errors.

A number PGD cycles for pericentric or paracentric inversions, deletions and duplications as well as various translocations have been reported in the ESHRE database (Harper *et al*, 2006, Sermon *et al*, 2007). PGD for pericentric inversions have been reported using one to three probes (Iwarsson *et al*, 1998, Escudero *et al*, 2001). The strategy for this has to include probes outside of the inversion and an optional probe

within the inverted region because of the risk of a duplication/deletion of the non-inverted segments if recombination occurs within the inversion. Paracentric inversions produce dicentric or acentric fragments if recombination occurs in meiosis, which will produce non-viable gametes and thus reduced risk of a viable unbalanced pregnancy. Alternatively, the inversion segment might be too small so the probability of recombination reduces within the inversion. The reproductive risk of paracentric inversion carrier is considered small and no PGD cases have been reported.

The first pregnancy from PGD for a microdeletion was reported by Iwarsson *et al* (1998). They used a dual probe designed for prenatal diagnosis to detect the deletion on chromosome 22 that causes DiGeorge syndrome. In addition, Malgrem *et al* (2006) reported FISH PGD for large deletions in the dystrophin gene for the detection of Duchenne Muscular Dystrophy (DMD) and Becker Muscular Dystrophy (BMD).

PGD for an intra-chromosomal insertion has been reported by Simopoulou *et al* (2003) where a healthy birth was accomplished. PGD for an insertional translocation has also been reported for chromosome 2 and 14 (Melotte *et al*, 2004). The authors suggest a minimum of 4 probes to be used in order to detect all abnormal outcomes. They used three probes on chromosome 2 (one within the insertion and two flanking it) and one telomeric probe for chromosome 14. The resulting embryos showed either 2:2 or 3:1 segregation. PGD workup for this kind of rare abnormality is not usually easy as probes within the insertion regions are seldom available.

In general, visualizing a structural chromosomal abnormality in an interphase nucleus from an embryo is accomplished by a variety of FISH strategies. However, limitations with interphase FISH or mosaicism, as with numerical abnormalities, can cause misdiagnosis; seven have been reported for FISH in the current ESHRE data (Sermon *et al*, 2007). A poor PGD strategy can result in the transfer of unbalanced embryos as in the case of a PGD for a carrier of the 11;22 translocation (Lim *et al*, 2004). Additionally, the high levels of mosaicism in preimplantation embryos can include chromosomes not involved in

the structural abnormality and aneuploidy screening with PGD for translocation carriers has been suggested (Pujol *et al*, 2006).

Several clinical studies have been reported on the outcome of PGD for structural chromosomal abnormalities (Munne *et al*, 2000a, Fridstrom *et al*, 2001, Pickering *et al*, 2003, Simopoulou *et al*, 2003, Grace *et al*, 2006, Feyereisen *et al*, 2007). Higher implantation rates and reduced spontaneous abortions after PGD for structural abnormalities were reported, compared with previous history of the couples involved (Munne et al, 2000a, Verlinksy et al, 2005). The pregnancy rate in these studies ranged from 24% to 38% per ET, which is within the same range as the current ESHRE data for chromosomal abnormalities; 24% per ET (Sermon *et al* 2007). One limitation for this type of PGD is the number of embryos suitable for transfer and their quality. From the above studies the pregnancy rate per OR ranges from 16% to 29%. This means that a lot of cycles had too few oocytes collected or embryos to biopsy or had no normal embryos to transfer. For carriers of structural chromosomal abnormalities, where a high number of unbalanced gametes will be produced, it is essential to have adequate number of embryos to test for PGD.

1.5 Aims of this study

Despite the information generated from studies in preimplantation embryos several questions still remain to be answered in respect to the genetic processes that cause abnormalities at gametogenesis and preimplantation stage of development. Very few reports on the outcome of PGS include full follow up studies to allow the distinction between meiotic and mitotic errors. Additionally, PGD and PGS methods and strategies are diverse and their efficiency cannot be easily measured. Moreover, there is little data on the segregation during oogenesis in carriers of translocations and none of carriers of rare types such as ring chromosomes. This study will try to address some of these problems and deficiencies. The aims of the study are:

Numerical chromosomal abnormalities- aneuploidy screening

- The development and clinical implementation of an efficient PGS protocol
- Evaluation of the PGS protocol in terms of its efficiency and specificity
- The determination of full chromosomal outcome from follow up analysis of untransferred embryos derived from PGS. To investigate the types of abnormalities, their origin and the mechanisms that produce them
- To assess the validity of PGS in relation to pregnancy outcome

PGD and structural chromosomal abnormalities

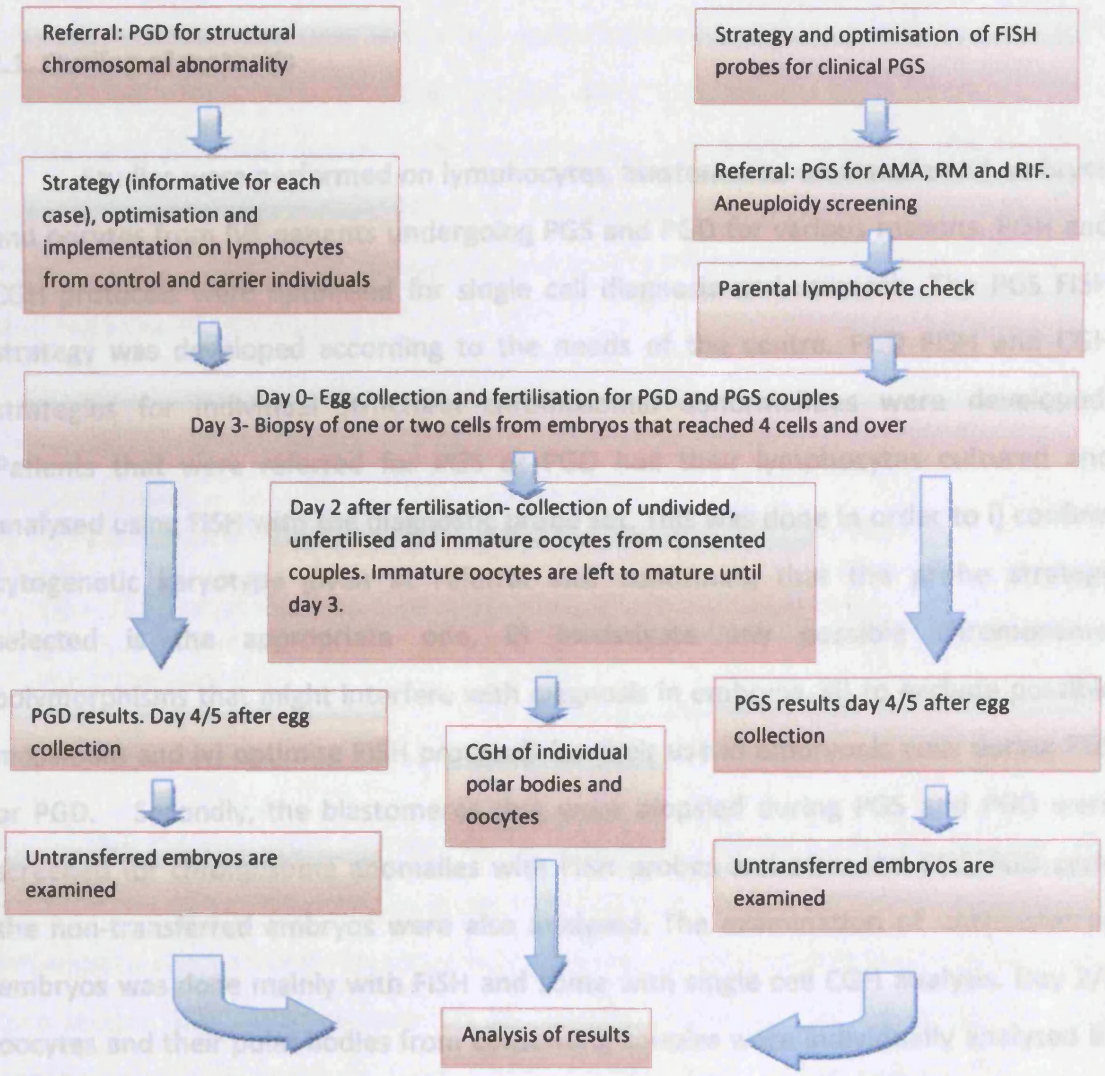
- To produce optimum strategies for PGD to help carriers of structural chromosomal abnormalities achieve a normal pregnancy
- To clinically implement and evaluate these strategies
- The determination of chromosomal outcome from follow up analysis of untransferred embryos derived from PGD. To investigate the types of abnormalities and their origin, in particular the segregation patterns at oogenesis of the rarer types of anomalies.

CGH investigation of oocytes and polar bodies

- The identification of abnormalities in female gametes derived from women undergoing PGS and PGD
- The examination of the origin of aneuploidy in these two groups of gametes and the investigation of the types of abnormalities arising during female meiosis. The correlation of the oocyte information to subsequent embryo abnormalities

1.6 Outline of study

The main methods used in this study were FISH and CGH. All protocols had to be optimized for single cell use. The following diagram shows a basic outline of the work involved in the study.



Chapter 2

Materials and Methods

2.1. Outline of methods

Studies were performed on lymphocytes, blastomeres, un-transferred embryos and oocytes from IVF patients undergoing PGS and PGD for various reasons. FISH and CGH protocols were optimised for single cell diagnosis and research. The PGS FISH strategy was developed according to the needs of the centre. PGD FISH and CGH strategies for individual structural chromosomal abnormalities were developed. Patients that were referred for PGS or PGD had their lymphocytes cultured and analysed using FISH with the diagnostic probe set. This was done in order to i) confirm cytogenetic karyotype given at referral also confirming that the probe strategy selected is the appropriate one, ii) investigate any possible chromosomal polymorphisms that might interfere with diagnosis in embryos, iii) to exclude possible mosaicism and iv) optimise FISH protocols for their use in embryonic cells during PGS or PGD. Secondly, the blastomeres that were biopsied during PGS and PGD were screened for chromosome anomalies with FISH probes and after the PGS/PGD cycle the non-transferred embryos were also analysed. The examination of untransferred embryos was done mainly with FISH and some with single cell CGH analysis. Day 2/3 oocytes and their polar bodies from consenting couples were individually analysed by CGH. Treatment and research on embryos and oocytes from PGS and PGD couples was carried out under licence from Human Fertilisation and Embryology Authority (HFEA). Informed written consent was obtained from all the couples.

2.2. Patient details, PGD/PGS cycles and sample collection

2.2.1 PGS referrals and FISH strategy for aneuploidy screening

PGS couples are divided into three major groups according to their indication for undergoing PGS. The first group (AMA) included those patients undergoing PGS for advanced maternal age only and in this study included females over 39 years of age. The second group (RM) included couples that had experienced three or more spontaneous abortions mostly from natural cycles and the third group (RIF) included couples that had experienced failure of implantation in routine IVF three or more times. Table 2.1 lists the FISH probe details for the PGS protocol followed. Figure 2.1 illustrates the PGS strategy.

Table 2.1. FISH probe details for PGS

1st Round probes	2nd Round Probes
CEP 18 a-satellite (D18Z1) in aqua LSI 13/21 dual probe, 13 in green, 21 in orange (part of the Aneuvysion kit) all from Abbott, UK	CEP15 a-satellite (D15Z4) in orange CEP 16 satellite II (D16Z3), in aqua (Abbott, UK) or spectrum orange combined with spectrum green (yellow) LSI 22 (22q11.2, Brc gene) in green All from Abbott, UK

The chromosomes included in the PGS FISH protocol were determined after study of the most commonly involved chromosomes in aneuploidy in embryos. The information came from various studies in prenatal and preimplantation genetic studies (see section 1.2). Chromosomes 13, 15, 16, 18, 21 and 22 were deemed the most appropriate. FISH protocols were optimised for each probe individually and in combinations. The final optimised protocol included two rounds of hybridisation. In the first instance chromosomes 13, 16 and 21 were studied in the first round and 15, 18 and 22 were studied in the second round. After initial problems with the probe of chromosome 16 in spectrum aqua it was replaced by two probes for chromosome 16 in spectrum orange and spectrum green. In simultaneous hybridisation this combination produced a yellow signal visible in filters that detected both these fluorochromomes. Chromosome 18 probe was then placed in the first round and

chromosome 16 probe in the second in order to detect abnormalities of all viable trisomies first.

PGS was performed for 76 couples that undergone 101 cycles. According to referral reason 26 cycles for 16 couples were for AMA, 19 cycles for 16 couples were for RM and 56 cycles for 44 couples were for RIF.

2.2.2 PGD referrals and diagnosis strategies

Couples were referred for PGD for structural chromosomal abnormalities include translocations, reciprocal and Robertsonian, a carrier of a ring chromosome 22 and a carrier for an interchromosomal insertion. Table 2.2 lists the karyotype, reproductive history, age and number of PGD cycles for each couple. There were 18 cycles for reciprocal translocation carriers, 9 cycles for Robertsonian translocation carriers and two cycles for a ring chromosome 22 carrier. Strategies for an interchromosomal insertion carrier were also evaluated with FISH and CGH.

Table 2.2. PGD referrals for structural chromosomal abnormalities

Carrier of Balanced Structural abnormality	Cycles	Average Maternal Age	Reproductive history
46,XX,t(8;12)(q11.2;12)	3	36	5 early miscarriages. Two of them showed adjacent-2 segregation (both 12+der12 in the oocyte). Sperm parameters normal
46,XY,t(9;15)(p12;q13)	1	38	5 previous ICSI cycles, 1 miscarriage with imbalance
46,XX,t(11;22)(q23.3;q11.2)	2	30	4 early miscarriages no cytogenetic data available
46,XX,t(X;4)(q26;p16.1)	3	32	Two previous first trimester induced abortions due to unbalanced karyotypes. Cytogenetic analysis showed unbalanced karyotypes due to adjacent-1 maternal meiotic segregation. One had partial trisomy Xq and partial monosomy 4p [46X, der(X), t(X;4)(q26;p16.1)mat]. The other had partial trisomy 4p and partial monosomy Xq [46XX, der(4), t(X;4)(q26;p16.1)mat].
46,XX,t(9;20)(p13;p11.2)	2	40	Four miscarriages and an ectopic pregnancy (no other info provided). One normal child.
46,XY,t(1;17)(q42.1;q25.3)	2	37	The couple have experienced 2 early miscarriages (10wks and 9wks) but no cytogenetic report on them has been given. They have one healthy girl. The carriers sister is also a carrier of the same translocation and she also had a number of early miscarriages but there is no live birth with an unbalanced karyotype.
46,XX,t(10;11)(q11.2;p15.3)	3	38	Two early miscarriages both in the 7th week. No cytogenetic data.
46,XY,t(1;18)(p32;q23)	1	36	6 years of infertility and 2 early spontaneous abortions. Sperm studies showed: 65% alternate segregation, 17% adjacent-1, 5% adjacent-2, 11% 3:1, 2% other.

46,XY,t(1;21)(q12;q22.1)	1	35	Infertility and no pregnancies, 2x failed IUI
Robertsonian translocations 45,--,t(13;14)(q10;q10)			
Couple 1 (XY)	3	38	Many years of infertility, poor sperm count. The carriers sister has the same translocation with a number of early miscarriages.
Couple 2 (XX)	1	38	1 miscarriage and infertility
Couple 3(XX)	1	39	Infertility no previous pregnancy
Couple 4 (XY)	1	47	Two previous cycles of PGD at diff. centre. 2nd one miscarried at 6/40. Naturally conceived blighted ovum.
Couple 5 (XY)	1	40	1 child. Several years of infertility
Couple 6 (XY)	2	36	Infertility no previous pregnancy
Other structural abnormalities carriers			
47,XX, del(22)(p10q12), +r(22)(q10q12)	2	37	Affected son with abnormal karyotype 47, XY, +r(22)(p11.2q11.2)/46,XY mosaic
46,XX, ins(14;4)(q13;q25q21.3)	0	37	Affected pregnancies with 46,XX, der(4), t(14;4)(q13;q25q21.3) and 46,XY, der(4), t(14;4)(q13;q25q21.3). Affected son with abnormal karyotype 46,XY, der(14), t(14;4)(q13;q25q21.3). Trisomy 21 pregnancy.

For each structural abnormality FISH or CGH strategies had to be optimized for single cell diagnosis. Some untransferred embryos and biopsied nuclei were re-examined for chromosomes unrelated to the original abnormality. FISH strategy for Robertsonian translocation usually included two locus specific probes. FISH strategies for reciprocal translocations varied according to the breakpoint of each translocation. Specific probes were determined for the ring chromosome 22 PGD case so that the balanced carrier embryos as well as the unbalanced ones could be identified during PGD. Table 2.3 lists the FISH probe details used in each PGD case.

Several strategies for PGD for the interchromosomal insertion were examined which were the use of commercially available probes, the use of custom made band specific probes and the use of single cell CGH. Additionally, immunofluoresence and FISH studies were performed for X-inactivation detection in lymphocytes and embryos from an X;autosome translocation carrier.

Table 2.3. FISH probe details for PGD for structural chromosomal abnormalities

PGD case	Probes	Additional investigations
46,XX,t(8;12)(q11.2;12)	CEP12 a- sat. (D12Z1) in orange and green CEP8 a-sat (D8Z1) in green 8q ter subtelomeric in red all from Abbott, UK	CEPX (green), CEPY(orange), CEP18 (aqua) three probe cocktail from Abbott, UK. CGH on 3 embryos
46,XY,t(1;18)(p32;q23)	1 sat.II/III, lab prepared in orange	Lab prepared centromeric probes: 12

	1pter, telomeric in green, from Abbott, UK CEP 18 a-satellite (D18Z1) in aqua from Abbott, UK	a-sat in green and 16 satIII in orange
46,XY,t(1;17)(q42.1;q25.3)	1 sat.II/III, lab prepared in green CEP 17 a-sat (D17Z1) in green and orange from Abbott, UK 1qter. Subtelomeric in orange from Abbott, UK	LSI 13/21 dual probe, 13 in green, 21 in red (Abbott, UK) CEP 18 a-satellite (D18Z1) in aqua
45,--,t(13;14)(q10;q10)	14qter, subtelomeric in orange form Abbott, UK LSI13, 13q14 in green from Abbott UK	CEP15 a-satellite (D15Z1) in orange CEP 18 a-satellite (D18Z1) in aqua CEP4 a-sat (D4Z1) in green All from Abbott, UK
46,XX,t(11;22)(q23.3;q11.2)	LSI "Di George" dual probe for 22 CEP 11 α-satellite for chromosome 11	16 centromeric β-satellite for chromosome 16, 15 centromeric satellite III for chromosome 15, CEP 18 centromeric α-satellite for chromosome 18 Cycle 2: CEP 18 centromeric α-satellite for chromosome 18
46,XX,t(10;11)(q11.2;p15.3)	CEP 10 a-sat (D10Z1) in green CEP11 a-sat (D11Z1) in aqua 10qter, subtelomeric in orange All from Abbott, UK	CEP 16 satellite II (D16Z3), in aqua (Abbott,UK) LSI 13/21 dual probe, 13 in green, 21 in red (Abbott, UK)
46,XX,t(X;4)(q26;p16.1)	CEP4 a-sat in green CEPX a-sat in orange and green CEPY sat III aqua Xq\Yqter, subtelomeric probe for the telomeres of X and Y orange. All from Abbott, UK	Immunofluorescence antibody for inactive chromosome X. Primary antibody: Anti-dimethyl-Histone H3 (Lys9) (Upstate, UK) Secondary antibody: Cy-3 con. AffinPure Donkey Anti-Rabbit IgG (Jackson Immunoresearch, UK)
46,XY,t(9;15)(p12;q13)	CEP15 spectrum aqua, CEP9 spectrum orange, 9pTel spectrum green, all from Abbott, UK	CGH on oocytes
46,XX,t(9;20)(p13;p11.2)	Cep 9 spectrum aqua 20ptel spectrum green 20qtel spectrum orange all from Abbott, UK	9ptel spectrum green Abbott,UK CGH on one oocyte
46,XY,t(1;21)(q12;q22.1)	LSI21spectrum orange, 1qtel spectrum green, all from Abbott, UK	CEP1 spectrum orange, Abbott, UK CGH on two embryos
47,XX, del(22)(p10q12), +r(22)(q10q12)	1st Round: DiGeorge dual band probe chr22 (Abbott, UK)	2nd Round: 14/22 centromere & 14q telomere (Qbiogene, UK) CGH on DNA and single buccal cells from unbalanced child
46,XX, ins(14;4)(q13;q25q21.3)	Band specific probe BSP4q23 (Qbiogene, UK) in green Single cell CGH	CGH on DNA and single buccal cells from unbalanced child

2.2.3. CGH oocyte study patient details

Oocytes and the corresponding polar bodies (PBs) were obtained from 15 females. 10 females were referred for PGS from whom 40 oocytes were studied, one routine IVF female from whom 2 oocytes were studied and 4 females that were referred for translocation PGD from whom 17 oocytes were studied. 104 samples, 59 oocytes and/or corresponding first and possibly second PBs were investigated during this study. Those that were unexposed to sperm were immature at the time of egg collection. Three were MI oocytes (left to mature but remained in MI), 2 GVs (left to mature but remained immature), 3 matured in vitro (2xMI, 1xGV) and had 1st polar body. The remaining oocytes were unfertilized after sperm exposure, 7 of which consisted of both first and second PBs, 28 had a 1st polar body and 17 did not show a PB. Table 2.4 lists the patient details and samples obtained.

Table 2.4. Oocyte study using CGH. Patient details

Case	Maternal age	Indication	No of oocytes and PBs
O	36	PGS	2 0PN/1PBs and 2 PBs
S1	26	PGS	4- 2 0PN/1PB and 2 PBs and 2 0PN/0PB
E1	36.5	PGS	10- 4 0PN/PB and 4 PBs and 4 0PN/0PB, 2 GV
W	42	PGS	5- 4 0PN/0PB and 4 PBs and 1 0PN/0PB
S2	42	PGS	5- 2 MI and 1 GV in vitro matured and 3 PBs, 1 0PN/2PB with 2 PBs, 1 0PN/0PB
C	42	PGS	3- 1 MI in vitro matured and 1PB, 1 1PN/1PB with 1 PBs, 1 0PN/1PB with 1 PB
M	26	PGS	3- 2 0PN/2PBs with 4 PBs and 1 0PN/1PB with 1 PB
E2	37	PGS	5- 1 MI, 1 0PN/1PB with 1 PB, 3 0PN/0PB
T	37	PGS	2- 1 0PN/2PB with 2 PBs, 1 0PN/1PB with 1 PB
E3	38	PGS	2- 1 0PN/1PB with 1 PB and 1 0PN/0PB
A1	37	Routine IVF	2- 1 0PN/1PB with 1 PB, 1 0PN
V	33	Non- carrier translocation 46, XY, t(1;4)(q11.1;q33)	4- 2 0PN/2PB with 4 PBs, 1 2PN/1PB with 1 PBs, 1 0PN/1PB with one PB
R	40	Carrier translocation 46,XX, t(9;20)(p13;p11.2)	1- 0PN/0PB
A2	31	Carrier translocation 46, XX, t(8;10)(p23;q24)	2 MI immature
S3	38	Non carrier translocation 46,XY,t(9;15)(p12;q13)	9- 1 1PN/2PB with 2PBs, 1 1PN/1PB with 1 PB, 2 0PN/2PB with 2PBs, 3 0PN/1PB with 3PBs, 2 0PN/1PB with no PBs.

2.3. IVF and PGS/PGD procedures, sample collection and preparation

Vaginal egg collection was performed at 37 hours post hCG injection. IVF or IVF/ICSI was performed at 40 and 41 hours post hCG respectively and was dependent on semen parameters and past fertilisation rates. Fertilisation was evaluated at 18-20 hours post insemination. Embryos were cultured in IVF medium (GIII series, Vitrolife).

On day 3, embryos were biopsied in Ca^{2+} -Mg^{2+} free biopsy medium (G-PGD, Vitrolife). One or two cells were removed from most embryos according to indication. For PGS one cell was usually removed unless technical difficulties or the presence of a binucleate cell produced the need for a second cell to be biopsied. For PGD, two cells were removed from embryos that had reached the 6 cell stage by day 3 post fertilisation. Biopsied blastomeres were spread onto microscope slides using the method described by Harper et al, (1994). Cells were washed in PBS and transferred to poly-L-lysine slides in spreading solution (0.01N HCL, 0.1% Tween 20) which was gently agitated until lysis occurred and the nuclei were clear of cytoplasm. The co-ordinates of the location of the cells were noted using an England Finder. The same technique was used for whole embryos. FISH was then performed.

Oocyte and polar body separation, blastomere and single cell separation and lysis for DNA amplification and CGH.

Acid Tyrode's (Sigma, UK) was used for removal of the zona pellucida and oocyte and corresponding PB separation. Oocytes and their PBs as well as single blastomeres, buccal cells and single fibroblast trisomic cells were washed in three 10µl droplets of PBS, 0.1% polyvinyl alcohol (PVA). They were then transferred to microcentrifuge tubes containing 1µl of sodium dodecyl sulphate (SDS, 17µM) (Sigma, UK), and 2µl of proteinase K (PK, 125µg/ml) (Roche, UK), and overlaid with light mineral oil. Cell lysis took place by incubation at 37 ° C for 1 h, followed by 15 min at 95 ° C.

Genomic DNA extraction for CGH use

DNA extraction from buccal cells, trisomic fibroblasts and lymphocytes were done with standard methods. Buccal cells DNA extraction was done for two carriers of unbalanced structural chromosomal rearrangements. One was from a male child with 47, XY, +r(22)(p11.2q11.2)/46,XY mosaic karyotype from the PGD couple referred for ring chromosome 22 [47,XX, del(22)(p10q12), +r(22)(q10q12)]. The other was from a male child trisomic for 4q21.3-q25 [46,XY, der(4), t(14;4)(q13;q25q21.3)] as a result of an inverted interchromosomal insertion in the mother with karyotype 46,XX, ins(14;4)(q13;q25q21.3).

Lymphocyte culture and counts

Lymphocyte cultures from both partners of PGS and PGD couples were by standard methods. For synchronization purposes, after the 48 hour incubation period 200µl of thymidine (30mg/ml, Sigma, UK) were added to each of the cultures which were then incubated at 37°C for a further 18 hours. Following this, 200µl of deoxycytosine (0.277mg/ml, Sigma, UK) were added to the culture flasks and incubated for 4 hours at 37°C. *2.5.3*

Lymphocyte fixation:

1-2 drops of fix (3:1 methanol: acetic acid) were added for several times in each tube. After each drop added the pellet in the tubes was resuspended by tapping each tube. This continued until the contents of the tubes stopped frothing and 10 ml of fix was added to each of the tubes. The tubes were centrifuged for 5 minutes at 1000 rpm, most of the supernatant was discarded and the pellet was resuspended. The fixation was repeated until a clear supernatant was obtained for each tube and the tubes were centrifuged for 5 minutes at 1000 rpm. The tubes, containing lymphocyte suspensions, were stored at -20°C.

Prefixation method: 8ml of 0.075M KCL pre-warmed at 37˚C were added and the pellet was resuspended and incubated at RT for 10 minutes. 1ml of fresh 3:1

methanol acetic acid fixative was added to each tube and inverted slowly. The tubes were then spun at 1100rpm for 10 minutes and the supernatant was removed. Fresh fix was slowly added up to 5 ml whilst mixing. The tubes were left in 4°C for 30 minutes then centrifuged at 1200rpm for 5 minutes and the fix was changed again as above. The tubes were left overnight at 4°C then checked and were transferred to -20°C the next day.

Lymphocyte slide preparation:

20ml of fresh fix and 10ml of 70% acetic acid were prepared. Most of the old fix in the lymphocyte preparations was discarded; a small amount of fresh fix was added, depending on the size of the pellet in the tube. The lymphocytes were resuspended. The glass slides were cleaned and moistened and a drop from the lymphocyte suspensions was dropped onto each slide. The slide was placed on the back of the hand to warm the nuclei and aid their spreading. The site of the nuclei was marked on the underside of the slide using a diamond marker. Once the slide was totally dry it was flooded with fix for 10-20 seconds. The fix was poured off and the slide was allowed to dry and flooded with 70% acetic acid for 10-20 seconds. The acetic acid was poured off; the slide was allowed to dry and observed under a light microscope using the x10 magnification lens in order to check the presence of the nuclei. Once good quality lymphocyte slides were obtained, they were dehydrated using through a 70%, 90% and 100% ethanol series for 5 minutes in each jar and air dried. Fresh fix was added to the initial lymphocyte suspension which was stored at -20°C.

2.4 Fluorescent In Situ Hybridisation

FISH experiments were undertaken in order to test and optimise conditions for all the probes in this study.

FISH for aneuploidy screening

Hybridisation to probes for six pairs of chromosomes was undertaken in two separate rounds; i) probes for chromosomes 13,16 & 21 were used in the first round and those for 15,18 & 22 were used in the second round ii) probes for chromosomes 13,18 & 21 were used in the first round and those for 15,16 & 22 were used in the second round. The slides with the fixed nuclei were incubated in 0.01N HCl and 0.5ml of 10mg/ml pepsin (Sigma, UK) at 37°C for 20 minutes. They were washed briefly in distilled water and PBS (Sigma, UK) and then fixed using 1% paraformaldehyde (Sigma, UK) in PBS for 10 minutes at 4°C and were washed first in PBS and then twice in water and were dehydrated through an ethanol series and air-dried. The first round probes, the LSI 13/21 dual probe, 13 labeled in Spectrum Green (SG), 21 in Spectrum Orange (SO and CEP 18 a-satellite (D18Z1) in aqua (all from Abbott, UK)were denatured separately from the slides at 75°C for 5min and were applied on to the slides and left to hybridise overnight at 37°C. The next day, the slides were washed in 50% formamide three times for 3 min each at 42°C then in 2x SSC three times for 3 min each at 42°C. They were then washed in 4x SSC/0.05% Tween twice for 5 min at room temperature. Finally, they were dehydrated through a 70, 90, and 100% ethanol series, dried in the dark and 6 μl of 0.2mg/ml 4',6'-diaminidino-2-phenyolindole (DAPI) (Vectar Laboratories, CA, USA) was applied under a coverslip and the slides were stored in the dark at 4°C ready for visualisation. After the scoring of the first round results, the probes were removed by washing the slides in 4x SSC/0.05% Tween two times for 5 minutes each and then in PBS for 10 minutes. The slides were then dehydrated as before and the second round probe mixture was applied which included CEP 16 satellite II (D16Z3), in SO and, CEP15 a-satellite (D15Z1) in SO, LSI 22 in SG all from Abbott, UK. The slides and probes were denatured and hybridised as before.

Hybridisation took place over the next four and half hours and in some cases overnight, depending on the day of transfer (day 4/5). Post hybridisation washes were as before but 40% formamide was used instead of 50%. The slides were examined under an epifluorescence Olympus microscope (Olympus BX 40) fitted with a photometrics cooled CCD camera utilising Smartcapture software (Digital Scientific, UK). DAPI stained nuclei were located using the blue filter. Using different colour filters the scoring of signals for each of the probes to the nuclei on the slides was possible with a good degree of accuracy. All scoring decisions were made directly by viewing signals under the microscope and by two independent observers in the case of biopsied cells.

FISH for structural abnormalities

Different FISH strategies were applied for each different structural abnormality. The basic principles of FISH are essentially the same as in the PGS protocol but different probe combinations required modifications that were applied in each PGD case.

Scoring and classification criteria of embryos according to FISH results

Strict scoring criteria were applied in order to classify the studied nuclei and embryos correctly according to Hopman *et al* (1988). i.e. i) Split signals: when a chromosome has two chromatids in interphase they may appear as doublets, which are equal in size and smaller than the normal signal. The split signals must be separated by less than the width of a normal signal in order to be classified as one chromosome. ii) Stretched or diffused signals must not present any interruption in order to be classified as one chromosome. iii) Nuclei with uniformly diploid signals were classified as normal. In addition: i) Embryos with blastomeres showing the same abnormality in at least 90% of cells was classified as uniformly aneuploid due meiotic error. ii) Embryos with cell lines showing different abnormalities were classified as mosaic (either aneuploid mosaic if all cell lines were aneuploid or diploid/aneuploid mosaic if there was a diploid cell line present) indicating errors mitotic in origin; reciprocal errors are recorded as due to mitotic non-disjunction; non-reciprocal errors in more than 20% of nuclei in an embryo were classified as chromosome loss or

chromosome gain. iii) Embryos with blastomeres showing different abnormalities in each nucleus, affecting at least three chromosome pairs, were classified as chaotic mosaics. iv) Embryos were fully chaotic if all cells were affected, otherwise they may be partially diploid or aneuploid and partially chaotic. v) Embryos were classified as diploid on follow up if they contained at least 90% diploid cells. vi) For mosaic embryos the existence of different cell lines was determined according to the developmental stage and the concordance between the biopsied cells and the follow up analysis; For example for 4 to 8 -cell stage embryos one abnormal cell (with an extra signal) in an otherwise diploid cell population constituted a postzygotic mitotic error. Loss of a signal in one cell could have been due to FISH error. One diploid cell in an otherwise uniformly aneuploid cell population was classed as a meiotic error with subsequent postzygotic "correction". The existence of a second diploid cell in the last case in a 8-cell stage embryo would be more complicated to interpret and would be subject to comparison of the biopsy results with the re-analysis data. In order to assess the mode of aneuploidy in embryos only those with definitive cell lines were included and we excluded those where there were doubts over the events that lead to aneuploidy.

Statistical analysis

The mean and standard deviation was calculated for the number of oocytes collected per cycle, embryos biopsied, and embryos with diploid result. X^2 distribution test was used to compare follow up results between different groups of data. The two sample t-test was used to compare differences in the number of embryos with meiotic errors between different maternal age groups in section 3.2.4 (figure 3.2).

2.5. Antibody for X-inactivation detection and FISH

Primarily the aim was to produce an immunofluorescence and FISH protocol to be used in nuclei from lymphocytes, oocytes and blastomeres. The X-inactivation antibody was used in order to detect X-inactivation patterns, if any, in preimplantation embryos from a carrier of a X;autosome translocation, 46,XX,t(X;4)(q26;p16.1), thus allowing some conclusion to be drawn about X-inactivation and the derivative X chromosome in this case. Fixation of samples was a crucial step in order to maintain protein structures in this study. Lymphocytes from normal controls (male and female) and

carrier were separated and cultured by standard cytogenetic methods. There were fixed in three ways i) methanol :acetic acid as described in a previous section, ii) resuspended in 0.075M KCl for 10 min and 0.5ml were cytospan onto glass slides, iii) 4% paraforlmadehyde.

Blastomere and oocytes were fixed as in Hodges and Hunt (2002) with some modifications. They were placed in 1% pronase (Sigma, UK) in culture media briefly to remove the zona pellucida. Glass slides were marked with a diamond marker to denote the position of the sample. A very small drop of paraformaldehyde /DTT (PF-DTT: 1% paraformadehyde in water, pH 9.2, 0.15% Triton-X, 3mM dithiothreitol, Sigma, UK) solution was placed on the slide. The oocytes or embryos/blastomeres were placed onto the slide and were left to dry a humid chamber at 37°C. Once dried, they were washed with 0.4% Ilfotol wetting agent (ILFORD, UK) in distilled water and dried at room temperature.

Immunofluorescence and FISH.

Protocol 1. Slides were incubated in KCM (120mM KCl, 20mM NaCl, 10mM TrisCL pH8.0, 0.5mM EDTA, 0.1% Triton-X) for 10 mins at RT. Serial dilution of primary antibody were prepared in KCM with 50µl total volume and were placed on the slides (Dilution 1:200 was used in blastomeres) covered with parafilm and incubated for 1hr at 37°C in a humid chamber. The slides were washed in PBS 3x5mins and were incubated with the secondary antibody in 1:40 dilution in KCM/1%BSA (Sigma, UK) for 30mins at RT. The slides were then washed with KCM briefly a fixed in KCM/4%paraformaldehyde for 5 min. They were then washed in water for 5 mins and counterstained with DAPI (Vectar Laboratories, CA, USA).

Protocol 2. Slides were washed in PBS (Sigma, UK) and permealised in PBS/0.5% Triton-X for 4 mins on ice. After another PBS wash they were placed in PBS/0.5%BSA for 15 mins. They were then incubated with serial dilutions of primary antibody in PBS/1%BSA for 1 hr in a humid chamber at 37°C. They were then washed in PBS and incubated with the secondary antibody at 1:40 dilution in PBS/1%BSA for 1hr at RT. After another wash with PBS the cells were counterstained with DAPI (Vectar Laboratories, CA, USA).

2.6. CGH for single cells

Degenerate Oligonucleotide Primed PCR (DOP-PCR)

Whole genome amplification was performed on the following: i) oocytes, ii) polar bodies, iii) blastomeres from day 4/5 embryos from some carriers of structural chromosomal abnormalities with known FISH result for day 3 and 5 iv) clumps of 2-5 buccal cells from karyotypically normal individuals and karyotypically abnormal individuals which were processed in exactly the same way as the oocytes, PBs and blastomeres; the normal buccal cells were used as the reference DNA sample with which the test sample was compared, v) diluted genomic female DNA was also used as reference with DOP amplification and vi) single trisomic fibroblast cell processed as before and used a positive control.

The Degenerate Oligonucleotide Primed PCR (DOP-PCR) was applied for the whole genome amplification of single cells (Wells *et al*, 2002, Fragouli *et al*, 2006b). Amplifications took place in a 50-µl reaction volume consisting of 10 mM dNTPs (Promega,UK), 2 µM DOP primer (CCGACTCGAGNNNNNNATGTGG) (Oswell, UK), 10× SuperTaq Plus buffer, and 2.5 U SuperTaq Plus (HT Biotechnologies, Cambridge, UK). Thermal cycling conditions were as follows: 94°C for 4.5 min; 10 cycles of 95°C for 30 s, 30°C for 1 min, and 68°C for 3 min; 40 cycles of 95°C for 30 s, 56°C for 1 min, and 68°C for 3 min; and a final extension at 68°C for 8 min. Amplification was carried out in a 9700 PE (Applied Biosystems, UK) thermocycler. At the end of each amplification 45 µl of product were further processed, whereas the remaining 5 µl were kept for agarose gel analysis.

Stringent precautions against contamination were taken during single cell lysis, and amplification (Wells and Sherlock, 1998). Negative control tubes containing 2 µl of the final drop of PBS/PVA into which single cells were washed, prior to their transfer into microcentrifuge tubes, along with the rest of the DOP-PCR reagents were included for each experiment.

DNA labelling

Incorporation of fluorescent labels was carried out enzymatically with the use of the Nick Translation kit (Vysis/Abbott, UK). Test DNA was labelled with Spectrum Green-dUTP (Vysis/Abbott, UK), whereas the reference DNA was labelled with Spectrum Red-dUTP (Vysis/Abbott, UK). Nick translation time was adjusted according to desired probe size. The latter was assessed by agarose gel analysis of 5 µl of labelled sample. Both red and green DNAs were co-precipitated with 30 µg of human Cot1 DNA (GIBCO/BRL, UK). Pellets were dried and resuspended in 6-10 µl of hybridization buffer (50% formamide, 2× saline sodium citrate SSC, 10% dextran sulphate, pH 7).

Comparative genomic hybridisation

Normal male metaphase spreads slides were used as targets (Vysis/Abbott, UK). Slides were dehydrated through an ethanol series (70%, 90%, 100%, 3 min in each), and were left to air dry. Slide denaturation took place in 70% formamide, 2× SSC in a water-bath set at 73°C for 5 min. Denaturation was stopped by placing the slides through ice-cold ethanol series. Denaturation of probes took place at 73°C for 10 min, followed by their cooling at room temperature. The fluorescent DNA samples were then placed on to slide target areas. Hybridization took place in a humidified chamber at 37°C for 48-72 h. Post-hybridization washes were carried out in the dark, in the following order: Post-hybridization washes were carried out in the dark, 2x SSC at 73 ° C, 4 x SSC, at 37 ° C, 4x SSC+ 0.1% Triton (Sigma, UK) at 37 ° C, 4x SSC at 37 ° C, and 2xSSC at room temperature, each of which lasted 5 min. Alternatively, once 0.4xSSC/0.3% NP-40 at 65-73°C for 2 min, once 2xSSC/0.1%NP-40 at RT for 2 min and 2xSSC at RT for 2 min whilst shaking. The slides were then dehydrated, air-dried and mounted in antifade containing diamidinophenylindole (DAPI-II, Vysis, UK) to counterstain the chromosomes.

Metaphase spreads were observed with the use of an Olympus BX 40 fluorescent microscope with a cooled charge-coupled device (CCD) system, and filters

for the fluorochromes used. Seven to ten metaphases were captured on average per hybridization. Analysis and interpretation of the captured images was feasible with the use of Vysis Quips CGH software (Vysis/Abbott, UK) that converted fluorescent intensities into a red-green ratio for each chromosome. Equal sequence copy number between the test and reference DNAs was seen as no fluctuation of the ratio profile from 1:1. Test sample under-representation was seen as fluctuation of the ratio profile in favour of the red colouration (below 0.80), whilst test sample over-representation was seen as fluctuation of the ratio profile towards the green colouration (above 1.20). Such fluctuations were respectively scored as losses or gains in the test sample, compared to the reference sample.

Distinction between loss of whole chromosomes and single chromatids was determined as in Fragouli *et al* (2006b) as follows: This was achieved by comparing the fluorescence intensity of the green fluorochrome (Test DNA) on the chromosome presenting loss with that on the euchromatic region of the Y chromosome. Since the Y chromosome is absent from both the test and reference DNA, the fluorescence observed on this chromosome could be attributed to background fluorescence. This acted as a point of reference of the amount of fluorescence expected on a chromosome that had been entirely lost. When some green fluorescence was visible and the chromosome in question was relatively bright compared to the Y chromosome, but fainter when compared to the rest of the chromosomes, then the loss would be due to a single chromatid. Gain of a single chromatid would only be distinguished from whole chromosome gain in cases where the corresponding cell was characterized as having lost this chromatid. Heterochromatic, centromeric and telomeric regions were excluded from analysis, as they tend to show an artefactual deviation of the ratio profile.

Chapter 3.

Results for Preimplantation Genetic Screening and numerical chromosomal abnormalities

The first aim for this study was to devise an efficient protocol for screening preimplantation embryos for aneuploidy and apply it clinically for couples at risk of aneuploidy. After evaluation of its efficiency, the protocol was used to screen for aneuploidy in blastomeres from day 3 preimplantation embryos as well as in follow up analysis of untransferred day 5/6 embryos. This study provided information on the validity of the protocol used for PGS. It also provides significant information about the aneuploidy mechanisms in preimplantation embryos from couples with poor reproductive history. 101 cycles of PGS were performed and the results are described in the following paragraphs. The author provided most of the biopsy and follow up information. Some of the follow up information was provided by Anastasia Mania, Leoni Xanthopoulou and Soha Taskandi. All the follow up information for each PGS cycle was checked by Prof. Delhanty. The author was responsible for data collection and analysis that defined the aneuploidy mechanisms.

3.1. Protocol for aneuploidy screening. Determination and optimization of PGS protocol.

The chromosomes which are mostly involved in aneuploidy were determined from a review of studies primarily in preimplantation embryos. These were chromosomes 13, 15, 16, 18, 21 and 22. The next step was to determine which protocol to use for screening for these chromosomes. The aim was to screen for the most common autosomal abnormalities without losing hybridization efficiency. The Aneuvision probe set (Abbott, UK) screened for 13, 18 21 and X and in two rounds of hybridization and it was one possibility since it was used in this centre for sexing of X-linked disorders and was highly optimized with probe efficiency of 90%. However, only 3 autosomes were screened.

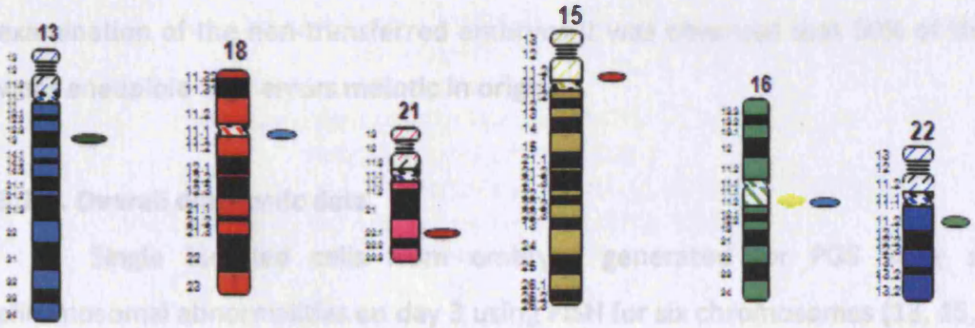
Next, the PB probe set (Abbott, UK) that screened for chromosomes 13, 16, 18, 21 and 22 in one hybridization round was evaluated in lymphocyte preparations. This probe set presented a good number of autosomes for screening and other chromosomes could be added in a second hybridization. However, several problems arose upon evaluation of its efficiency. Problems included wide variations in efficiency between different the probes within or between experiments, bleeding of different signals between filters and difficulty in locating embryonic nuclei without using blue counterstain. Individual probe efficiencies within the PB set were acceptable (if counted individually without the error rate of the other probes in the same nucleus); however the efficiency of the combination of probes ranged from 50-75% in normal lymphocytes. Individual probes within the PB set showed variation in efficiency. The best possible conditions for the PB set produced the following efficiencies: 70% for chromosome 18, 94% for chromosome 16, 95% for chromosome 22, 77% for chromosome 13 and 69% for chromosome 21. The probability of one of these 5 probes failing to hybridise in a single nucleus was very high so it was excluded from consideration. This led to the development of a custom protocol that screened for three chromosomes in each hybridization round thus reducing the possibility of hybridization failure.

Six chromosomes (13, 15, 16, 18, 21 & 22), in two rounds of FISH appeared to give the most efficient and effective results. Details for specific probes used can be found in chapter 2. Hybridisation efficiency ranged from 88% to 95% for the whole probe set for both rounds in normal and abnormal nuclei. All FISH conditions were optimized for single cell work in untransferred spare IVF embryos. The sex chromosomes were omitted for screening since the mild phenotype that sex chromosome abnormalities produce compared to the autosomes made them a low priority for the couples at risk of severe chromosomal abnormalities in their embryos. Also, there is no raised incidence with maternal age overall. Additionally, the addition of two extra probes would have reduced the FISH efficiency and a third round of hybridization would have decreased efficiency since embryonic nuclei tend to degenerate in multiple rounds of FISH. The sex

chromosomes were only screened for if there was a specific reason for an increased risk of sex chromosome aneuploidy such as sex chromosome mosaicism.

The final optimized PGS protocol allowed for flexibility in the probes used in each round. After the HFEA license was granted it was applied to clinical PGS cases. Some problems arose with some probes, in particular for chromosomes 16 and 18 that were both in Spectrum Aqua. The chromosome 18 probe when used in the second round in a single biopsied nucleus faded very quickly, this did not happen in whole embryo FISH. Chromosome 16 probe had similar problems in the second round. This problem was solved by placing chromosome 18 in aqua in the first round. Chromosome 16 was placed in the second round but with a mix of spectrum orange and spectrum green 16 producing a yellow colour upon hybridization which did not fade in the second round. Lower formamide and lower temperature in the post washes of the second round seemed to give even better results by reducing DNA damage to the biopsied nucleus (Detailed protocol in chapter 2). Figure 3.1 shows the ideogram of the chromosomes for this PGS protocol and the location of the probes used in this study.

Figure 3.1. Chromosomes tested in PGS cycles and their probe locations



3.2. Numerical chromosomal abnormalities in embryos from couples undergoing PGS

3.2.1 Lymphocyte and FISH efficiency results

Couples referred for PGS first had their karyotypes checked by a routine clinical cytogenetics laboratory in order to exclude any existing structural chromosomal abnormalities. In our laboratory lymphocyte slide preparations from both partners were analysed after FISH with the diagnostic probe set in order to exclude mosaicism or polymorphic chromosomal areas that could interfere with the screening of day 3 blastomeres. The efficiency of the PGS protocol in both partners was calculated. The overall efficiency (percentage of cells with diploid signals) of FISH in lymphocyte slides ranged from 88% to 96% and no polymorphisms were found in any of the PGS couples. There were however some unusual findings. The lymphocyte preparations from three males that were referred from recurrent miscarriage couples showed considerably increased aneuploidy compared with the controls for the same experiments. The normality rate was below 70% in all and as low as 58% in one case. All three couples had previous aneuploid conceptions and there were no abnormal sperm parameters. One of the couples proceeded to PGS and had a normal live birth with their first cycle; upon examination of the non-transferred embryos it was observed that 50% of these embryos were aneuploid with errors meiotic in origin.

3.2.2. Overall diagnostic data

Single biopsied cells from embryos generated for PGS were screened for chromosomal abnormalities on day 3 using FISH for six chromosomes (13, 15, 16, 18, 21 & 22). All un-transferred embryos (apart from 8 that were cryopreserved) were spread on slides and studied using the same probe set after embryo transfer on day 5.

Table 3.1 summarises the diagnostic information from the PGS cycles. In total, 101 PGS cycles were performed for 76 couples, with average maternal age of 37.8. 1281 oocytes were collected, and 935 embryos were biopsied with an average of 9.3 (±4.1)

embryos per cycle. 90% of the biopsied blastomeres gave results. From these, 18.6% were found to be diploid for the tested chromosomes and 81.5% were abnormal. The overall pregnancy rate was 29.7% per oocyte retrieval, 33.3% per embryo transfer and 39.5% per couple. Implantation rate was 24.6%. There were 26 deliveries of healthy singletons, and 5 very early miscarriages (16% miscarriage rate). All miscarriages were detected at 7 weeks of gestation but no follow up karyotype analysis was done due to lack of material. There were 17 cycles out of 101 that had more than two normal embryos available for transfer.

Table 3.1. Overall results from PGS cycles carried out on day 3 of embryo development

No. of couples	76
Average maternal age	37.8
No. Cycles to biopsy	101
No. oocytes	1281, Average 12±4.1
No. fertilised (2pn)	906 (71%)
No. abnormally fertilised	237
No. of embryos biopsied	935*, Average 9.3± 3.4
No. of embryos with result on biopsy	846 (90%)
Normal for chromosomes tested on biopsy	157/846 (18.6%), Average 1.6± 1.1
Cycles with more than 2 normal embryos	17/101
Embryos transferred	134 (maximum 2)
Embryos cryopreserved	8
No. of embryos abnormal on biopsy	689/845 (81.5%)
Cycles with embryo transfer	89
No. pregnancies	30
Pregnancy rate	
per egg collection with biopsy	29.7%
per embryo transfer	33.3%
Miscarriages	16% (5, all before 7 weeks)
Miscarriage rate per embryo transfer	5.6% (5/89)
Deliveries	27
Ongoing	0
Twin pregnancies	3
	Two delivered twins
	One delivered singletons
Implantation rate	24.6%

**Included 74 embryos derived from abnormally fertilized oocytes, 54 of them were 0PNs which are routinely included in the IVF procedures due to the possibility of missed pronuclear stage. There were also 9 1PNs included because pregnancies have been reported from 1PN embryos (explained as asynchrony in pronuclei formation). For the sake of completion we have included 11 3PN embryos.*

3.2.3. Overall follow up data

Table 3.2 summarises the overall results from the follow up of un-transferred embryos. Follow up results were obtained for 596 of the 787 embryos available for reanalysis (76%). Among these 596 embryos, 53.4% were fully chaotic mosaic and 40.3% were classified as other mosaic types. The most prevalent of the other mosaic types were the aneuploid mosaics (31.9%) followed by those that were diploid/chaotic (26.1%) and aneuploid/chaotic (17.2%). Biopsied embryos resulting from 37 0PN, 5 1PN and 8 3PN were also included in the follow up studies since the pronuclear classification does not always predict accurately the chromosomal status of subsequent embryos.

105 mosaic embryos had diploid cells on follow up and were diagnosed as abnormal on biopsy. In these embryos the diploid cell lines constituted a range of 10% to 65% of the total. In all, only 5.7% of the embryos were uniformly abnormal where all the cells carried the same abnormalities; three quarters of these were aneuploid and the remainder was haploid. Parental origin errors (meiotic) were identified in 16.9% of all embryos. There were 9 embryos that were diploid on biopsy and were not transferred. Six of these were confirmed normal on follow up and three of these were abnormal. Three embryos that were found to be abnormal on biopsy were normal on follow up. From the embryos with no result on biopsy, follow up was obtained in 55 out of 89, eight had been transferred undiagnosed as there was no alternative but no pregnancy resulted from any undiagnosed transfers, and two were frozen as they reached blastocyst on day 5 and were of good quality. The remaining 24 could not be analysed further mostly due to being degenerate by day 5/6.

Table 3.2. Overall follow up data from FISH analysis on embryos not transferred after PGS	
Number of embryos with follow up	596/787 (76%)
Normal on follow up	6 (normal on biopsy- over 95% diploid on follow up)
	3 (abnormal on biopsy- over 90% diploid on follow up)
Abnormal on follow up	3 (normal on biopsy)
	587 (abnormal or no result on biopsy)
Uniformly abnormal incl. haploid	34/590 (5.7%)
Fully chaotic mosaic	315/590 (53.4%)
Other Mosaic (total)	238/590 (40.3%)
Other Mosaic types	
Aneuploid mosaic	76/238 (31.9%)
Diploid/chaotic mosaic	62/238 (26.1%)
Aneuploid/chaotic mosaic	41/238 (17.2%)
Diploid/aneuploid mosaic	20/238 (8.4%)
Diploid /Aneuploid/chaotic	23/238 (9.7%)
Ploidy mosaics	16/238 (6.7%)
Embryos with meiotic errors	100/590 (16.9%)

The follow up results from the abnormally fertilized embryos were included in the overall follow up data. Table 3.3 shows the follow up results of the abnormally fertilized embryos separately.

Table 3.3. Follow up data from biopsied embryos classified as abnormally fertilised at the pronuclear stage.	
Number of abnormally fertilised embryos	74
Abnormally fertilized embryos with result on follow up	41
0PN with result on follow up	27
	<u>0PN follow up results</u>
	Chaotic mosaic: 19
	(8 in RIF, 7 in RM, 4 in AMA)
	Mosaic (no diploid cell line): 6
	(4 in RM, 1 in RIF, 1 in AMA)
	Mosaic (with diploid cell line): 2
	(1 RM and 1 AMA)
1PN with result on follow up	6
	(2 were haploid, 1 chaotic and 1 aneuploid mosaic for RIF, 1 was haploid/chaotic mosaic for RM and 1 chaotic for AMA)
3PN with result on follow up	8
	(4 chaotic and 1 triploid/chaotic mosaic for RIF, 1 chaotic for RM and 2 chaotic for AMA)

Table 3.4 lists the errors detected in this study from the aneuploidy screening of day 3 blastomeres and day 5 embryos. Although the exact abnormalities detected on biopsy were not necessarily present in the embryo after follow up, in cases of reciprocal

mitotic non-disjunction or of chaotic mosaicism for example, it was considered that only seven of the 596 embryos had false positive results, where an error for one of the chromosomes tested did not show in the follow up as expected. Three of those were clinically significant since the embryos were almost entirely diploid on follow while they were not considered for transfer due to the false abnormal result in biopsy. This gives a false positive rate of 1.18%. There were three false negative results which would have resulted in the transfer of two chaotic embryos and one embryo with trisomy 13. The false negative rate is 0.5%. Only one of these errors was due to a clear extra signal of chromosome 18. Unclear results on biopsy were the main cause of these errors due to split signals, poor quality of the nucleus and overlapping signals.

Table 3.4. Errors	
False positive	3/596 One trisomy 18 on biopsy (clear on biopsy)-90% diploid on follow up One trisomy 21 on biopsy (unclear- split signals)- 95% diploid on follow up One trisomy 13 on biopsy (unclear- obstruction in view of nucleus)- diploid on follow up
False negative	3/596 Two normal on biopsy (one clear, one with degenerate nucleus)- fully chaotic on follow up One normal on biopsy (unclear-split signal scored)- Trisomy 13 on follow up
Other errors (in embryos confirmed abnormal with other abnormalities)	4/596 Two for chromosome 18- Trisomy 18 scored on biopsy (not clear- split signals, normal for 18 on follow up One for chromosome 15- Monosomy 15 scored on biopsy (not clear- degenerate nucleus)- Normal for 15 on follow up One for chromosome 16- Monosomy 16 on biopsy, normal for 16 on follow up
Total	10/596
Error rate	1.8%

3.2.4. Maternal age and embryo chromosomal abnormalities

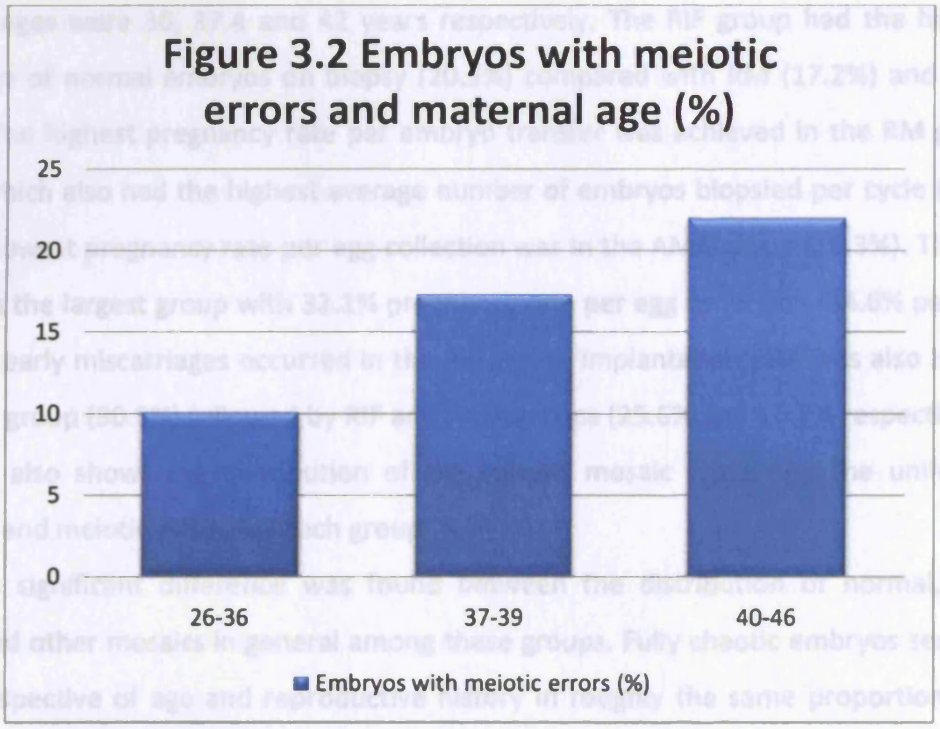
Table 3.5 shows the results of PGS cycles and chromosomal abnormalities found in embryos in relation to maternal age. The results are divided into three age groups 26-36, 37-39 and 40-46 years of age. The distribution of normal and different types of abnormal embryos was investigated within the three age groups. This analysis was done in order to investigate if maternal age alone (irrespective of referral reason for PGS) was a significant risk factor for certain embryo abnormalities. As The normality rate of embryos was lower for the oldest age groups (15.1%) while it was similar for the other two age groups (20.4% and 22% respectively). The pregnancy rate per embryo transfer was higher in the younger age group (42.9%) and dropped with increasing maternal age. This trend was also observed for the implantation rate (32.1%, 17.9% and 16%). The follow up data showed that there were no significant differences between the distribution of uniformly abnormal, mosaic and chaotic mosaic embryos between the three age groups. Within the mosaic groups however there were significant differences in the distribution of the aneuploid mosaics ($p< 0.005$) which was higher in the older age groups reflecting the increase in the meiotic errors with increasing maternal age. The distribution of diploid chaotic mosaics was also significantly different with a higher rate in the younger age group ($p<0.01$).

Table 3.5. Maternal age and chromosomal abnormalities in embryos

Maternal age (y)	26-36	37-39	40-46
No. of couples	32	14	30
No. of cycles.	39	19	43
Average maternal age (y)	33.4	37.7	41.8
No. of embryos biopsied	373, Average 9.5±3.4	175, Average 9.2±3.2	387, Average 9±3.6
No. of embryos with results	333	164	349
Normal on biopsy	68 (20.4%), Average 1.7±1.1	36 (22%), Average 1.9±1.3	53 (15.1%), Average 1.2±0.8
Embryos transferred	56	28	50
No. of cycles with no ET	4	1	6
Pregnancy rate per EC to biopsy	38.5% (15-3 twin pregnancies)	31.2% (6)	18.6% (8)
Pregnancy rate per ET	42.9%	33.3%	21.6%
Pregnancy rate per couple	46.9%	42.8%	27%
Miscarriages	4-early	1- early	0
Deliveries	11, One set twins, 10 single	5	8
Implantation rate	18/56 (32.1%)	5/28 (17.9%)	8/50 (16%)
Result on follow up (abnormal)	219	104	264
Uniformly abnormal incl. haploid	12/219 (5.5%)	5/104 (4.8%)	17/264 (6.4%)
Fully Chaotic mosaics	125/219 (57.1%)	47/104 (45.2%)	143/264 (54.2%)
Other mosaic types	91/219 (41.6%)	46/104 (44.2%)	101/264 (38.2%)
Aneuploid mosaic	15/91 (16.5%)[a]	23/51 (45%)[a]	38/101 (37.6%)[a]
Aneuploid/chaotic mosaic	13/91 (14.2%)	7/51 (13.7%)	21/101 (20.8%)
diploid/aneuploid mosaic	10/91 (11%)	4/51 (7.8%)	6/101 (6%)
Diploid/Chaotic mosaic	34/91 (37.3%)[b]	9/51 (17.6%)[b]	19/101 (18.8%)[b]
Other	19/91 (20.8%)	3/51 (5.9%	17/101 (16.8%)
Embryos with meiotic errors	24/219 (10.1%) *Average 0.6±1.1	18/104, (17.3%) *Average 0.95±1.6	58/264, (22%) *Average 1.3±2.1

*ET- embryo transfer. [a]Significant difference p<0.005 in the distribution of aneuploid mosaic embryos in the three age groups, [b]significant difference p<0.01 in the distribution of diploid/chaotic mosaic embryos in the three groups. *Average number of embryos with meiotic errors per cycle.*

Figure 3.2 shows the percentage of embryos with meiotic errors in relation to maternal age. A general trend of percentage of embryos with meiotic errors increasing with maternal age can be observed. The rate of embryos with meiotic errors was higher for the older maternal age group (22%). A significant difference was found in the number of embryos with meiotic errors when the youngest (26-36) and the oldest groups (40-46) were compared at the 5% confidence level ($t=2.02 > t=1.99$ at 80 degrees of freedom). No significant difference was found in the number of embryos with meiotic abnormalities between the other age groups (26-36vs37-39: $t=0.25$ df56, 37-39vs40-46: $t= 0.76$ df60).



Although certain differences were observed when the data were analysed by maternal age alone these findings might also reflect a bias towards the most common PGS referral group and not be a true representation of the effect of maternal age on embryos. The predominance of the RIF group in the younger age groups in these data series can affect the results and affect the differences in embryo abnormalities. This limitation can

obscure the true maternal age effect in embryo aneuploidy. Analysis of data according to referral reason is therefore more useful in looking at embryo abnormalities.

3.2.5 Referral groups and embryo abnormalities

Table 3.5 summarises the data from the RIF, RM and AMA referral groups. Average maternal ages were 36, 37.4 and 42 years respectively. The RIF group had the highest percentage of normal embryos on biopsy (20.3%) compared with RM (17.2%) and AMA (17.8%). The highest pregnancy rate per embryo transfer was achieved in the RM group (36.6%) which also had the highest average number of embryos biopsied per cycle (9.9 ± 3.4). The lowest pregnancy rate per egg collection was in the AMA group (19.3%). The RIF group was the largest group with 32.1% pregnancy rate per egg collection (34.6% per ET). Four very early miscarriages occurred in the RIF group. Implantation rate was also higher in the RM group (30.8%) followed by RIF and AMA groups (25.6% and 16.7% respectively). Table 3.6 also shows the distribution of the various mosaic types and the uniformly abnormal and meiotic errors for each group.

No significant difference was found between the distribution of normal, fully chaotic and other mosaics in general among these groups. Fully chaotic embryos seem to occur irrespective of age and reproductive history in roughly the same proportion. The distribution of less severe mosaicism appears to differ. While in the AMA and RM groups aneuploid mosaics dominate (35.9% and 41.9% respectively) in the RIF group aneuploid/ diploid/chaotic mosaics were most frequent. Within the mosaic types there was no statistically significant difference between the distribution of aneuploid mosaics but there was a significant difference in the distribution of diploid/chaotic ($p<0.05$) embryos in the groups; there were more diploid/chaotics in the RIF group. There was a significant statistical difference between the distribution of uniformly abnormal embryos ($p<0.05$) and embryos with meiotic abnormalities ($p<0.005$) in these groups. An almost threefold

increase in the percentage of embryos with meiotic errors is evident in the AMA and RM groups versus the RIF group.

Although the number of couples was too low to make any significant comparisons within the RM group, the rate of embryos with meiotic abnormalities did not seem to vary significantly between age groups (within the RM group). For RM women up to 37 years the error rate was 17% and for older women (38-42 years) it was 22%. The largest group overall in this study was RIF. Meiotic abnormalities within the RIF did not differ significantly within the age groups. For women up to 37 years the error rate was 7.9% and for older women it was 9.4%.

Table 3.6. Chromosomal abnormalities in embryos according to indication for PGS

Indication for PGS	RIF	RM	AMA
No. of couples	44	16	16
No. of cycles.	56	19	26
Average maternal age (y)	36 (range 26-44)	37.3 (29-42)	42 (range 39-46)
No. of embryos biopsied	507, Average 9±3.5	188, Average 9.9±3.4	240, Average 9.2±3.5
No. of embryos with results	462	168	215
Normal on biopsy	94 (20.3%), Average 1.6±1.1	29 (17.2%), Average 1.5±1.1	34 (15.8%), Average 1.3±0.9
Embryos transferred	78	26	30
No. of cycles with no ET	4	3	4
Pregnancy rate per EC to biopsy	32.1% (18)	36.6% (7)	19.3% (5)
Pregnancy rate per ET	34.6%	43.8%	21.7%
Pregnancy rate per couple	41%	43.8%	31%
Miscarriages	4	0	1
Deliveries	14	8, one set of twins delivered	4
Implantation rate	20/78 (25.6%)-2xTwin- single births	8/26 (30.8%)	5/30 (16.7%)
Result on follow up (abnormal)	300	118	169
Uniformly abnormal incl. haploid	8/300 (2.7%)[a]	11/118 (9.3%)[a]	15/169 (9%)[a]
Fully Chaotic mosaics	178/300 (59.3%)	61/118 (51.7%)	76/169 (45%)
Other mosaic types	114/300 (38%)	46/118 (39%)	78/169 (46.1%)
Aneuploid mosaic	29/114 (25.4%)	19/46 (41.3%)	28/78 (35.9%)
Aneuploid/chaotic mosaic	15/114 (13.2%)	9/46 (19.6%)	17/78 (21%)
diploid/aneuploid mosaic	11/114 (9.6%)	4/46 (8.7%)	5/78 (6.4%)
Diploid/Chaotic mosaic	39/114 (34.2%)[b]	8/46 (17.4%)[b]	15/78 (19.2%)[b]
Other	20/114 (17.5%)	6/46 (13%)	13/78 (16.7%)
Embryos with meiotic errors	26/300 (8.7%)[c], Average 0.46±0.7	27/118 (22.9%)[c], Average 1.4±2	47/169 (27.8%)[c], Average 1.8±2.5

EC- egg collection, ET- embryo transfer. [a]Significant difference p<0.05 in the distribution of uniformly abnormal embryos within the three groups, [b]significant difference p<0.05 in the distribution of diploid chaotic mosaic embryos within the three groups, [c]significant difference p<0.005 in the distribution of embryos with meiotic errors within the three groups

3.2.6. Insemination method and chromosomal abnormalities

The data were also investigated in relation to the insemination method for each cycle. There were 56 IVF cycles (41 couples) and 47 ICSI cycles (35 couples). For the ICSI group the average maternal age was 36 and for IVF it was 39. There were a slightly higher percentage of normal embryos found in the ICSI group (21%) than the IVF group (17%). There was no significant difference in the distribution of normal, mosaic & chaotic embryos between these two groups. Simple mosaicism was almost identical in the two groups at around 40%.

Embryos with meiotic errors were around 22.5% in the IVF cycles and 9.5% in the ICSI cycles ($p<0.05$), however, average maternal age was higher in the IVF group than in the ICSI group. Similarly, there was a significant difference in the distribution of uniformly abnormal embryos ($p<0.005$), (29/380 in the IVF group vs. 3/210 in the ICSI group), aneuploid mosaic ($p<0.05$) higher in the IVF group and diploid/chaotic mosaics ($p<0.005$) being higher in the ICSI group. However, these results may be mainly the result of the difference in maternal age between the two groups.

Since the ICSI group consisted mostly of RIF couples (36/47 total ICSI cycles were performed for RIF couples) to investigate whether insemination method had any effect data only for the RIF group was used to compare insemination methods. Within the RIF group there were 36 ICSI and 20 IVF cycles. The average maternal age was 37 for the IVF group and 35 for the ICSI group. The information is displayed in table 3.7.

Table 3.7. Chromosomal abnormalities and insemination method within the RIF group		
Follow up information	ICSI-RIF	IVF-RIF
Average maternal age (y)	35	37
Normal (%)	23.4	15.7
Chaotic (%)	53.6	66.4
Uniformly abnormal (%)	2.3	2.2
Mosaic excluding chaotic (%)	45.1[a]	27.6[a]
Aneuploid mosaic (%)	18.4[b]	33.3[b]
Diploid chaotic mosaic (%)	46[c]	8.8[c]
Embryos with meiotic errors (%)	9.6	7.5
Pregnancy (%) per ET	32.3 (2m/s)	38.9 (2m/s)

[a]Significant difference p<0.05 in the distribution of mosaic embryos within the two groups, [b]significant difference p<0.05 in the distribution of aneuploid mosaic embryos within the two groups, [c]significant difference p<0.001 in the distribution of diploid/chaotic mosaic embryos within the two groups.

No significant difference was found in any of the follow up data between the IVF and ICSI cycles in the RIF group for normal, chaotic mosaic and embryos with meiotic errors which shows that these parameters are not affected by the insemination method. However, a significant difference was found in the difference in distribution of mosaic embryos ($p<0.05$), the distribution of aneuploid mosaic embryos ($p<0.05$) and the distribution of the diploid chaotic mosaic embryos ($p<0.001$). Aneuploid mosaics are found to be higher in the IVF-RIF group possibly reflecting the increased maternal age within this group.

In order to see which factor, age, indication or insemination method, is indicative for these observed differences in diploid/chaotic mosaic embryos certain variables have to be excluded. Since the RIF group was the largest studied, comparisons within the RIF group would exclude the variable of the referral reason. Within the RIF group, the distribution of diploid chaotic mosaic embryos differed significantly between two age groups with 44% occurring in the younger age group (up to 37 years) and 24.4% occurring in the older group (38 and older). This also relates to the age specific differences found in the previous section when only maternal age was considered.

Next the insemination method had to be considered within the RIF group. The ICSI-RIF group had significantly higher diploid/chaotic mosaic embryos than the IVF-RIF group. Finally, to exclude indication and insemination method the diploid chaotic mosaic

embryos were compared between younger and older females within the ICSI-RIF group. There was no significant difference between the younger (up to 37 years) and the older age group (38 and older) age group in the distribution of diploid chaotic mosaic embryos within the ICSI-RIF group (41.6.% and 45.6% respectively). This is also true for general simple mosaicism within the ICSI-RIF group between younger and older women (47.7% and 35.5% respectively).

In conclusion the above observations show that the occurrence of simple mosaic embryos appears to be increased in the ICSI-RIF group irrespective of maternal age. Secondly, the diploid/chaotic mosaics are also increased in the ICSI-RIF irrespective of maternal age. Although the number of cycles in each age group in the ICSI-RIF group is relatively low these observation appear to be indicating that the insemination method might play a role in the formation of mosaicism and some types of mosaicism. Therefore paternal sperm factors that caused ICSI to be used might be responsible for certain types of mosaicism within the RIF group.

3.2.7. Specific chromosomes and mechanisms of aneuploidy

The overall data for the specific chromosomal errors where a mechanism could be established are shown in table 3.8. Meiotic abnormalities are the largest identifiable group, because the errors were universal in the embryonic nuclei and the aneuploidy was clearly seen to be of parental origin prior to fertilisation. Errors most commonly affected chromosomes 21 (30%), 22 (18%), and 18 (16%), and trisomy appeared to be more frequent than monosomy although not by a large margin (53% vs 47%). The mechanisms of mitotic abnormalities were less obvious in most cases due to the high frequency of chaotic mosaicism but overall they seemed to affect chromosomes 13 (20%), 15 (19%) and 22 (18%) more often. Mitotic non-disjunction was the most easily identifiable mechanism of post zygotic errors and hence appeared most prevalent (65%), followed by chromosome loss (22%) and lastly chromosome gain (13%). Overall the meiotic and mitotic chromosome error frequency is, in order, 21 (21%), 22 (18%), 18 (17%), 13 & 15 (15%) and 16 (14%).

Table 3.8. Individual chromosomal abnormalities (%)*

Indication	RIF					RM					AMA					Overall							
Chromosomes	Mitosis			Meiosis		Mitosis			Meiosis		Mitosis			Meiosis		Mitosis				Meiosis			Total
	MND	CL	CG	T	M	MND	CL	CG	T	M	MND	CL	CG	T	M	MDN	CL	CG	All	T	M	All	
c13	4.1	4.1	2.7	1.4	2.7	6.3	0.0	0.0	6.3	4.2	5.7	3.8	0.0	3.8	1.9	5.3	3.1	0.9	9.2	3.5	2.6	6.1	15.4
c15	6.8	0.0	0.0	0.0	4.1	12.5	0.0	2.1	0.0	2.1	5.7	1.9	0.0	6.6	3.8	7.5	0.9	0.4	8.8	3.1	3.5	6.6	15.4
c16	8.1	2.7	2.7	4.1	2.7	4.2	0.0	0.0	0.0	4.2	1.9	1.9	0.9	2.8	3.8	4.4	1.8	1.3	7.5	2.6	3.5	6.1	13.6
c18	2.7	1.4	2.7	5.4	2.7	6.3	0.0	0.0	8.3	4.2	5.7	1.9	1.9	2.8	4.7	4.8	1.3	1.8	7.9	4.8	3.9	8.8	16.7
c21	2.7	2.7	1.4	5.4	4.1	4.2	0.0	0.0	14.6	10.4	3.8	0.0	0.0	6.6	10.4	3.5	0.9	0.4	4.8	7.9	8.3	16.2	21.1
c22	5.4	4.1	0.0	8.1	5.4	2.1	0.0	0.0	6.3	2.1	5.7	1.9	2.8	5.7	1.9	4.8	2.2	1.3	8.3	6.6	3.1	9.6	18.0
Total types	29.7	14.9	9.5	24.3	21.6	35.4	0.0	2.1	35.4	27.1	28.3	11.3	5.7	28.3	26.4	30.3	10.1	6.1	46.5	28.5	25.0	53.5	100.0
Total	54.1			45.9		37.5			62.5		45.3			54.7									

MND- mitotic non-disjunction, CL-chromosome loss, CG- chromosome gain

**Mitotic and meiotic embryos can co-exist in the same embryos as well as errors of two or more chromosomes in a single embryo. This table presents errors and mechanisms of aneuploidy of individual chromosomes only and therefore the exact embryo number could not be specified without increasing the complexity of this analysis. This table includes: the total number of embryos with meiotic errors (100 embryos) and 129 embryos with mitotic errors where the mechanism of aneuploidy could be positively identified.*

For the RIF group, the most frequent mitotic errors involve chromosome 16 (25%), 13 (20%) and 22 (17.5%) with mitotic non disjunction being the prevalent mechanism (55%). In meiosis, most frequent errors were observed for chromosomes 22 (29%), 21 (20%) and 18 (18%) while trisomy is in almost equal ratio with monosomy.

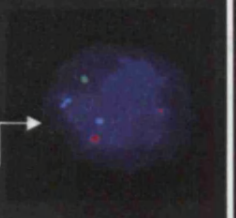
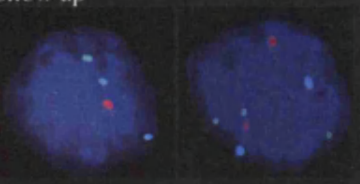
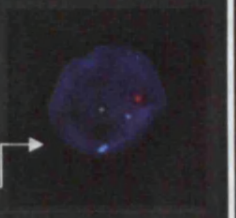
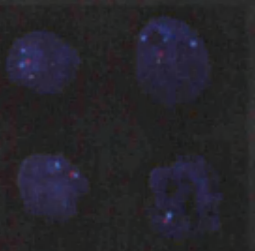
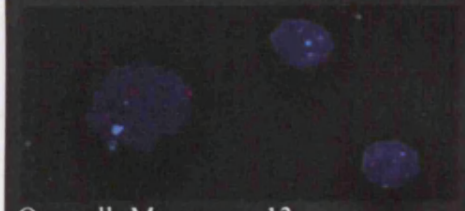
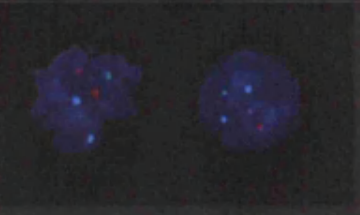
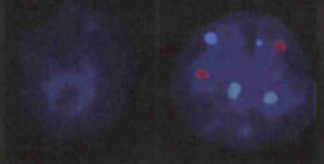
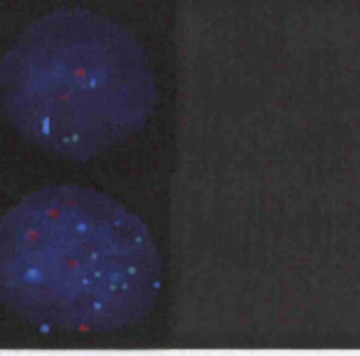
Meiotic errors in the RM group involved mostly chromosomes 21 (40%), 18 (20%) followed by 13 (17%). Trisomy and monosomy was seen almost in a 1:1 ratio. Mitotic non-disjunction was almost the only mechanism of mitotic aneuploidy in the RM group (94%). Most common mitotic abnormalities were observed for chromosome 15 (39%) and 13 (28%).

In the AMA group, most meiotic errors involved chromosomes 21 (31%), 15 (19%) and 18 and 22 (14%). Trisomy was in 1:1 ratio with monosomy. Mitotic non-disjunction was the most common mitotic abnormality (62%). Mitotic errors were most commonly observed for chromosomes 22 (23%), 13 and 18 (21%).

Figure 3.3 shows examples of abnormalities found their mechanisms of aneuploidy. The most frequent errors for individual chromosomes are as follows;

- Chromosome 13- mitotic non-disjunction
- Chromosome 15- mitotic non-disjunction
- Chromosome 16- mitotic non-disjunction
- Chromosome 18- mitotic non-disjunction and meiotic trisomy
- Chromosome 21- meiotic monosomy and meiotic trisomy
- Chromosome 22- meiotic trisomy and mitotic non-disjunction

Figure 3.3. Embryo abnormalities on biopsy and follow up

Biopsy result	Follow up	
 Monosomy 13		Mosaic aneuploid embryo chromosome loss of 21 and 18 and mitotic non-disjunction of chromosome 13
 Monosomy 21		Mosaic aneuploid embryo Meiotic monosomy 21 and Chromosome loss of both copies of 13.
 One cell- Monosomy 13 One binucleate cell- Monosomy 13 (x2)		Uniformly abnormal embryo Embryonic metaphase and interphase showing meiotic monosomy 13 as in biopsy
 Binucleate cell- One nullisomy for all chromosomes, one – diploid.		Mosaic diploid/chaotic embryo. The embryos had normal and highly aneuploid cells on follow up.

3.2.8. Embryo classification and developmental potential

Overall, 192 out of 935 non transferred embryos (20.5%) reached the blastocyst or morula stage by days 5/6 (on day 5/6 all untransferred embryos were spread irrespective of their developmental stage). All the untransferred embryos were scored by an embryologist before their spreading and analysis. Of these embryos 73 were blastocyst and the rest (119) were morulas or cavitating morulas; 96% of blastocysts were mosaic. Table 3.9 shows the chromosomal constitution of these mosaic blastocysts in relation to referral group. For the AMA group 9.5% of embryos reached the blastocyst stage, and most frequent were aneuploid mosaic (23.8%) or fully chaotic (23.8%). In the RM group 11.7% embryos reached the blastocyst stage. Most frequent were the diploid chaotic (18%) and aneuploid chaotic (18%) blastocysts. In the RIF group 5.5% of embryos progressed to the blastocyst stage. One third of these blastocysts (33.3%) were diploid chaotic mosaic followed by aneuploid chaotic (18.5%).

Of the mosaic blastocysts 34/70 (49%) had a diploid cell line as well as chaotic or aneuploid lines and the rest were either fully chaotic or had no diploid cell lines. The diploid cell lines were ranging from 10%- 65% of the total cells within each embryo. There were only three uniformly abnormal embryos and 4 diploid/polyploidy or diploid/haploid mosaic blastocysts.

Table 3.9. Blastocysts and chromosomal abnormalities				
Indication for PGS	RIF	RM	AMA	Overall
Blastocyst Morula	28/507 (5.5%)	22/188 (11.7%)	23/240 (9.5%)	73/935 (7.8%) 119/935 (12.7%)
Uniformly abnormal blastocysts	1		2(m18, t15), (t13, t15)	3
Mosaic blastocysts	27	22	21	70
		Mosaic types		
Diploid/chaotic % of diploid cells present	9 30-60%	4 25-50%	2 40-50%	15 30-60%
Diploid/Aneuploid % of diploid cells present	5 35-65%	1 50%	2 33-60%	8 33-65%
Diploid/polyploid or haploid % of diploid cells present	1 40%	3 25- 35%	0	4 25-40%
Aneuploid mosaic Aneuploidies seen	3 m22, mnd21,cl22	3 m21, m18 mnd 15	5 m15/t15,t22, m13, 2 ploidy mosaic	11
Aneuploid/chaotic Aneuploidies seen in mosaic embryos	5 t16, t22, triploid/chaotic	4 3 multiploid/chaotic, m13,	2 m21, t21	11
Diploid/aneuploid/chaotic % of diploid cells present	1 20%	2 15-32%,	4 10-30%	7 10-32%
Fully chaotic	1	2	5	8
No result	2	3	1	6

m-monosomy, t-trisomy, mnd-mitotic non-disjunction, cl-chromosome loss

Brief summary of findings for this section

- An efficient PGS protocol for chromosomes 13, 15, 16, 18, 21 and 22 was applied clinically.
- A large number of embryos were studied with detailed follow up data where meiotic and mitotic abnormalities were distinguished as well as subtype of mosaic embryos.
- The error rate due to technical limitations was found to be 1.8%.
- Significant differences were found in the distribution of certain mosaic embryos in relation to maternal age. Aneuploid mosaic embryos were found to increase significantly in frequency for women over 37 years compared to women under 36 where diploid/chaotic mosaic embryos were significantly increased. Embryos with meiotic errors were increasing with maternal age.
- Significant differences were found in the distribution of uniformly abnormal embryos, embryos with meiotic errors and diploid/chaotic mosaic embryos in relation to referral group. Uniformly abnormal embryos and embryos with meiotic errors were significantly decreased in the RIF group compared to the RM and AMA groups. Diploid chaotic mosaic embryos were found in higher frequency in the RIF group.
- Within the RIF group significant differences were observed in the distribution of diploid/chaotic mosaic embryos and aneuploid mosaic embryos in relation to insemination method. The RIF-ICSI group had highly significant increase in the diploid/chaotic mosaic embryos. While the RIF-AMA group presented with aneuploid mosaic embryos more frequently.
- Specific chromosomal errors and their mechanism of aneuploidy were identified overall and for each referral group. Differences in specific chromosome susceptibilities were found in relation to the mechanism of aneuploidy overall and for each referral group. Overall, trisomy and monosomy were seen in 1:1 ratio. Mitotic non-disjunction was the most commonly identified mitotic error. The chromosomes most commonly affected were 21, 22, 18 followed by 13, 15 and 16.

Chromosomes 13, 15 and 16 seem to present mitotic non-disjunction most frequently. Chromosomes 21 presents meiotic errors most frequently and chromosomes 18 and 22 can present meiotic and mitotic errors in equal proportions.

- The developmental potential of the untransferred embryos was investigated overall and in relation to chromosomal abnormalities and referral groups. The RIF group had the lowest number of embryos reaching the blastocyst stage among the three groups while the RM group had the highest.

Chapter 4.

Results from studies of preimplantation embryos from carriers of structural chromosomal abnormalities

Twenty seven cycles of PGD for structural chromosomal abnormalities were investigated. 18 cycles were for balanced reciprocal chromosomal translocations, 9 for Robetsonian translocations and 2 for a ring/deleted 22 abnormality. A PGD strategy was also investigated for an inverted interchromosomal insertion. X-inactivation was investigated in embryos from an X;autosome translocation carrier.

4.1.1. Studies of embryos from carriers of balanced reciprocal translocations

Each reciprocal translocation PGD required a specific FISH probe strategy and optimization of each FISH protocol both in the control and carrier lymphocytes for interphase and single cell use. The FISH efficiency was determined by counting 200 interphase nuclei for each protocol. The ideograms and the probe strategies used for each translocation as well as other related details are illustrated in section 2 of the Appendix. As a general rule three to four commercially available probes were used for each translocation. Table 4.1.1 shows the biopsy results from embryos of carriers of balanced reciprocal translocations (RCT). Overall, 18 cycles were performed for 9 couples. The average maternal age was 35.6 years. An average of 9.7 embryos were biopsied per cycle and results were obtained for 93% of embryos of which 22.8% were found to be normal for the chromosomes tested. In 30% of cycles more than two normal embryos were found on biopsy. The pregnancy rate was 33.3% per embryo transfer and the implantation rate was 22.2%.

Table 4.1.1 Overall results from PGD for balanced RCT carriers. Cycles carried out on day 3 of embryo development

No. of couples	9
Average maternal age	35.6
No. Cycles to biopsy	18
No. oocytes	258
No. fertilised (2pn)	158 (61.2%)
No. of embryos biopsied	174, Average 9.7±4
No. of embryos with result on biopsy	162 (93%)
Normal for chromosomes tested on biopsy	37 (22.8%), Average 2±1.4
Cycles with more than 2 normal embryos	6
Embryos transferred	27, Average 1.5
Embryos cryopreserved	5
No. of embryos abnormal on biopsy	125 (71.6%)
Cycles with embryo transfer	14
No. pregnancies	5
Pregnancy rate	
per egg collection with biopsy	26.3%
per embryo transfer	33.3%
Miscarriages	2
Deliveries	3
Ongoing	0
Twin pregnancies	1, One singleton birth
Implantation rate	22.2%

4.1.2. Follow up information for RCTs

Table 4.1.2 shows the follow up information from embryos of RCT carriers for each individual translocation. Overall, 33.9% were chaotic mosaic, 26% were simple mosaic and 17.9% were uniformly abnormal. In some cases chromosomes not involved in the translocation were checked. There was one false positive result where an abnormal embryo on biopsy was 100% normal on follow up. Upon re-examination the error was attributed to signal overlap in the first cell and poor quality nucleus in the second cell. There were no other misdiagnoses. All of the pregnancies that delivered healthy babies came from cases where the female was a carrier and had experienced a number of miscarriages previously. Only one pregnancy was achieved when there was a carrier male parent and that ended in an early miscarriage.

TABLE 4.1.2. Follow up studies in embryos from reciprocal translocation carries									
Carrier of Balanced Reciprocal Translocation	Reproductive history	PGD Cycles	Maternal age	PGD Cycles follow up information					
				PGD pregnancy	Normal (%)	Chaotic	Mosaic	Uniformly Abnormal	Total
46,XX,t(8;12)(q11.2;12)	5 early miscarriages. Two had adjacent-2 segregation	3	36	Yes 2 in 3 cycles one miscarried, one delivered	10 (23)	17	10	5	42
46,XY,t(9;15)(p12;q13)	5 previous ICSI cycles, 1 miscarriage with imbalance	1	38	No	1 (33)	1	0	1	3
46,XX,t(11;22)(q23.3;q11.2)	4 early miscarriages no cytogenetic data available	2	30	Yes 1 in 2 cycles delivered	2 (18)	6	2	1	11
46,XX,t(X;4)(q26;p16.1)	Two previous first trimester induced abortions due to unbalanced karyotypes. Both adjacent-1	3	32	No	7 (25)	6	5	5	23
46,XX,t(9;20)(p13;p11.2)	Four miscarriages and an ectopic pregnancy. One normal child.	2	40	No	3 (25)	0	5	3	11
46,XY,t(1;17)(q42.1;q25.3)	The couple has experienced 2 early miscarriages. They have one healthy girl.	2	37	NO	2 (14)	7	2	3	14
46,XX,t(10;11)(q11.2;p15.3)	Two early miscarriages and infertility. No cytogenetic data.	3	38	Yes 1 in 3 cycles delivered	9 (24)	11	16	5	41
46,XY,t(1;18)(p32;q23)	6 years of infertility and 2 early spontaneous abortions.	1	36	No	1 (8)	3	1	5	10
46,XY,t(1;21)(q12;q22.1)	Infertility and no pregnancies, 2x failed IUI	1	35	Yes Early miscarriage	2 (15)	6	3	2	13
Total		18	35.6	5	37 (22)	57 (34%)	44 (26)	30 (18%)	168*

**Includes unbiopsied embryos*

The chromosomes affected by aneuploidy were mostly the translocation chromosomes. Specifically:

- For 46,XX,t(8;12)(q11.2;12) cycle 1- Re-FISH with X, Y and 18 showed that chaotic embryos were abnormal for the non-translocation chromosomes as well as the chromosomes involved in the translocation.
- 46,XX,t(11;22)(q23.3;q11.2) cycles 1 and 2- Re-FISH with 15, 16, 18 and X, Y showed that only chaotic embryos were abnormal for the non-translocation chromosomes.
- 46,XX,t(X;4)(q26;p16.1) cycle 2- Re-FISH with chromosome 11 and 17 probes showed that they were affected only in 1/3 chaotic embryos. Two more embryos were tetraploid (one tetraploid chaotic, one tetraploid) and one chaotic started as hexaploid. In the polyploid embryos chaotic divisions were only observed for the translocation chromosomes.
- 46,XY,t(1;17)(q42.1;q25.3) cycle 1- Re-FISH with chromosome 13, 18 and 21 probes showed that they were affected only in 1/3 of chaotic embryos.
- 46,XX,t(10;11)(q11.2;p15.3) cycle 1- Re-FISH with chromosome 13, 16 and 21 probes showed that they were affected in 4/6 chaotic embryos.
- 46,XY,t(1;18)(p32;q23) cycle 1- Re-Fish with chromosomes 21 and 16 showed that they were affected for 2/3 chaotic embryos.
- 46,XY,t(1;21)(q12;q22.1) cycle 1- CGH was performed for two embryos - Embryo 1 (1 cell) had monosomy 1q, trisomy 21, 2, 3, monosomy 20, Trisomy 16p/Monosomy 16q and was XO; this cell was classed as chaotic. Embryo 2 (was mosaic- results from 3 cells) had monosomy 1q or monosomy 1, trisomy 21 or trisomy 21q,2.2 and was XY indicating an adjacent-2 segregation). Figures 4.1.1 and 4.1.2 shows examples of the CGH results in single blastomeres for this translocation. Overall, CGH confirmed the biopsy result and revealed other errors.

Figure 4.1.1. Embryo 1 CGH result from 46,XY,t(1;21)(q12;q22.1) PGD. Highly chaotic chromosomal constitution can be observed for many chromosomes. Reference DNA was 46, XY. This embryo appears to be XO.

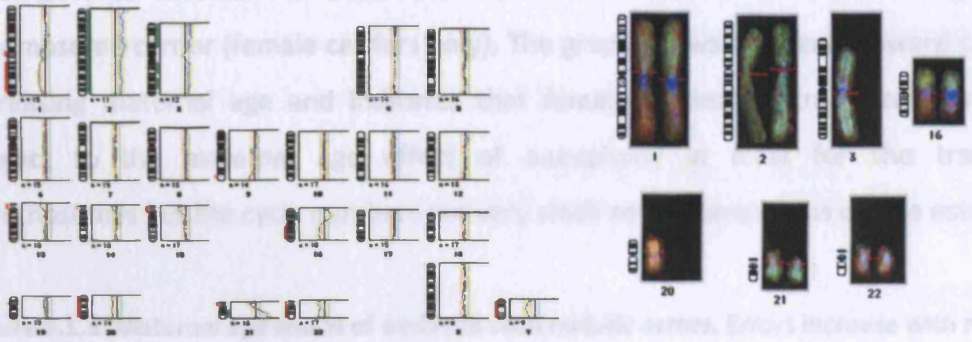


Figure 4.1.2. Embryo 2 cell 3. CGH result from 46,XY,t(1;21)(q12;q22.1) PGD. Reference DNA was 46, XY. This embryo appears to be XY. It also shows monosomy for chromosome 1 and trisomy for chromosome 21. Other chromosomes were not affected in this embryo (3 cells studied by CGH).

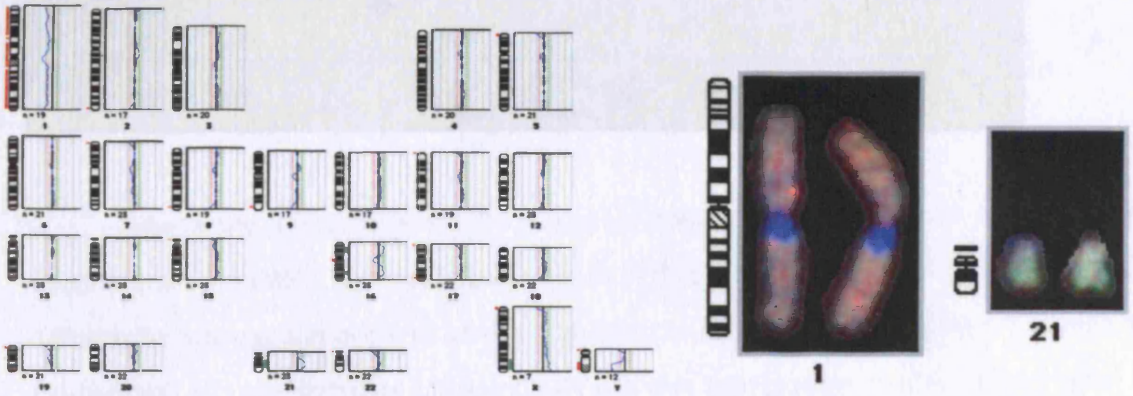


Figure 4.1.3 shows the percentage of embryos with meiotic errors for the chromosomes involved in each translocation in relation to maternal age of the chromosome carrier (female carriers only). The graph shows a general upward trend with increasing maternal age and indicates that female carriers of translocations are also subject to the maternal age effect of aneuploidy at least for the translocated chromosomes but the cycle numbers are very small so no correlations can be established.

Figure 4.1.3. Maternal age and % of embryos with meiotic errors. Errors increase with maternal age.

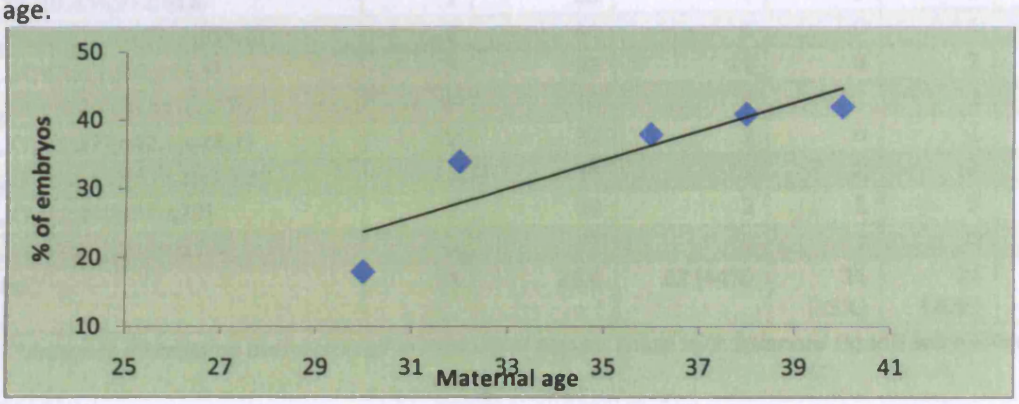


Table 4.1.3 shows the observed segregation patterns of the translocation chromosomes at meiosis for each PGD case. All modes of segregation can be seen. In total, the alternate segregation was the most frequent (44%) followed by 3:1 (22.5%), adjacent 1 (20%) and adjacent 2 (13.5%). Only in one case adjacent-1 segregation was more frequent than the alternate [46,XY,t(1;18)(p32;q23)].

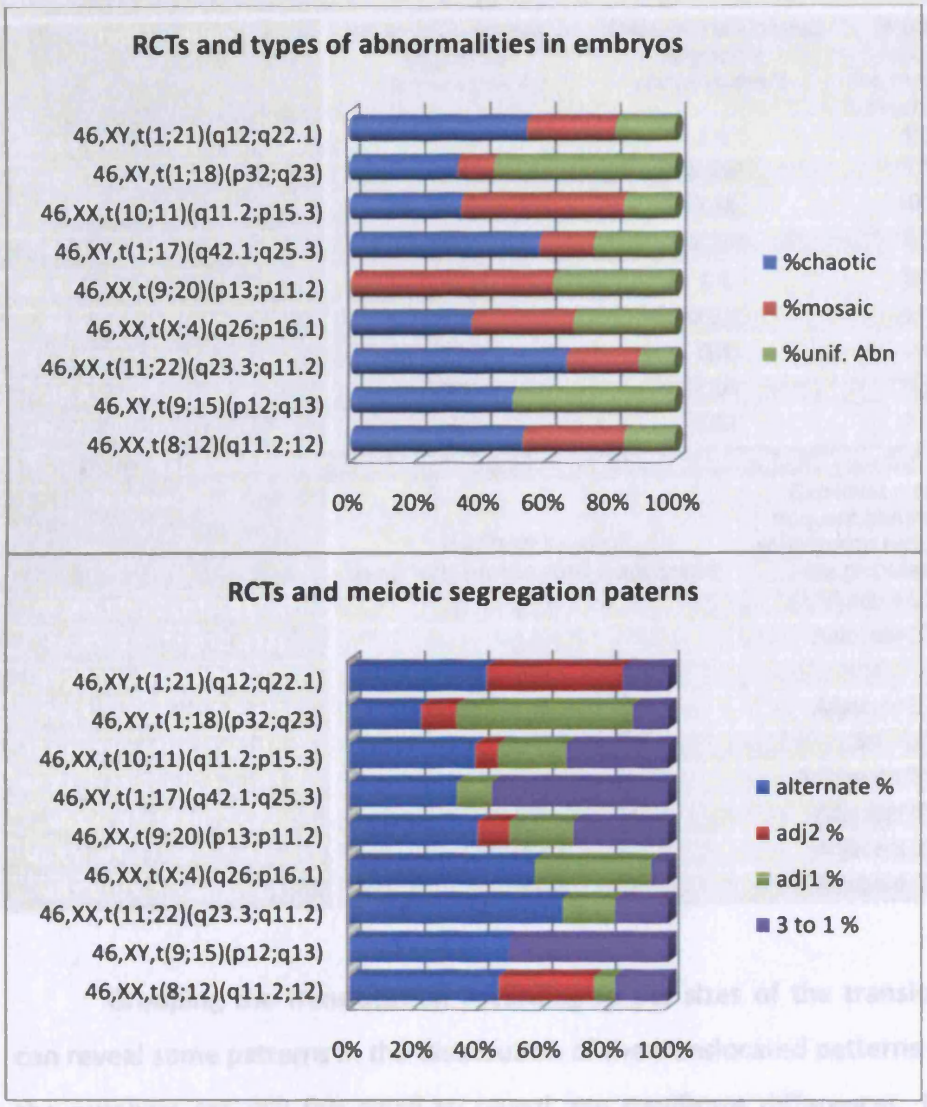
Table 4.1.3. Segregation mode in embryos from reciprocal translocation carriers

Carrier of Balanced Reciprocal Translocation	Cycles	Segregation mode during meiosis					
		Mat. age	Alternate*	adj2	adj1	3 to 1	Total
46,XX,t(8;12)(q11.2;12)	3	36	14	9	2	5	30
46,XY,t(9;15)(p12;q13)	1	38	1	0	0	1	2
46,XX,t(11;22)(q23.3;q11.2)	2	30	4	0	1	1	6
46,XX,t(X;4)(q26;p16.1)	3	32	11	0	7	1	19
46,XX,t(9;20)(p13;p11.2)	2	40	4	1	2	3	10
46,XY,t(1;17)(q42.1;q25.3)	2	37	3	0	1	5	9
46,XX,t(10;11)(q11.2;p15.3)	3	38	11	2	6	9	28
46,XY,t(1;18)(p32;q23)	1	36	2	1	5	1	9
46,XY,t(1;21)(q12;q22.1)	1	35	3	3	0	1	7
Total	18	35.6	53 (44%)	16 (13%)	24 (20%)	27 (23%)	120

**Assumes no crossing over occurred in interstitial region. Those with balanced signals were scored.*

There was great variability in the percentage of normal, chaotic mosaic, simple mosaic and uniformly abnormal between each individual translocation. This variability also affects the segregation patterns at meiosis. This can be observed in figure 4.1.4 were the percentage of chromosomal abnormalities and the segregation modes can be seen for each translocation cycle. For some translocations chaotic embryos are very frequent while for others they are very much reduced. The same applies to the proportion of mosaic embryos. Although the alternate (balanced) segregation appears to be most frequent type in most RCTs in only 3/9 cases the alternate is more than 50% of the total segregation. In the rest, unbalanced segregation constitutes more than 50% of the total. There does not seem to be any relation between the type abnormalities seen in the follow up and the reproductive history of these couples.

Figure 4.1.4. Chromosome abnormalities in embryos from PGD cycles for reciprocal translocations.
The first graph shows the % of chaotic, mosaic and uniformly abnormal embryos in relation to each translocation. The second graph shows the % of each segregation pattern at meiosis for each translocation



In order to see if there is any connection between the chromosomal abnormalities of the embryos and the size of the translocated segments, the % of haploid autosomal length (HAL) was used as a measure of each translocated and centric segment. Table 4.1.4 shows the %HAL of each segment for each translocation and the most frequent observed segregation patterns. It also shows the relative ratio of the sums of centric and

translocated fragments for each translocation as well as expected frequencies of abnormal segregation according to Jalbert *et al* (1980).

4.1.4. HAL, relative ratio of translocated segments and most frequent segregation mode in RCTs

Translocation	%HAL of translocated segments chromosome A	%HAL of translocated segments chromosome B	Relative ratio of translocated segments	Observed most freq.
46,XX,t(11;22)(q23.3;q11.2)	0.75	1.1	1:1	2:2 alt
46,XX,t(X;4)(q26;p16.1)	NA	0.25	1:1	2:2 adj-1
46,XY,t(1;21)(q12;q22.1)	4.16	0.42	10:1	2:2 alt+adj-2
46,XY,t(1;18)(p32;q23)	1.65	0.21	8:1	2:2 adj-1
46,XX,t(10;11)(q11.2;p15.3)	3	1.1	3:1	2:2 alt
46,XX,t(8;12)(q11.2;12)	3.15	3.26	1:1	2:2 alt
46,XY,t(1;17)(q42.1;q25.3)	0.62	0.3	2:1	3:1
46,XY,t(9;15)(p12;q13)	3.04	2.28	1:1	Alt+ 3:1
46,XX,t(9;20)(p13;p11.2)	3	1.92	2:1	Alt+ adj-2

Expected frequencies of abnormal segregation according to Jalbert *et al*, 1980

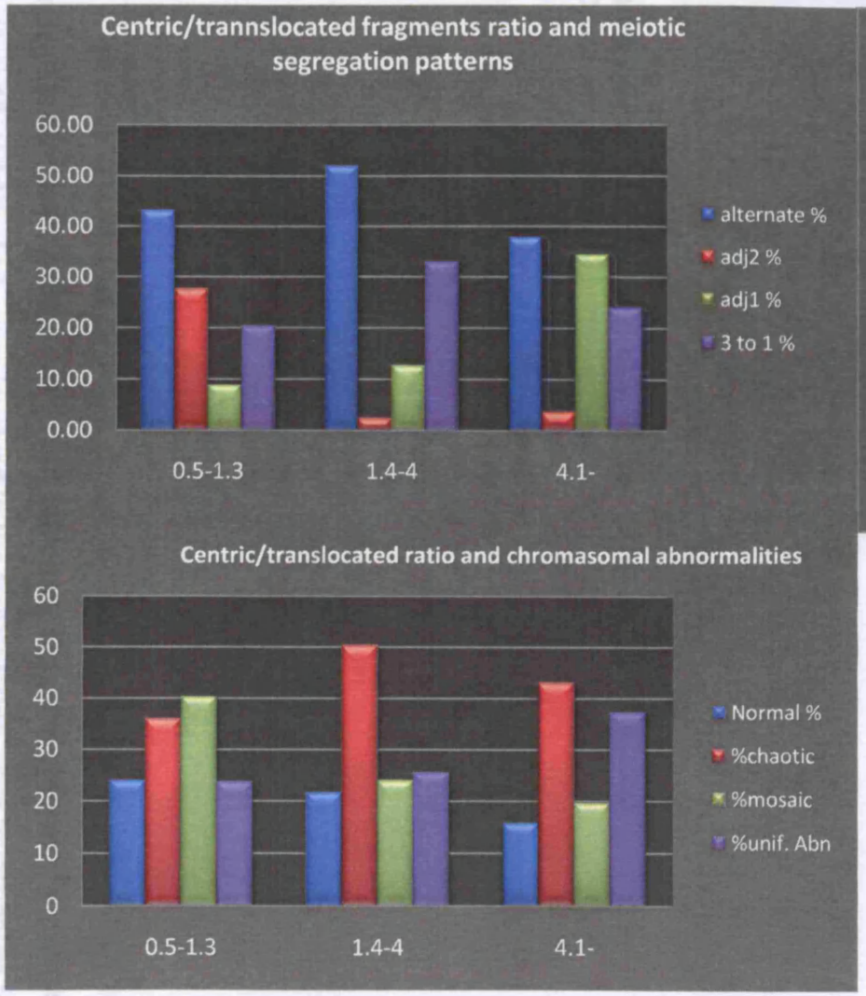
Translocation	**Ratio of sum of centric/translocated fragments**	Expected most frequent abnormal segregation pattern in the gametes	Observed most freq. abnormal
46,XX,t(9;20)(p13;p11.2)	0.50	Adjacent-2	Adj-2
46,XY,t(9;15)(p12;q13)	0.55	Adjacent-2	3:1
46,XY,t(1;21)(q12;q22.1)	1.26	3:1	Adj-2
46XX, t(8;12)(q11.2;q12)	1.50	Adjacent-2	Adj-2
46XX, t(10;11)(q11.2;p15.3)	2.73	3:1	3:1
46XX, t(11;22)(q23.3;q11.2)	3.59	Adjacent 1/3:1	Adj-1/3:1
46XY, t(1;18)(p32;q23)	6.11	Adjacent 1	Adj-1
46XY, t(1;17)(q42.1;q25.3)	12.71	Adjacent 1	3:1
46XX, t(X;4)(q26;p16.1)	25.20	Adjacent 1	Adj-1

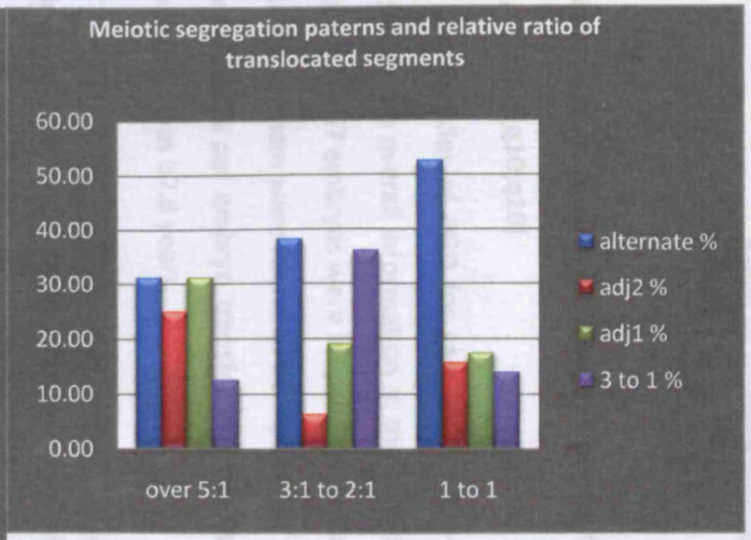
Grouping the translocation according to the sizes of the translocated fragments can reveal some patterns in the distribution of the translocated patterns of RCTs although the numbers are still too small to reveal any significant differences. However, certain trends can be observed. Figure 4.1.5 shows the different segregation patterns when the data are divided according to the size of the translocated and centric segments and their relative ratio.

If the data are divided in three groups according to the size of the centric and translocation segments (sum of centric fragments/sum of translocated fragments) the following observations could be seen. The alternate segregation was more frequent in all groups and higher when the sum of centric and the translocated fragments are roughly the same size. Adjacdent-1 and 3:1 segregation can be found in almost equal proportions when the centric fragments are much larger than the translocated sum, while adjacent-2 is more frequent when the sum of the translocated fragments is much bigger than the centric ones.

Another classification is shown in the second graph of figure 4.1.5 were the % of embryos with each segregation pattern can be seen in relation to the relative ratio of the translocated fragments (HAL of translocated A/HAL of translocated B) shown in table 4.1.5. This graph shows that when very large and very small segments are translocated the frequency of the alternate is almost equal to that of adjacent-1 followed by adjacent-2. When the ratio of the translocated fragments is between 2:1 and 3:1 the alternate segregation is almost equal in frequency to that of the 3:1 segregation, while adjacent 2 is the least frequent. Finally, when the two translocated fragments are almost equal in size and hence in 1:1 ratio, the alternate segregation is by far the most frequent while all other types exist in equal proportions.

Figure 4.1.5. Meiotic abnormalities from RCT embryos and size of imbalance.
The first graph shows the % embryos with specific segregation mode in relation to the size of the translocated segments involved. The second graph shows % of meiotic segregation modes in embryos according to the relative ratio of translocated segments.





4.2.1 Results from studies on embryos from carriers of Robertsonian translocation

45, --, t(13;14)(q10;q10)

Nine cycles of PGD for carriers of 45, --, t(13;14)(q10;q10) were studied. Table 4.2.1 shows the overall information at the time of biopsy. Overall, there were 9 cycles for 6 couples and 67 embryos were biopsied. Results were obtained for 66 embryos on biopsy and 34.8% of them were found to be diploid for the chromosomes tested. The pregnancy rate was 28.6% per embryo transfer and the implantation rate was 21.4%. The average maternal age was 37.8 years.

Table 4.2.1. Overall results from PGD for balanced Robertsonian translocation carriers. Cycles carried out on day 3 of embryo development

No. of couples	6
Average maternal age	37.8
No. Cycles to biopsy	9
No. oocytes	104
No. fertilised (2pn)	74
No. of embryos biopsied	67 (Average 7±3)
No. of embryos with result on biopsy	66 (98.5%)
Normal for chromosomes tested on biopsy	23 (34.8%), Average 2.5±2
Cycles with more than 2 normal embryos	4
Embryos transferred	14, Average 1.5
Embryos cryopreserved	0
No. of embryos abnormal on biopsy	43
Cycles with embryo transfer	7
No. pregnancies	2
Pregnancy rate	
per egg collection with biopsy	22.2%
per embryo transfer	28.6%
Miscarriages	0
Deliveries	3
Ongoing	0
Twin pregnancies	1, One boy, one girl
Implantation rate	21.4%

The overall follow up data are shown in table 4.2.2. There were 32.4% mosaic embryos, 26.4% chaotic embryos and 7.4% uniformly abnormal embryos. Chromosomes other than 13 and 14 which were involved in the translocation were investigated for 4/9 cycles. In couples 1, 4 and 5 other chromosomes were affected in most cells. All couples

had several years of infertility and very few natural pregnancies. Only couple 5 had a child and then had infertility for several years. Most of the couples needed ICSI; even in cases where the female was a carrier, the male had poor sperm parameters.

Table 4.2.2. Follow up data for PGD of Robertsonian translocation carriers

Carrier of Balanced Robertsonian Translocation		Cycles	PGD outcome	Maternal age	Follow up information on PGD cycles				
					Normal (%)	Chaotic	Mosaic	Uniformly abnormal	Total
Couple 1 (XY)	ICSI- poor sperm count	3	Yes Twins delivered	38	10 (33)	6	11	2	19
Couple 2 (XX)	ICSI- poor sperm parameters	1	No	38	2 (40)	1	0	2	3
Couple 3(XX)	ICSI- poor sperm count	1	No	39	5 (50)	2	3	0	5
Couple 4 (XY)	IVF	1	No transfer	37	0	2	1	0	3
Couple 5 (XY)	ICSI- maturity arrest	1	No transfer	40	0	2	0	1	3
Couple 6 (XY)	ICSI- poor sperm count	2	Yes ongoing	36	6 (35)	5	7	0	12
Overall		9		37.8	23 (34%)	18 (26%)	22 (32%)	5 (7%)	68*

**includes embryos with no result on biopsy as well as unbiopsied embryos.*

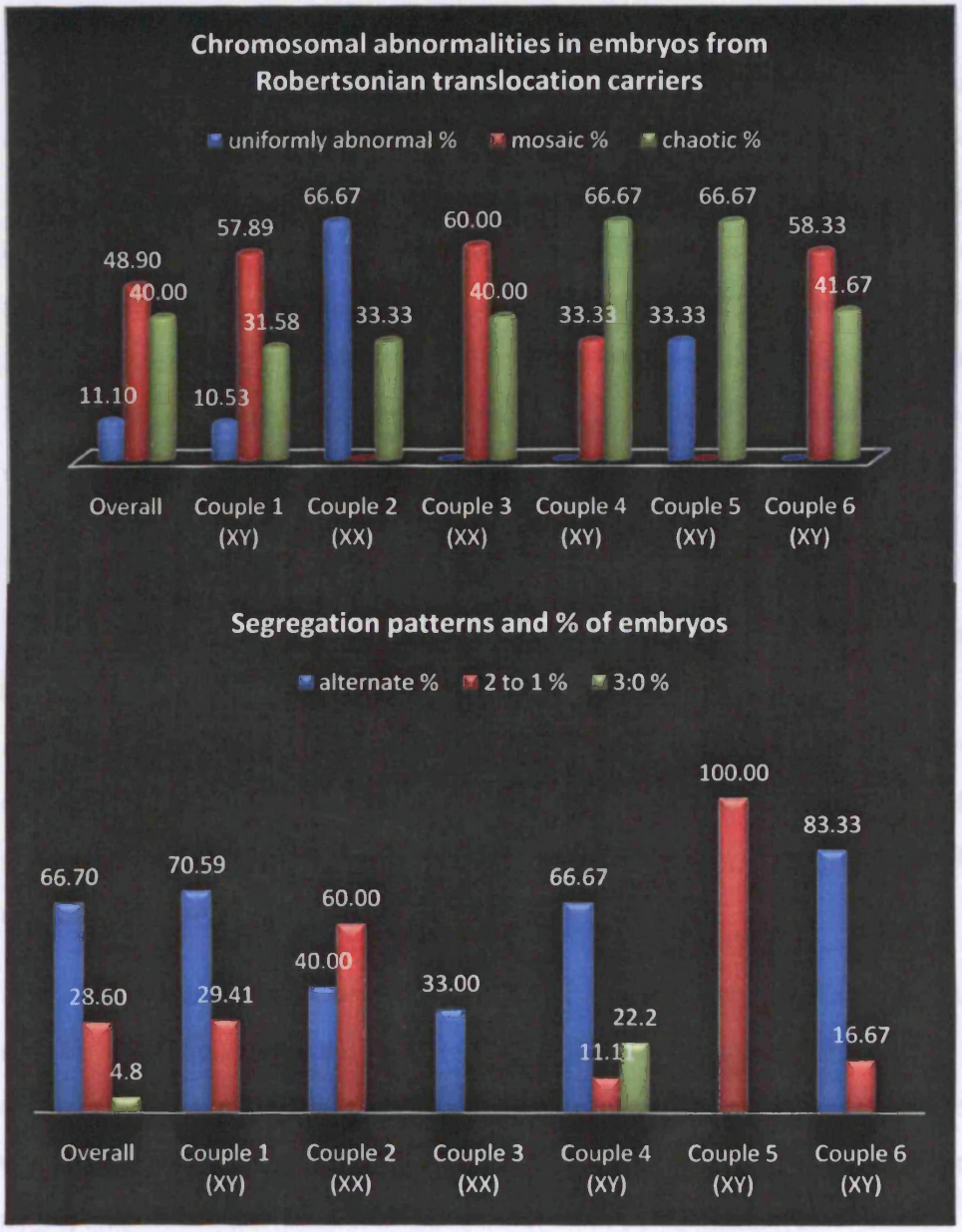
Table 4.2.3 shows the meiotic segregation patterns that were found in embryos. The most frequent was the alternate segregation (69%) followed by 2:1 (27%) and 3:0 (4.4%).

Table 4.2.3. Meiotic Segregation mode in embryos from Robertsonian translocation carriers

Carrier of Balanced Robertsonian Translocation		Alternate	2: 1	3:0	Total
Couple 1 (XY)	ICSI	12	5	0	17
Couple 2 (XX)	ICSI	2	3	0	5
Couple 3(XX)	ICSI	6	1	2	9
Couple 4 (XY)	IVF	1	0	0	1
Couple 5 (XY)	ICSI	0	1	0	1
Couple 6 (XY)	ICSI	10	2	0	12
Overall		31 (69%)	12 (27%)	2 (4%)	45

Although all the carriers have basically the same translocation (it might differ slightly molecularly) there are differences in the distribution of normal and abnormal types of embryos as well as in the distribution of meiotic segregation modes between individuals. Unfortunately, the number of carriers from the different sexes is too small for comparisons. The percentage of uniformly abnormal, chaotic and mosaic embryos varies significantly between individuals as shown in the first graph of figure 4.2.1. Couples 3, 4 and 6 have no uniformly abnormal embryos while couple 2 only presents with uniformly abnormal and chaotic mosaic embryos. The segregation patterns vary between individuals. This is shown in the second graph of figure 4.6. Couples 2 and 5 only present unbalanced embryos from 2:1 segregation while 3:0 appears to be more frequent than 2:1 in couple 3. These differences show that certain individual factors are also responsible for the production of chromosomal abnormalities in embryos from carriers of Robertsonian translocations undergoing PGD.

Figure 4.2.1. Chromosomal findings in embryos from Robertsonian translocation carriers.



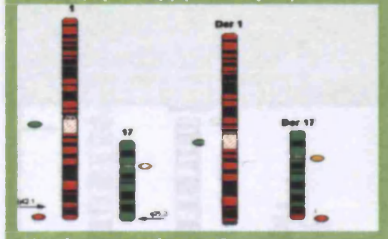
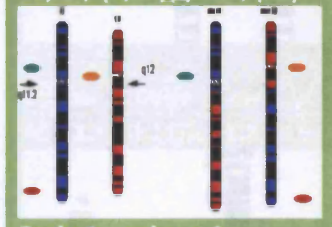
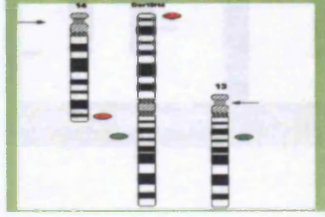
4.2.2. Follow up of embryos from translocation carriers diagnosed as mosaic normal/abnormal on biopsy during PGD.

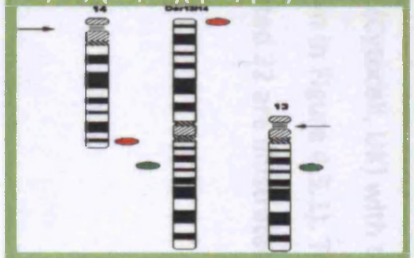
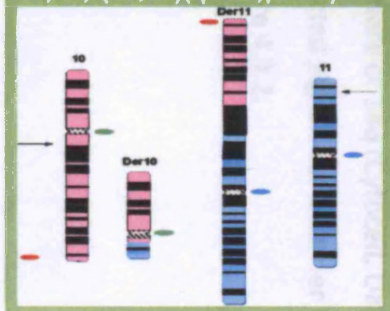
The frequent embryo mosaicism observed during the PGD cycles could have been a source of error. More specifically, embryos where two cells were studied during biopsy sometimes presented a balanced cell and an unbalanced one. This can be explained either by mosaicism or failure of a FISH probe and made any decisions about the chromosomal status of the embryos very difficult. Follow up data from such embryos were evaluated from the PGD cycles for the reciprocal and Robertsonian translocations mentioned above. Table 4.2.2.1 shows the biopsy and follow up results of embryos that presented with normal/abnormal result on biopsy.

In all cases where follow up was available, a result of balanced/unbalanced cells on two cell biopsy was always confirmed. This gives greater confidence in that these mosaic results obtained during diagnosis correspond to a true finding of mosaicism and not an error of the FISH protocol. Two cell biopsy for these PGD embryos in combination with efficient protocols should provide a good representation of the chromosomal status of the rest of the embryo.

As it can be observed from table 4.2.2.1 in most cases an unbalanced gamete resulted in the creation of a mosaic embryo. In these cases the creation of the balanced cell line was a random by-product of the post-zygotic errors. In two cases the gamete could have been balanced and post zygotic errors resulted in a balanced/unbalanced embryo.

Table 4.2.2.1. Follow up of embryos with normal/abnormal result in biopsy for carriers of structural chromosomal abnormalities

Chromosomal abnormality	Biopsy result	Progression Day 4/5	Follow up result	Comment
46,XY,t(1;17)(q42.1;q25)  Cycle 1- embryo 6	Nucleus A. Balanced Nucleus B. Partial trisomy 1qtel	Morula	6 nuclei- Balanced 4 nuclei- Partial trisomy 1qtel 2 nuclei- Partial monosomy 1qtel 1 nucleus - Monosomy 1 1 nucleus- Partial monosomy 1q & trisomy 17 *(ReFISH for 13, 18 and 21- all nuclei normal)*	Biopsy result was confirmed. Balanced/unbalanced mosaic embryo. Possibly started with adjacent-1 segregation and chromosome breakage followed post-zygotically. Gamete Adjacent-1 with chromosomes 1 and der17
46,XX,t(8;12)(q11.2;q12)  Cycle 1- embryo 4	Nucleus A. Trisomy 12 Nucleus B. Balanced	10 cells (8 analysed with FISH).	5 nuclei- balanced 3 nuclei- Partial monosomy 8centric. *(ReFISH for X, Y and 18 - all nuclei normal)*	Biopsy result confirmed Balanced/unbalanced mosaic embryo. Possibly the embryo started with 3:1 segregation where 12, der8 and der12 all segregated together. Successive post zygotic chromosome loss followed. Gamete 3:1 with chromosomes 12, der8 and der12
46,XY,t(13;14)(q10;q10) 	Nucleus A. Balanced Nucleus B. Trisomy 14	Morula	15 nuclei- Trisomy 13 4 nuclei- Balanced 3 nuclei- Trisomy 13 and 14 3 nuclei- Trisomy 14 2 nuclei- Monosomy 13 *(ReFISH for chromosome 11- all nuclei normal)*	Biopsy result confirmed Balanced/unbalanced mosaic embryo. There is evidence of mitotic non-disjunction for chromosome 13. The embryo could have started with the alternate segregation and chromosome gain

Couple 1/Cycle 3- embryo 6				of chromosome 14 followed. Alternatively, the embryo could have started with trisomy 14 and chromosome loss followed. Gamete Balanced or 2:1 with chromosomes 14 and der13/14
46,XY,t(13;14)(q10;q10)  Couple 1/Cycle 3- embryo 7	Nucleus A. Balanced Nucleus B. Monosomy 14	Morula	15 nuclei- Trisomy 13 4 nuclei- Monosomy 14 2 nuclei- Balanced *(ReFISH for chromosome 11- all nuclei normal)*	Biopsy result confirmed Balanced/unbalanced mosaic embryo. The embryo possibly started with the alternate segregation. Chromosome loss of 14 and chromosome gain of 13 followed. Gamete Possibly balanced
46,XX,t(10;11)(q11.2;p15.3)  Cycle 1- embryo 1	Nucleus A. Balanced Nucleus B. Trisomy 10 (centric fragment)	Morula	10 nuclei- Trisomy 10 (centric fragment) 1 nucleus- Balanced 3 nuclei- chaotic *(ReFISH for chromosome 13, 16 and 21- all nuclei normal apart from chaotic in which they showed various signals)*	Biopsy result confirmed Mainly unbalanced/chaotic mosaic embryo. The embryo possibly started with 3:1 segregation (10, 11 and der10). The mitotic loss of the small der10 chromosome can result in a balanced karyotype in some cells. Gamete 3:1 with chromosomes 10, 11 and der10

4.3. Studies for carriers of rare chromosomal abnormalities and special investigations.

4.3.1 Ring/deleted chromosome 22

This couple underwent two cycles of preimplantation genetic diagnosis for a rare ring chromosome abnormality. The female partner is a carrier of a ring chromosome 22 and a deleted chromosome 22 with a karyotype 47, XX, del(22)(p10q12), +r(22)(q10q12) producing a balanced state overall. In both cycles there was no embryo transfer due to all the embryos being affected or carriers of the maternal chromosomal abnormality. When considering the appropriate probes for this particular type of abnormality it was decided that the balanced carriers of the maternal rearrangement need to be detected due to the high risk of mosaicism and instability that is associated with this particular abnormality. The size of the ring chromosomes is around 14Mb. The % of HAL for imbalance of the ring chromosome is 0.6% which is within the viability limits in monosomic and trisomic form.

Cytogenetic workup in parental lymphocytes with commercially available probes for chromosome 22 showed that the centromere of chromosome 22 was split between both recombinant chromosomes 22 in the female carrier. The lymphocyte workup was done with the help of Mariana Apergi. Consequently, two rounds of FISH were used in order to detect all the unbalanced and balanced carriers in the resulting embryos. The DiGeorge probe (Abbott, UK) was used for the first round and the centromeric probe for chromosome 14/22 (Cytocell, UK) with the telomere of 14q (Cytocell, UK) were used in the second round (Shown in Figure 4.3.1). The expected signals for a carrier and non carrier of the ring 22 and deleted 22 are illustrated in Figure 4.3.2.

Figure 4.3.1. *Ideogram and probe strategy of the ring 22/del22 carrier and the PGD probe strategy. First round: DiGeorge- dual band probes (22q11.2 orange/22q13.3 green. Second round: Centromere 14/22 red and 14qtel in green (not shown).*

47,XX,del(22)(p10q12),+r(22)(q10q12)

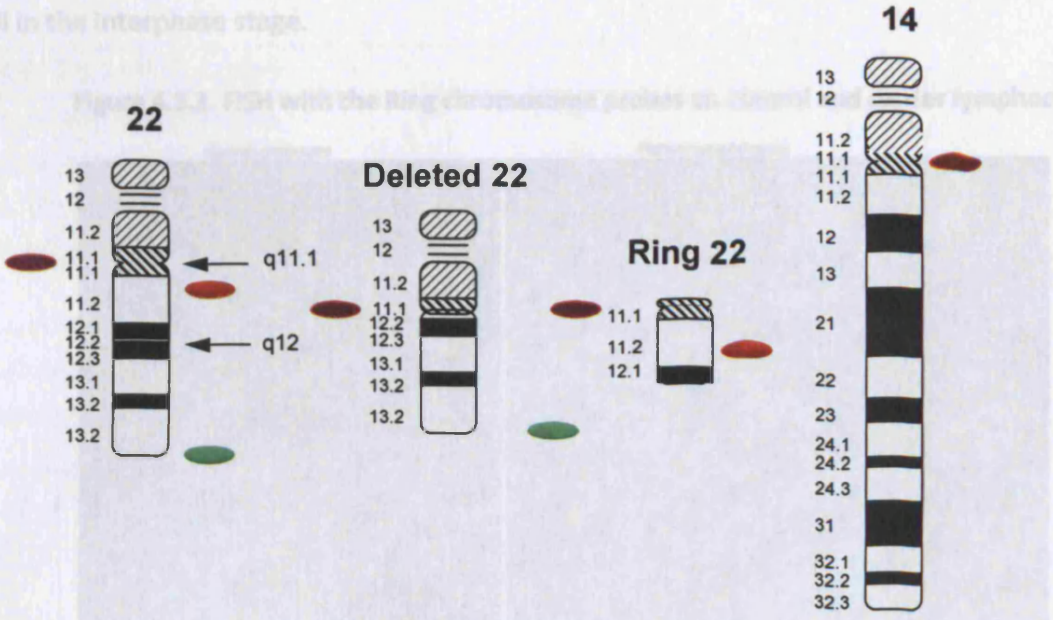
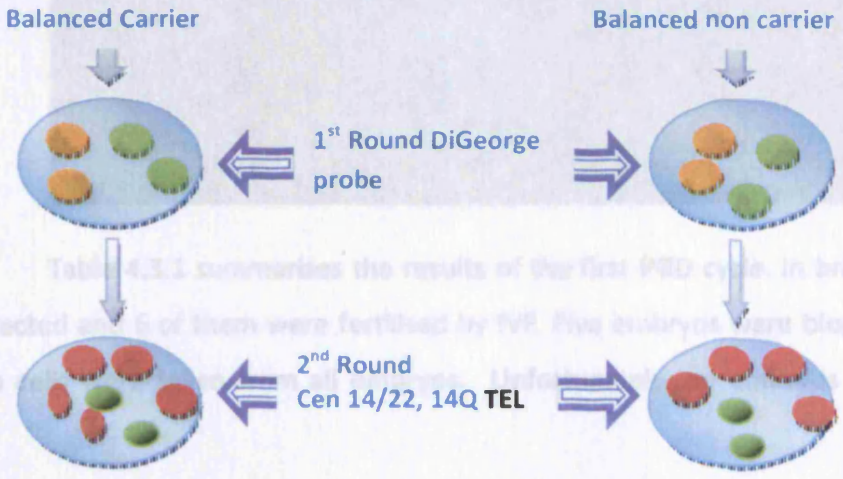


Figure 4.3.2. Expected FISH signals in embryonic nuclei of balanced carriers and normal embryos (non carriers of the ring chromosome 22). No difference in the number of FISH signals can be detected in the first round. While in the second round four equal sized red signals are observed for the non-carrier, the carrier presents five signals three of which are of equal size and two that are smaller. The former combination denotes the splitting of one of the signals for chromosome 22 and thus the existence of the ring chromosome.



The combined FISH probe efficiency on control lymphocytes was 90% and on patient lymphocytes was 95%. Figure 4.3.2 shows the FISH results on control and patient lymphocytes for both metaphase and interphase nuclei. Figure 4.3.4 shows an example of the FISH results on embryonic nuclei from biopsied and untransferred embryos which are all in the interphase stage.

Figure 4.3.3. FISH with the Ring chromosome probes on control and carrier lymphocytes.

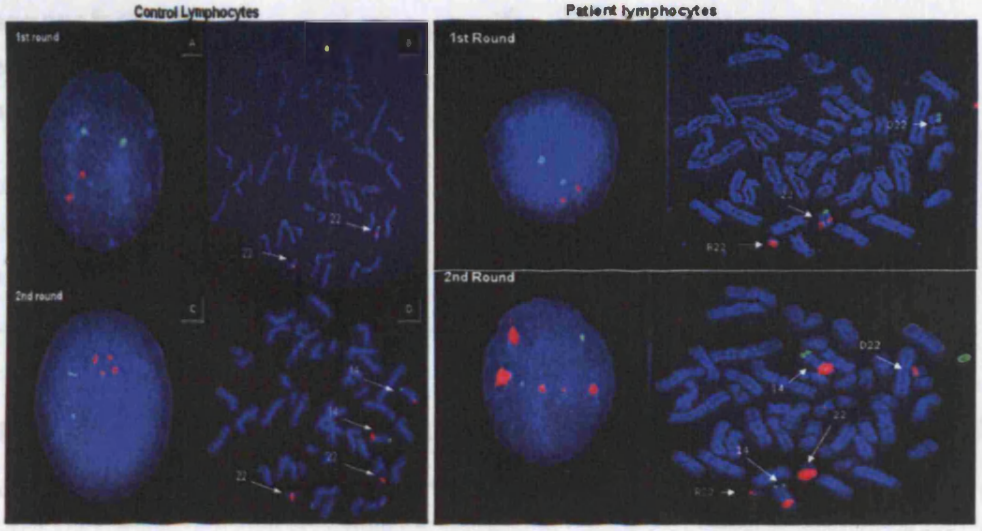


Figure 4.3.4. Results of FISH on biopsied blastomeres from cycle 1 of PGD. *A. First round FISH result blastomere showing loss of ring chromosome 22. B. Normal FISH signals for chromosome 22, the embryo could be a carrier. C. Second round FISH result with Cep14/22 shows balanced carrier of the ring and deleted 22.*

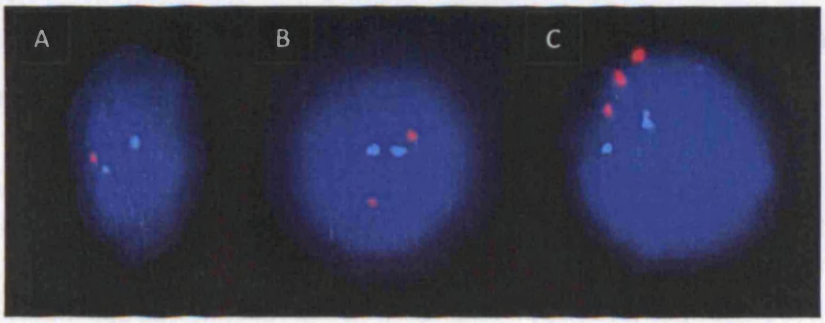


Table 4.3.1 summarises the results of the first PGD cycle. In brief, 7 oocytes were collected and 6 of them were fertilised by IVF. Five embryos were biopsied on day 3 and two cells were taken from all embryos. Unfortunately, no embryos were available for

transfer. A balanced carrier of the ring was found but due to instability of the ring 22 during cell division, the couple decided not to have it transferred. All embryos were spread on slides on day 5 for the follow up. Four out 5 embryos had reached the blastocyst stage by day 5.

Table 4.3.1. PGD cycle 1	
No. of oocytes collected	7
No. of oocytes fertilised	6
No. of embryos biopsied	5
No. of normal or balanced embryos on biopsy	1 balanced carrier (not transferred)
No. of unbalanced embryos on biopsy	4
Embryos with follow up result	4
Embryo progression on day 5	4 blastocysts 1 morula

Results were obtained on follow up for 4 out of 6 embryos (Table 4.3.2). Three embryos were confirmed as aneuploid mosaics. The embryo that was diagnosed as a balanced carrier on day 3 had become mosaic by day 5 with loss of the ring in 50% of the cells. The meiotic segregation of the recombinant and normal 22 chromosomes in the oocytes were determined according to the follow up and biopsy results. One out of the five oocytes started with the ring and deleted 22, two out of five started with only the deleted chromosome 22 present and the remaining two started with the normal 22 and the deleted 22 present (Table 4.3.5).

Table 4.3.2. Follow up results for PGD cycle 1

Embryo no.	Biopsy result	Embryo characterisation after follow up	Mechanism of mosaicism	Theoretical oocyte content for chromosome 22
1	Partial monosomy 22: 46, --, del22(p10q12)	Aneuploid/Chaotic mosaic embryo Aneuploid cell line 46, --, del(22)(p10q12)	Meiotic error and chaotic cell divisions	
2	Balanced carrier of r(22) and del(22): 47, --, del(22)(p10q12), +r(22)(q10q12)	Balanced/aneuploid mosaic embryo. 47, --, del(22)(p10q12), +r(22)(q10q12)[7]/ 46, --, del(22)(p10q12)[7]/chaotic[3]	Loss of r(22) postzygotically	
4	Partial monosomy 22: 46, --, del(22)(p10q12)	Aneuploid mosaic embryo 46, --, del(22)(p10q12)[10]/45,--, -22 [47]	Meiotic error and loss of del(22) postzygotically	
5	Partial trisomy 22: 47, --, +del(22)(p10q12)	Chaotic/aneuploid mosaic embryo. 47, --, +del(22)(p10q12)[5]/chaotic[9]	Meiotic error and chaotic cell divisions	
6	Partial trisomy 22: 47, --, +del(22)(p10q12)	No result on follow up	Meiotic error	

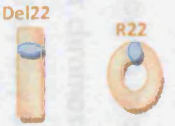
Karyotypes shown assume normality for all other chromosomes

Table 4.3.3 summarises the results of the second PGD cycle. During this cycle, 10 oocytes were collected 8 of which were normally fertilised by IVF. Seven embryos were biopsied, two cells were taken from three embryos and one cell was taken from 4 embryos. No normal or balanced embryos were found in this cycle. All embryos were arrested at the 3-10 cell stage by day 5. Follow up results were obtained for six un-transferred embryos.

Table 4.3.3. PGD cycle 2	
No. of oocytes collected	10
No. of oocytes fertilised	8
No. of embryos biopsied	7
No. of normal or balanced embryos on biopsy	0
No. of unbalanced embryos on biopsy	6 1 gave no result
Embryos with follow up result	6
Embryo progression on day 5	All under 10 cells

Table 4.3.4 shows the biopsy and follow up results for this cycle. Four embryos were fully chaotic. One embryo was aneuploid mosaic, one embryo appeared to be haploid with a single intact copy of chromosome 22 and a single copy of chromosome 14. One embryo was partial monosomy 22 but that was based on only one cell with results. The follow up results helped determine the meiotic segregation of chromosome 22 in the oocytes. Two out of seven oocytes had the ring and the deleted chromosome 22 present. Two out of seven oocytes had the deleted chromosome 22 only and another two had the ring chromosome 22 only leading to partial monosomy 22 after fertilisation. In one embryo there was insufficient information in order to determine the chromosomal complement of the oocyte (Table 4.3.5).

Table 4.3.4. Follow up results for PGD cycle 2

Embryo no.	Biopsy result	Embryo characterisation after follow up	Mechanism of mosaicism	Theoretical oocyte content for chromosome 22
1	Partial monosomy 22: 46, --, r(22)(q10q12)	Aneuploid/chaotic mosaic embryo. 46, --, r(22)(q10q12)[10]/chaotic [9]	Meiotic error and chaotic chromosome divisions	
2	Mosaic partial trisomy 22/Partial monosomy 22 47, --, +del(22)(p10q12)[10]/45,--, -22 [47]	Chaotic embryo	Meiotic error and chaotic cell divisions	
3	Monosomy 22 and 14, normal 22 is present.	Haploid embryo	Possibly one parental genome was present.	Unknown
4	Monosomy 22, ring and deleted 22 are present in the nuclei. 46, --, del(22)(p10q12)r(22)(q10q12)	Chaotic embryo	Chaotic cell divisions	
5	Partial monosomy 22: 46, --, del(22)(p10q12)	Chaotic embryo	Meiotic error and chaotic cell divisions	
7	Partial monosomy 22: 46, --, r(22)(q10q12)	Chaotic embryo	Meiotic error and chaotic cell divisions	

Karyotypes shown assume normality for all other chromosomes

In total, 12 embryos were biopsied, and follow up information was obtained for 11 embryos. No embryos were normal or balanced for chromosome 22 by day 5. There was only one balanced embryo out of 12 biopsied and by day 5 postzygotic errors lead to a mosaic karyotype with half the cells having lost the ring chromosome by the blastocyst stage. In addition, according to the follow up studies, 2 oocytes possibly started with a balanced chromosome complement but both had the deleted and the ring 22 and not the intact chromosome 22. After fertilisation these oocytes accumulated postzygotic errors for chromosome 22 with an end result of being either mosaic or chaotic. The rest of the oocytes are thought to have started with unbalanced products of meiosis. Post-zygotic errors in the resulting embryos were wide ranging and very frequent in almost all the embryos. Table 4.3.5 summarises the theoretical chromosomal complement in oocytes that resulted in PGD embryos and natural pregnancies. Both natural ongoing pregnancies resulted from oocytes that had an extra ring chromosome (24, X, +r22) but none of the PGD embryos presented this combination. Embryo 3 in the second cycle represents the only possibility of an oocyte with an intact chromosome 22 but it was not possible to determine the origin of that single chromosome 22.

Table 4.3.5. Meiotic Segregation in oocytes

Theoretical meiotic segregation in oocyte						
Cycle no.1	0	1	2	0	2	0
Cycle no.2	0	2	2	2	0	0
Natural pregnancies	0	0	0	0	0	2
Total	0	3	4	2	2	2

4.3.2. X;4 Translocation 46,XX,t(X;4)(q26;p16.1) and X-inactivation

Studies of a carrier of an X;autosome translocation were undertaken. The couple had two previous first trimester induced abortions due to unbalanced karyotypes. Cytogenetic analysis of the products of conception showed unbalanced karyotypes due to adjacent-1 maternal meiotic segregation. One had partial trisomy Xq and partial monosomy 4p [46X, der(X), t(X;4)(q26;p16.1)mat]. The other had partial trisomy 4p and partial monosomy Xq [46XX, der(4), t(X;4)(q26;p16.1)mat]. The ideogram and the probe strategy are shown in Figure 4.3.5

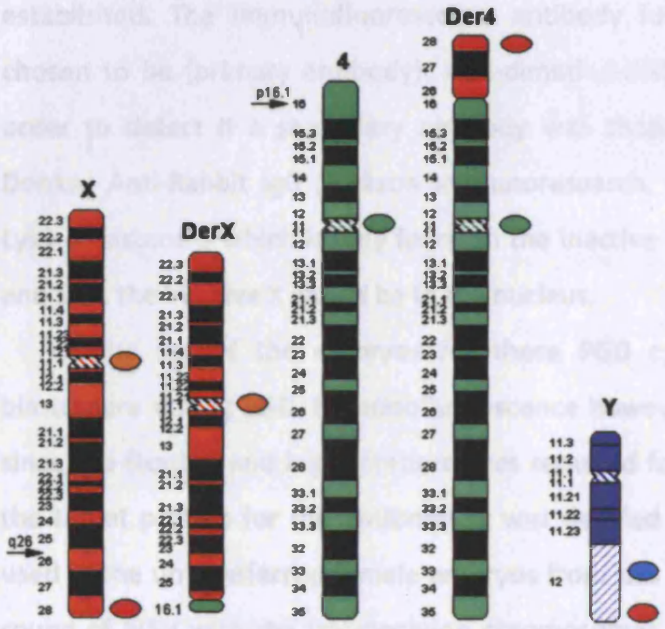


Figure 4.3.5.
Ideogram and PGD probe strategy for 46,XX,t(X;4)(q26;p16.1). The probes used were CEP4 a-sat in green, CEPX a-sat in orange and green, CEPY sat III aqua, Xq\Yqter, subtelomeric probe for the telomeres of X and Y in orange. All from Abbott, UK

The outcome of three cycles of PGD is detailed in section 4.2 among the cycles for reciprocal translocation carriers. Unfortunately, none of the PGD cycles resulted in a pregnancy for this couple. The added complexity for this translocation was X-inactivation and the extent to what this would affect the PGD outcome or any future progeny. The couple was counselled in respect of the PGD outcome and the role of X-inactivation. Any male carrier embryos might have future infertility problems while female carriers might have varying degrees of phenotypic abnormalities depending on X-inactivation. In these

carriers, the normal X might be preferentially inactivated in order to allow the autosomal genes on the derX to function properly. The other alternative is that if random X-inactivation occurs then it might be incomplete so it would not affect the autosomal genes on the derX. X-inactivation studies in this carrier and the embryos produced were attempted in order to establish more information about the patterns of X-inactivation in the preimplantation stage.

In order to attempt to look into X-inactivation in embryos first the sex of the embryo needed to be established using FISH and then immunofluorescence needed to be performed to check if the inactive X was the derivative or the normal one in the embryos. At the same time the patterns of the X-inactivation in the carrier female had to be established. The immunofluorescence antibody for the inactive chromosome X was chosen to be (primary antibody): Anti-dimethyl-Histone H3 (Lys9) (Upstate, UK) and in order to detect it a secondary antibody was chosen to be Cy-3 conjugated AffinPure Donkey Anti-Rabbit IgG (Jackson Immunoresearch, UK). The primary antibody binds to Lys9 of histone 3 which is only found in the inactive X. The localization of histone 3 (lys9) and thus the inactive X would be in the nucleus.

The sex of the embryos for these PGD cycles was checked in the biopsied blastomere during PGD. Immunofluorescence however could not be done after the FISH since the fixation and high temperatures required for FISH would cause degeneration of the target protein for the antibody. It was decided that immunofluorescence would be used in the untransferred female embryos from this couple combined with a subsequent round of FISH with the translocation chromosomes. For this to be achieved, a different spreading method had to be established for embryos other than the tween/HCL method or methanol acetic acid that were currently used. This would also allow antibodies and FISH to be used in the same samples. This method can then be used for this and any subsequent immunofluorescence studies with any antibody on human embryos.

Because the protocol developed for immunofluorescence use all the cells and nuclear structures had to be preserved in human embryos. This would allow detection of targets in the cytoplasm and nucleus of blastomeres. The method chosen (using a

combination of paraformaldehyde and DTT) had been used in mouse oocytes and embryos (Hodges and Hunt, 2002) but not in human embryos. So optimization for human embryos use had to be done first. The protocol was tested in human oocytes and embryos donated from routine IVF patients. Initially, FISH was performed after fixation in the tested samples in order to establish the integrity of the nucleus until the immunofluorescence protocol was ready. Figure 4.3.6 shows some examples of FISH on oocytes and embryos after fixation.

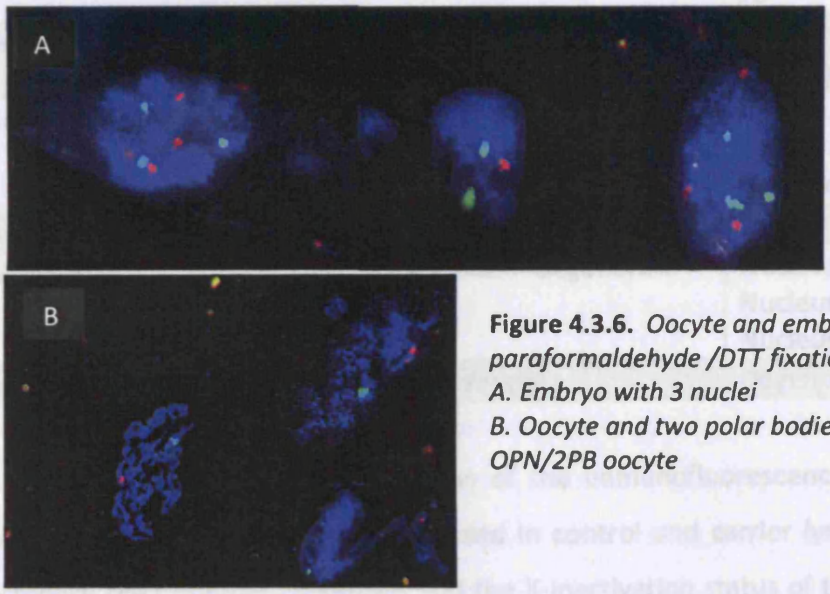


Figure 4.3.6. *Oocyte and embryo FISH with paraformaldehyde /DTT fixation.*
A. Embryo with 3 nuclei
B. Oocyte and two polar bodies from a OPN/2PB oocyte

In general the protocol produced good results especially for the oocytes. Results were produced even in degenerate oocytes. In embryos the results were more varied although FISH signals were visible the integrity of the structures was not optimum. However by omitting the digestion step of the FISH protocol the structures appeared less damaged. Table 4.3.6 shows the results of these experiments.

Table 4.3.6. Results of fixation and FISH in oocytes and embryos

Sample	Fixation result (paraformaldehyde/DTT)	FISH result CEP16 (o), Cep 7 (g)
Oocyte1-0PN/2PB	2 PBs seen	Oocyte chromosomes not found PB1- 2x16, 2x7 PB2- 1x16, 1x7
Oocyte2- 0PN/2PB	Not found	
Oocyte3- 0PN/2PB	Oocyte chromosomes seen and 2 PBs	Oocyte. 1 x16, 1 duplet x7 PbA. 1 duplet x16, 1 duplet x7 PbB. 1 single x7, 1x single 16
Oocyte4-2PN/2PB	Oocyte and PBs seen	Only one PB found- 1x16, 1 duplet x7
Oocyte 5- 0PN/2PB	Oocyte and PB seen	Oocyte. 1 x16, 1x7 PbA. 1 duplet x16 PbB. 1 duplet 7, 1 x 16
Oocyte 6- 0PN/0PB	Oocyte seen	Oocyte only- 2x16, 2x7
Embryo1	3 cells found- degenerate	Nucleus 1. 1x16, 1x7 Nucleus 2. 3x16, 3x7 Nucleus 3. 3x16, 3x7
Embryo2	2 nuclei found	Degenerate with no signals

At the same time optimization of the immunofluorescence protocol was taking place. First the protocol had to be used in control and carrier lymphocytes in order to establish the optimum conditions and the X-inactivation status of the carrier. This part of the study was done by Vinita Shrivastava. First a fixation method had to be established that would allow immunofluorescence and then FISH to be used. The lymphocytes were either, first fixed with paraformaldehyde or methanol after culture or "cytospun" on to slides immediately after culture and then fixed with paraformaldehyde or methanol. The resulting slides were checked and those that contained adequate number of cells underwent FISH primarily to check if the structures were adequate. Unfortunately, the most of the above fixation procedures did not produce good quality cells and after FISH with the translocation probes up to 50% of the nuclei did not contain the correct signals.

As the carrier blood was not easy to obtain each time a new fixing technique was tested this limitation lead to focus on optimizing the immunofluorescence protocol in lymphocytes from the carrier that had already been fixed in 3:1 methanol acetic acid. Although the acetic acid might damage the histone structure a literature review showed that this type of histone might not be damaged by this fixing method. The protocol was optimized and it was found that protocol 2 for immunofluorescence listed in Chapter 2 (PBS/BSA protocol) was the most appropriate to use in lymphocytes and subsequently in embryos from the PGD cycle for this translocation. The lymphocyte study indicated that random and probably incomplete X-inactivation had taken place in the lymphocytes of the carrier of the translocation although the results were not very conclusive due to high fluorescent background artefacts that were contributed to by the fixing method. However, there was a tendency for the normal X to be inactivated in more cells than the derivative X (60%vs40%). If the above pattern was happening, this meant that the autosomal genes might not be switched off when the derivative X was inactivated in that tissue.

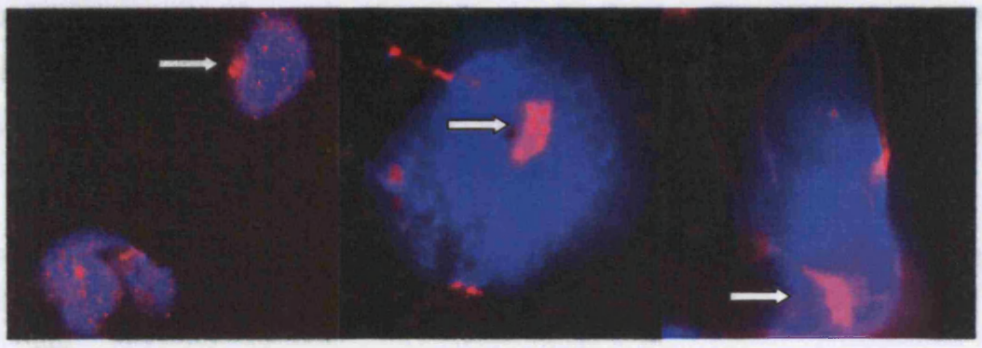
Untransferred embryos from cycle 3 of PGD for this couple were used. They were fixed for immunofluorescence and FISH using the protocol mentioned above. The results are listed in table 4.3.7. The blastomeres were mostly in interphase so co-localization of antibody and the X probe meant that the inactive X was present. Absence of the Xq telomere or the presence of the 4p telomere near the localized antibody on an interphase nucleus meant that the inactive X is the derivative X. There results were varied for each embryo and X-inactivation was not consistently detected. Additionally, although the nuclei after immunofluorescence were intact after FISH they were degenerating. Some nuclei were also lost after the FISH procedure. As a result the outcome of this study was not clear. Figure 4.3.7 shows some nuclei after immunofluorescence for this PGD cycle. Additionally, the embryos in this cycle were not of good quality and were arrested at the 2- 6 cell stage by day 5.

Table 4.3.7. Results of Immunofluorescence and FISH on human day 5 embryos

Embryo number	Biopsy result	Immunofluorescence result	FISH result	Comment
1 Grade 2+ No. cells d3: 6 Day 4: 6	Partial trisomy X & monosomy 4	2n- + ve for antibody	9n 7n- no signals 2n- 3x4, 1xXqYq, 1xX	Expected as trisomy X was seen on biopsy Unfortunately positive nuclei had no signals after FISH and no further information can be obtained.
3 Grade 2+ No. cells d3: 7 Day 4: 5	Pentasomy X, hexasomy 4	-ve for antBX	5n- 6x4, 2xXqYq, 1xX, 1xY	Expected- Male embryo
4	Not biopsied	1n- +ve for antBX	3n- no signals	Degenerate nuclei after FISH.
5 Grade 1- No. cells d3: 3 Day 4: 2	No result	-ve for antBX	4n All chaotic with no Y signal. Multiple X signals	Chaotic embryo, negative for X-inactivation. Not expected.
6 Grade 1- No. cells d3: 6 Day 4: 6	Monosomy X & trisomy 4	1n +ve for antBX	2n 1n- 3x4, 2xXqYq, 1xX 1n- 1x4, 2xXqYq, 3xX	Expected in triple X nucleus only.
8 Grade 2 No. cells d3: 3 Day 4: 2	No result	-ve for antBX	10n 8n-2x4, 3xXqYq, 1xX, 1xY 2n-2x4, 1xXqYq, 1xX, 1xY	Expected as male embryo. There was also multinucleation of cells
10	Not biopsied	-ve for antBX	1n- 1x4, 2xXqYq, 1xX	Expected as only one whole X was present in cell.
11 Grade 1- No. cells d3: 4 Day 4: 4	XXY nucleus	-ve for antBX	4n- 2x4, 3xXqYq, 1xX	Not expected as one X should have been inactive.

antBX- antibody for the inactive chromosome X

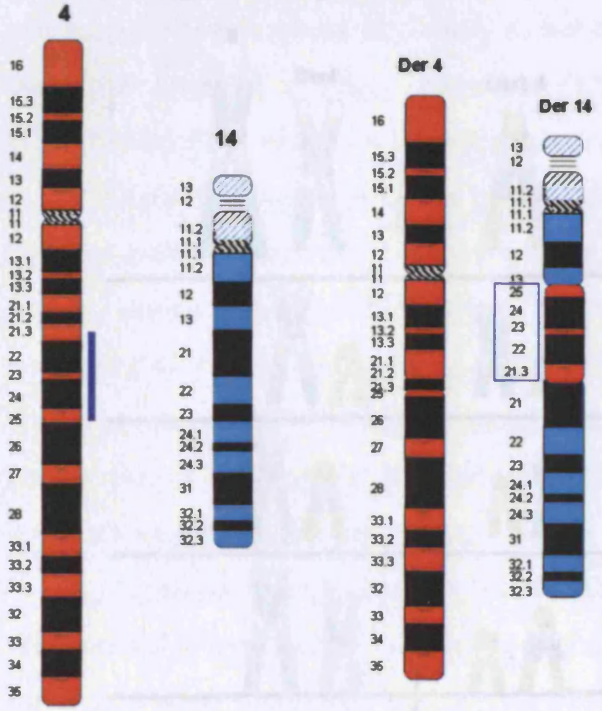
Figure 4.3.7. *Immunofluorescence with X-inactivation antibody for embryos of an X;autosome translocation. Arrows indicate the possible present of the inactive X and are scored as positive stained nuclei.*



4.3.3. PGD protocol development for an interchromosomal insertion carrier

A couple was referred for PGD after having affected pregnancies with 46,XX, der(4), ins(14;4)(q13;q25q21.3) and 46,XY, der(4), ins(14;4)(q13;q25q21.3) effectively monosomic for chromosome 4q21.3-q25. The mother is a carrier an interchromosomal insertion of 46, XX, ins(14;4)(q13;q25q21.3). During the time taken to develop a PGD protocol she also had an affected son with the abnormal karyotype 46,XY, der(14), ins(14;4)(q13;q25q21.3) who was trisomic for chromosome 4q21.3q25. She subsequently also had a trisomy 21 pregnancy. The ideogram for the carrier is illustrated in figure 4.3.8.

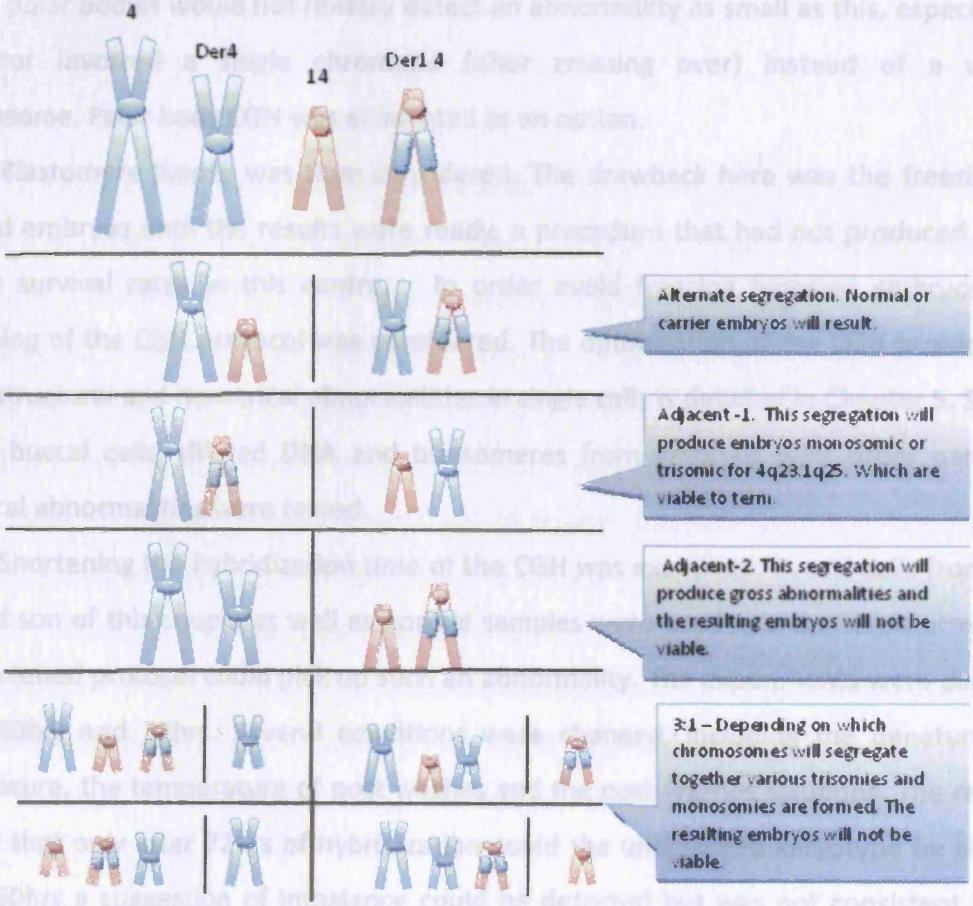
Figure 4.3.8. Ideogram of carrier of insertional translocation 46, XX, ins(14;4)(q13;q25q21.3). The translocated segment is also inverted.



The size of the translocated fragment is around 51.3Mb and constitutes 0.59% of the haploid autosomal length which means that either the deleted or the trisomic state of the translocated segment will produce viable unbalanced pregnancies. In order for the PGD strategy to be informative for all possible meiotic outcomes it had to detect copy changes of the chromosomal region 4q21.q25 segment. The possible meiotic outcomes of

this translocation are illustrated in figure 4.3.9. The difficulty was that there was no commercially available FISH probe for that section of chromosome 4. Two alternatives were considered. Firstly, to construct a FISH probe especially for this segment of chromosome 4 and combine it with commercially available centromeric and telomeric probes for chromosomes 4 and 14. The second option was to use either polar body CGH or blastomere CGH to detect abnormalities in single cells. The abnormality constituted 24% of chromosome 4 and theoretically could be picked up by CGH. As single cell CGH require 72hrs hybridization the results of polar body CGH would be available by day 5 while blastomere CGH would require freezing of embryos until the results were obtained or a shortened CGH protocol.

Figure 4.3.9. Meiotic segregation patterns for the interchromosomal insertion carrier



The first option required a specially made probe for this specific abnormality. As the making of a specific FISH probe would have been very time consuming to construct in-house it was decided the probe would be provided by a company that provides custom made band specific probes. Qbiogene offered this service and a band specific probe for the chromosomal 4q23 band was supplied. However, when this probe was used there were some problems. The main one was that although in metaphase chromosomes the probe could be seen at the correct location, in interphase the signal was very big and very diffuse. This did not allow for correct counting of the probe signals and thus this approach was deemed inappropriate for use in PGD.

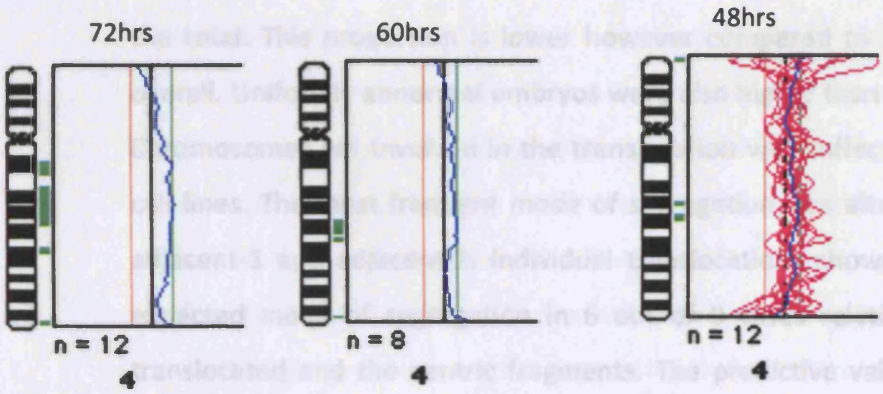
The second option was to use single cell CGH. Polar body CGH was explored since it would avoid freezing of the embryos. However, there were concerns that CGH on first and second polar bodies would not reliably detect an abnormality as small as this, especially if the error involved a single chromatid (after crossing over) instead of a whole chromosome. Polar body CGH was eliminated as an option.

Blastomere biopsy was then considered. The drawback here was the freezing of biopsied embryos until the results were ready, a procedure that had not produced good embryo survival rates in this centre. In order avoid freezing biopsied embryos the shortening of the CGH protocol was considered. The optimization of the CGH protocol to detect structural and numerical abnormalities in single cells is detailed in Chapter 5. Single control buccal cells, diluted DNA and blastomeres from embryos with other parental structural abnormalities were tested.

Shortening the hybridization time of the CGH was examined. Buccal cells from the affected son of this couple as well as normal samples were used in order to determine if the shortened protocol could pick up such an abnormality. The experiments were done at 48hrs, 60hrs and 72hrs. Several conditions were changed, including the denaturation temperature, the temperature of post washes and the post washes solutions. The results showed that only after 72hrs of hybridization could the unbalanced karyotype be picked up. At 60hrs a suggestion of imbalance could be detected but was not consistent in all metaphases from the same experiments. At 48hrs the imbalance was barely visible in each

experiment along with various other artefacts. When more stringent post washes were used the imbalance was not visible at all. Figure 4.3.10 shows some examples of CGH with different hybridization times.

Figure 4.3.10. CGH of single buccal cells of 46,XY, der(14), ins(14;4)(q13;q25q21.3)



The final conclusion from this investigation was that CGH could only be used for PGD for this carrier in single day 3 blastomeres. The abnormality was clearly visible in unbalanced single cells only at 72hrs after hybridisation. This would mean that all embryos would have to be frozen while CGH was carried out. Fortunately, at that time freezing embryos by vitrification was implemented in this centre. This appeared to give good survival rates for frozen embryos at the blastocyst stage. So CGH with 72hrs hybridization and vitrification was chosen as the appropriate course of action for this PGD case. The couple however was fortunate to then have a natural pregnancy with a normal carrier baby and did not require PGD. This case however paved the way for CGH to be developed for clinical use in PGD for chromosomal abnormalities that were not possible by using FISH and a license application is currently being considered by the HFEA.

Summary of main findings for this chapter

- Efficient probe strategies were developed and applied clinically for each PGD for reciprocal and Robertsonian translocation carriers.
- Detailed follow up analysis showed extensive mosaicism (60%) in the embryos from reciprocal translocation carriers. Chaotic mosaic embryos constituted 34% of the total. This proportion is lower however compared to that of the PGS group overall. Uniformly abnormal embryos were also higher than the PGS group at 18%. Chromosomes not involved in the translocation were affected only in the chaotic cell lines. The most frequent mode of segregation was alternate followed by 3:1 adjacent-1 and adjacent-2. Individual translocations showed preference for the expected mode of segregation in 6 out of 9 cases relating to the size of the translocated and the centric fragments. The predictive value of the outcome of meiosis relative to the size of the translocated and centric fragments was investigated and some predictive patterns could be seen.
- Embryos from Robertsonian translocation carriers showed a high number of normal embryos produced (35%) in comparison to those of reciprocal translocation carriers. The level of mosaicism was the same (60%) as in the reciprocal group. But uniformly abnormal embryos decreased (7.4%). The most frequent segregation was alternate (67%) followed by 2:1 (27%) and 3:0 (4.7%). However, the same translocation exhibited different meiotic and mitotic cell division patterns in different individuals. Chromosomes not involved in the translocation were affected mainly in chaotic cell lines.
- Balanced/unbalanced mosaicism in embryos could result in errors in diagnosis after biopsy. The biopsy of two cells reduces the probability of these errors occurring. Follow up analysis showed that this type of mosaicism should be taken as a true finding for the translocation chromosomes. Post-zygotic errors can lead to a balanced cell line in a originally unbalanced embryo.
- A PGD protocol was developed for a carrier of a rare chromosomal abnormality ring/deleted 22. Follow up of embryos revealed for the first time the behaviour of

a ring chromosome during preimplantation development. It revealed the highly unstable nature of the ring and deleted 22 in the embryos while it was very stable in the maternal carrier. The theoretical content of the oocytes (revealed by the follow up embryo studies) showed that the segregation of the normal 22 alone was very rare while most frequently the deleted 22 alone was found.

- A protocol was established for fixation of human oocytes and embryos for use with immunofluorescence and FISH. The X-inactivation patterns in a X;autosome carrier were partially established to be slightly skewed in favour of the normal X being inactivated. To detect if any inactivation was happening in embryos and if the derivative X was inactivated an X-inactivation antibody of Histone 3 (Lys9) was used. In embryos, the X-inactivation antibody could be seen in a limited number of cells within some embryos. The degenerate nature of the embryos contributed to the failure of FISH after immunofluorescence in some nuclei. Inconclusive results were obtained in this study.
- The use of PGD for an interchromosomal insertion carrier was investigated. FISH was not possible as there were no readily available probes for the inserted region. Finally, single cell CGH was optimized to detect the imbalances in blastomeres from day 3 embryos from this carrier. An HFEA licence application was completed.

Chapter 5

Results from studying human oocytes and polar bodies using comparative genomic hybridization (CGH)

Oocytes and their corresponding polar bodies (pbs) from females going through PGS, PGD and routine IVF were studied using CGH in order to ascertain the level of abnormality in these patients and study the mechanisms of aneuploidy during meiosis. The CGH protocol which is detailed in chapter 2 was optimized for single cell use in diluted DNA, buccal cells, trisomic fibroblast single cells and blastomeres with a known abnormality.

5.1. Optimisation of CGH protocol for use in oocytes

The CGH protocol to detect aneuploidy in embryos was followed according to Fragouli *et al* (2006b). However, several checks had to done first to allow the author to confidently identify abnormalities at the single cell level. Additionally, slight modifications had to be done to compensate for variable reagent quality and general experimental conditions. CGH was first used on genomic DNA and when optimized at this level was used on clumps and single buccal cells; these constituted the negative controls. Positive controls were single trisomic fibroblasts. The results for these experiments can be seen in figures 5.1, 5.2 and 5.3 respectively.

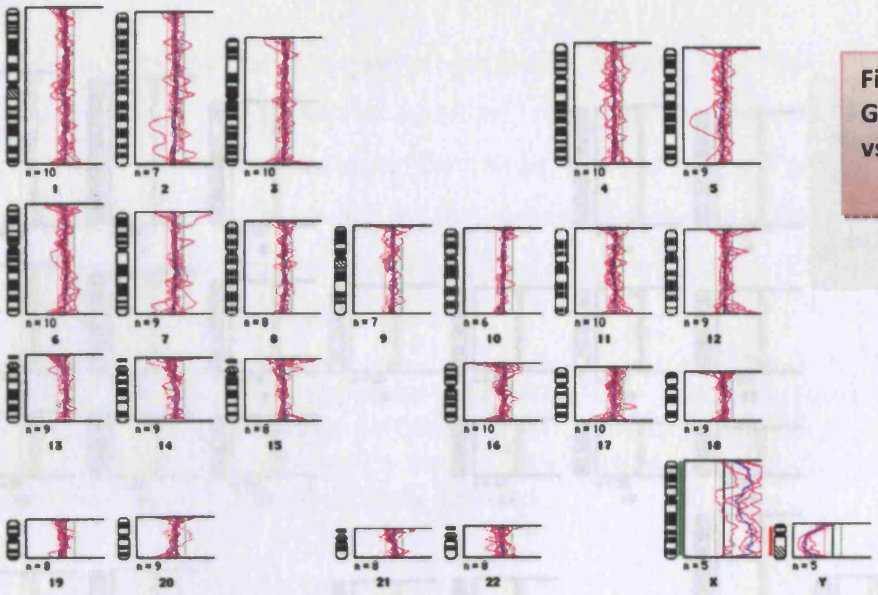


Figure 5.1.
Genomic 46,XX (green) vs Genomic 46,XY (red).

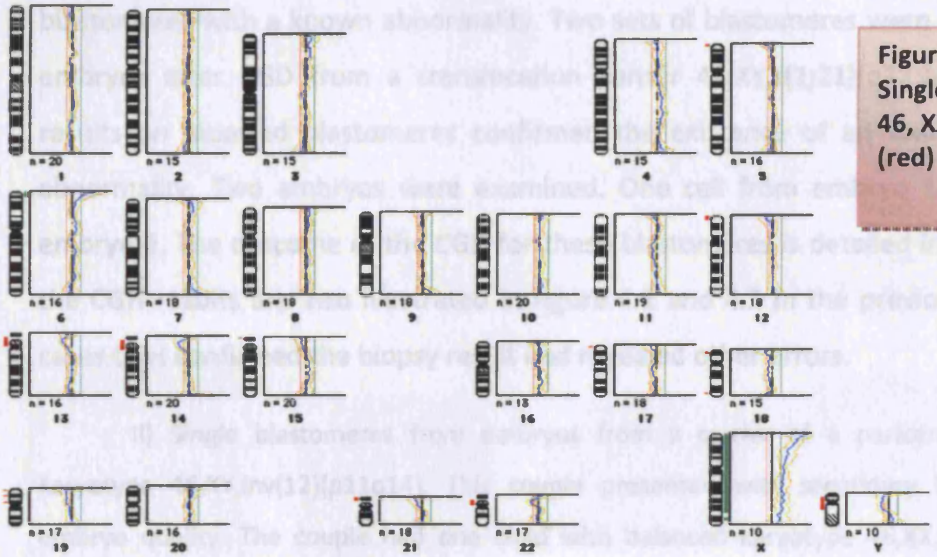


Figure 5.2.
Single buccal cells CGH 46, XX (green) vs 46, XY (red)

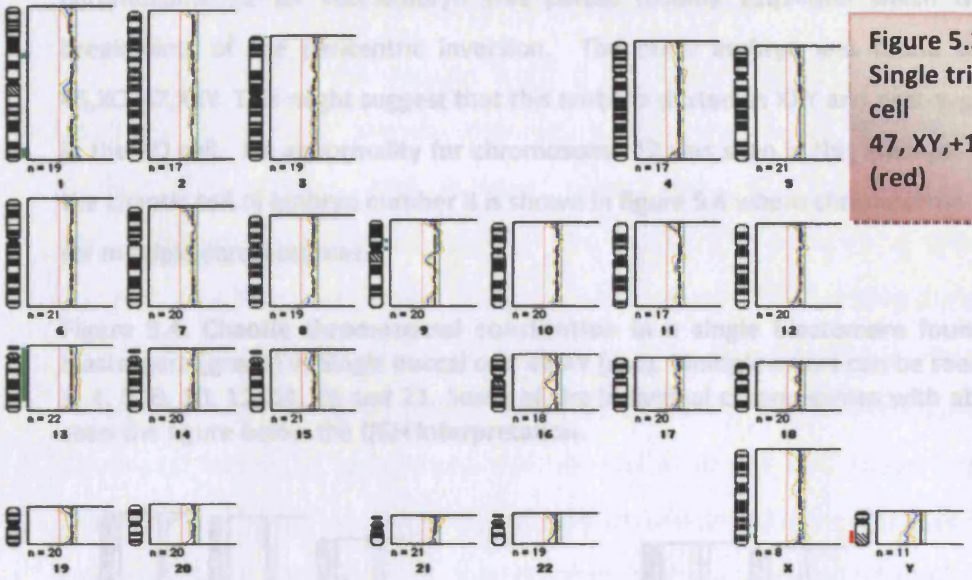


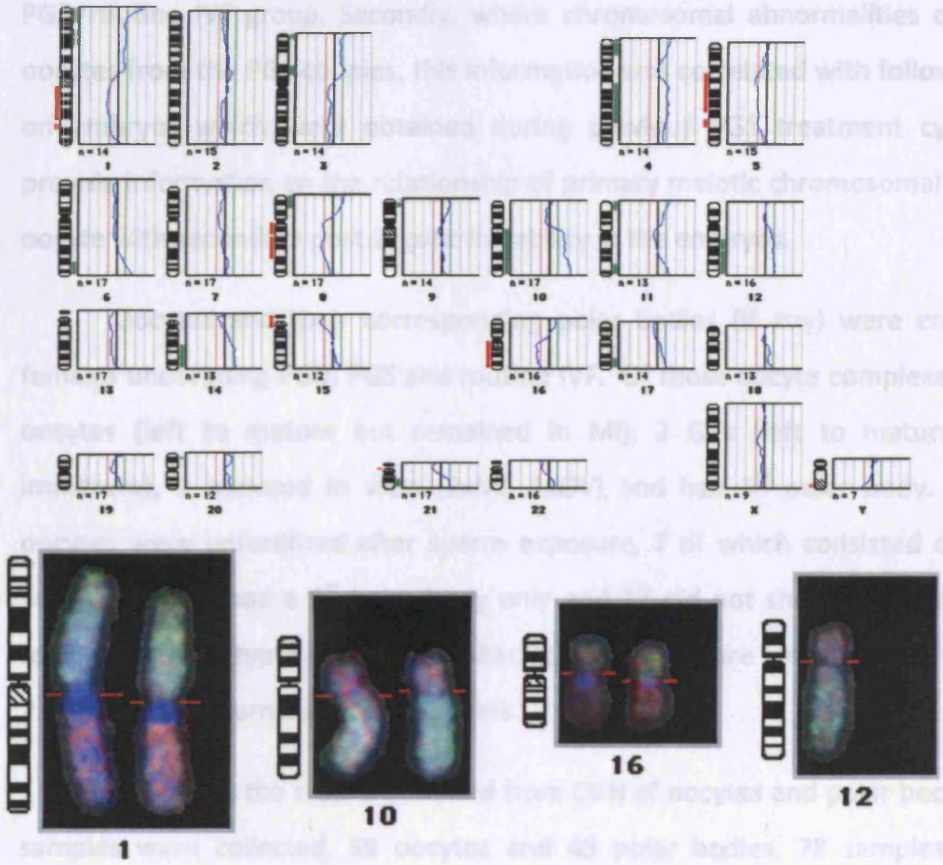
Figure 5.3.
Single trisomy 13 fibroblast cell
47, XY,+13 (green) vs 46,XY (red)

After the control experiments were established CGH was used on single blastomeres with a known abnormality. Two sets of blastomeres were examined. I) From embryos after PGD from a translocation carrier 46,XY,t(1;21)(q12;q22.1) where FISH results on biopsied blastomeres confirmed the existence of an unbalanced structural abnormality. Two embryos were examined. One cell from embryo 1 and 3 cells from embryo 2. The outcome of the CGH for these blastomeres is detailed in section 4.1.2 and the CGH results are also illustrated in figure 4.1 and 4.2 in the previous chapter. In all cases CGH confirmed the biopsy result and revealed other errors.

II) Single blastomeres from embryos from a carrier of a pericentric inversion with karyotype 46,XY,inv(12)(p11q14). This couple presented with secondary infertility and poor embryo quality. The couple had one child with balanced karyotype 46,XX,inv(12)(p11q14)pat. Analysis of routine IVF embryos with FISH (probes 12, 13 and 21) and CGH showed a high number of embryos were found to be chaotic and aneuploid mosaics with only 2 out of 9 embryos tested showing a diploid result for the chromosomes tested; additionally, one embryo showed trisomy for the centromere of chromosome 12. CGH in 5 single blastomeres from two embryos was performed (3 cells from embryo no. 8 and 2 cells from embryo no. 9). One embryo was found to be diploid and chaotic mosaic with XY sex chromosomes. One cell had multiple abnormalities including for chromosome 12 and the other two were diploid (46, XY). The abnormality for

chromosome 12 for this embryo was partial trisomy 12q14qtel which is near one of the breakpoints of the pericentric inversion. The other embryo was found to be a mosaic for 45,XO/47,XXY. This might suggest that this embryo started as XXY and post-zygotic errors resulted in the XO cell. No abnormality for chromosome 12 was seen in this embryo. The CGH result for the chaotic cell of embryo number 8 is shown in figure 5.4 where chromosome breakage is evident for multiple chromosomes.

Figure 5.4. Chaotic chromosomal constitution in a single blastomere found by CGH. Single blastomere (green) vs Single buccal cell, 46,XY (red). Multiple errors can be seen for chromosomes 1, 4, 5, 8, 10, 12, 14, 16 and 21. Some of the individual chromosomes with abnormalities can be seen the figure below the CGH interpretation.



5.2. CGH results from oocytes and polar bodies

The aims in this section were i) the identification of abnormalities in female gametes derived from women undergoing PGS compared to those oocytes derived from routine IVF females and females undergoing PGD for structural chromosomal abnormalities, ii) the examination of the origin of aneuploidy in these two groups of gametes and the investigation of the types of abnormalities arising during female meiosis and iii) the correlation of the oocyte information to subsequent embryo abnormalities. Firstly, the degree and types of chromosomal abnormalities in the studied oocytes were compared between two groups, the oocytes from the PGS group and those from the PGD/routine IVF group. Secondly, where chromosomal abnormalities could be seen in oocytes from the PGS couples, this information was correlated with follow up information on embryos which were obtained during previous PGS treatment cycles. This could provide information on the relationship of primary meiotic chromosomal instability in the oocyte with secondary post-zygotic instability in the embryos.

Oocytes and their corresponding polar bodies (if any) were collected from 15 females undergoing PGD, PGS and routine IVF. Of those oocyte complexes three were MI oocytes (left to mature but remained in MI), 2 GVs (left to mature but remained immature), 3 matured in vitro (2xMI, 1xGV) and had 1st polar body. The remaining oocytes were unfertilized after sperm exposure, 7 of which consisted of both first and second PBs, 28 had a 1st polar body only and 17 did not show a PB. The details of the females and the types of oocytes collected from them are summarised in chapter 2 table 2.4. Average maternal age was 36 years.

Table 5.1 lists the results obtained from CGH of oocytes and polar bodies. Overall, 104 samples were collected, 59 oocytes and 45 polar bodies. 78 samples worked (75%). Results were not obtained from 6 oocyte/polar body complexes (one with 1st and second polar body), 6 oocytes and 7 polar bodies. Results were obtained for 27 oocyte/polar body complexes (1st and/or 2nd polar body), 20 single oocytes and 3 single polar bodies. Out of 78 samples studied, 13 showed chromosomal abnormalities (18%). These included 5

oocyte/polar body complexes where the oocyte and the polar body were abnormal and 3 oocyte only abnormalities (3 without polar bodies and 1 where the polar body was normal). Out of 47 oocytes abnormalities were seen in 8 of them giving an error rate in this sample of 17%.

From the PGS group 53 samples from 10 patients gave results; 20 oocyte/polar body complexes, 13 single oocytes and one polar body. Errors observed were 5 involving both the oocyte and the polar body and 2 the oocyte only. With two exceptions the expected reciprocal results were seen in the MII oocyte and first polar body. This gives an error rate of 21%. The average maternal age was 36.3 years.

Table 5.1. Oocyte CGH study results with patient details

Case	Maternal age	Indication	No of oocytes and PBs with results	Normal (23, X)	Abnormalities seen and karyotype	Oocyte outcome post-fertilisation
O	36	PGS/RIF	2 oocytes and 2 PBs	All	No	2 oocytes- Normal
S1	26	PGS/RIF	4 oocytes and 2 PBs	All	No	4 oocytes- Normal
E1	36.5	PGS/RIF	8 oocytes and 2 PBs	7 oocytes and 1 polar body	One complex (0PN/1PB) Oocyte- 22, X,-12 PB- 24, X, +12	7 oocytes- Normal **Abnormal oocyte** showed meiotic non-disjunction of whole chromosome 12. At risk of **monosomy 12 post** fertilisation.
W	42	PGS/AMA	3 oocytes 1 PB	All	No	3 oocytes- Normal
S2	42	PGS/AMA	4 oocytes and 4 PBs	2 oocytes and 2 polar bodies	Two complexes A. (MII- matured in vitro) Oocyte- 22,- PB- 24, XX B. (0PN/2PB) Oocyte- 22, - PB1- not worked PB2- 23, del(X)(q10qter)	2 oocytes- Normal **Abnormal Oocytes A and B.** Meiotic non-disjunction of chromosome X. Post fertilization with X-bearing sperm would result in monosomy X. With a Y-bearing sperm would result in a non-viable conception.
C	42	PGS/AMA	2 oocytes and 3PBs	All	No	2 oocytes- Normal

M	26	PGS/RIF	2 oocytes and 3 PBs	All	No	2 oocytes- Normal
E2	37	PGS/RIF	3 oocytes and 1 PB	2 oocytes and 1 polar body	One oocyte only (0PN/0PB)- 24,XX	2 oocytes- Normal **Abnormal oocyte** showed disomy X. At risk of trisomy X or XXY post fertilisation.
T	37	PGS/RIF	2 oocytes and 2 PBs,	1 oocyte and 1 polar body	One complex (0PN/2PB) Oocyte- 23, X, dup(20)(q11.2q13.3) PB1- 23,X, del(20)(q12q13.1) PB2- not worked	1 oocyte- Normal **Abnormal oocyte** showed chromosome breakage seen in reciprocal form in the oocyte and PB. At risk of partial trisomy 20q12-q13.1 post fertilization.
E3	38	PGS/RM	2 oocytes and 1 PB	No	Two oocytes and 1 PB A. (0PN/0PB) Oocyte only- 23, dup(X)(q26q28) B. (0PN/1PB) Oocyte 23, dup(X)(p21p22.3), dup10 (p11.2p14) PB- 23, dup(X)(p21p22.3)(q25q28)	**Abnormal oocyte A.** partial duplication for chromosome X. At risk of partial trisomy q26-q28 in an XX zygote or partial disomy in an XY zygote. **Abnormal oocyte B.** Partial duplication for chromosome X and 10. At risk of partial trisomy Xp21-p22.3 and trisomy 10p11.4-p14). The polar body shows the same duplication at Xp21p22.3 and an additional one at the q25q28 as in oocyte A. This might suggest that both X duplications happened before the onset of meiosis.
A1	37	Routine IVF	2 oocytes and 1 PB	2 oocytes and 1 polar body	No	2 oocytes- Normal
V	33	PGD/Non-carrier of translocation 46, XY, t(1;4)(q11.1;	3 oocytes and 3 PBs	All	No	3 oocytes- Normal

		q33)				
R	40	PGD/Carrier translocation 46,XX, t(9;20)(p13;p 11.2)	1 oocyte	No	Oocyte-23,X, dup(20)(p11.2p13)	Abnormal oocyte- Expected structural abnormality seen for the translocated chromosome 20. At risk of partial trisomy 20p11.2-p13.
A2	31	PGD/Carrier translocation 46, XX, t(8;10)(p23;q 24)	2	2 oocytes	No	2 oocytes- Normal
S3	38	PGD/Non carrier translocation 46,XY,t(9;15) (p12;q13)	7 oocytes and 6 PBs	6 oocytes and 6 polar bodies	Oocyte fertilized with unbalanced sperm from translocation 46,XY,t(9;15)(p12;q13). The oocyte was probably normal. Corresponding PB- normal	6 oocytes- Normal One oocyte was normal and was fertilised by abnormal sperm. The resulting zygote had – duplication 9p12-pter, deletion 9q12-qter and duplication 15q13-pter, deletion 15q13-qter.

RIF- repeated implantation failure, AMA- advance maternal age, RM- recurrent miscarriage, PB- polar body, PN- pronucleus

Table 5.2 relates the oocyte results with the findings in embryos from the PGS couples in the cases were there was follow up information. Five out of seven abnormalities observed in the PGS group involved chromosome X. Four of the X-chromosome abnormalities were from the patients with a history of AMA and recurrent miscarriage (patients S2 and E3 respectively). There were two meiotic non-disjunction events visible in both the oocytes and the corresponding polar body; two were reciprocal. One involved non-disjunction of a whole chromosome 12 (patient E1). The second one involved non-disjunction of a chromosome X (S2, oocyte A). Oocyte B from patient S2 showed nullisomy X in the oocyte and one of the polar bodies showed partial nullisomy Xq10-qter. The other polar body of this complex did not show any results. Theoretically, this could be explained if the abnormality involved non-disjunction of a chromatid X in meiosis I that could result in an hypohaploid oocyte and an hyperhaploid polar body. In meiosis II malsegregation of the remaining chromatid X in the second polar body and chromosome breakage during division could result in the present abnormality in the second polar body.

Table 5.2. Embryo follow up information from PGS cycles of females with oocyte information

Case	Maternal age	Indication	Embryo follow up	Abnormalities in oocytes
O	36	PGS/RIF	ICSI: 88% mosaic, 12% chaotic, 25% meiotic abnormalities. Pregnancy-delivered	No
S1	26	PGS/RIF	ICSI: 100% chaotic	No
E1	36.5	PGS/RIF	ICSI: 50%mosaic, 46% chaotic, 4% meiotic Pregnancy-delivered	Yes. Chromosome 12 in one oocyte
W	42	PGS/AMA	IVF: 60% mosaic, 20% chaotic, 60% meiotic	No
S2	42	PGS/AMA	ICSI: 40% mosaic, 60% chaotic, 0% meiotic Pregnancy-delivered	Yes. Chromosome X in two oocytes
C	42	PGS/AMA	ICSI: 100% chaotic	No
M	26	PGS/RIF	ICSI: 75% mosaic, 25% chaotic, 0% meiotic Pregnancy- miscarried	No
E2	37	PGS/RIF	ICSI: 67% mosaic, 33% chaotic, 0% meiotic	Yes. Chromosome X in one oocyte.
T	37	PGS/RIF	ICSI: 100% chaotic	Yes. Partial chromosome 20 in one oocyte.
E3	38	PGS/RM	ICSI: 100% chaotic	Yes- Partial chromosome X in two oocytes. Partial chromosome 10.

Results from CGH on oocytes

There were four structural chromosomal abnormalities seen. Three X structural chromosomal abnormalities came from two oocytes in one female undergoing PGS with a history of recurrent miscarriage, and involved the terminal regions of chromosome X in the p and the q arms. Additionally, chromosome 10 was also affected in one of these oocytes. Another structural abnormality involved chromosome 20 which was seen in reciprocal form in both the oocyte and the corresponding polar body. This suggests that chromosome breakage or abnormal recombination or duplication took place in the oocyte during meiosis I.

Twenty five samples of non PGS oocytes/pbs were studied from 4 PGD patients and one having routine IVF. These included 7 oocyte/polar body complexes, 7 single oocytes and 2 single polar bodies. The average maternal age was 35.8 years. There was one abnormality seen from an oocyte of a translocation carrier 46,XX, t(9;20)(p13;p11.2) showing 23,X, dup(20)(p11.2p13). One more abnormality was detected in an oocyte from a partner of a carrier for translocation 46,XY,t(9;15)(p12;q13) (Female S3, Table 5.1) in the oocyte and not the corresponding polar body. This abnormality however was due to unbalanced sperm fertilizing the oocyte and thus the oocyte was considered normal. No X chromosome aneuploidy similar to that seen in the PGS group was observed in these oocytes.

Some of the abnormalities visible with CGH in the study are shown in the figures 5.5, 5.6, 5.7, 5.8, 5.9, 5.10 and 5.11.

Figure 5.5. *Meiotic non-disjunction of chromosome X visible in an oocyte/polar body complexA from female S2. Reference DNA is 46, XX labelled in red in both cases. The oocyte lost an X and the PB has an extra X chromosome*

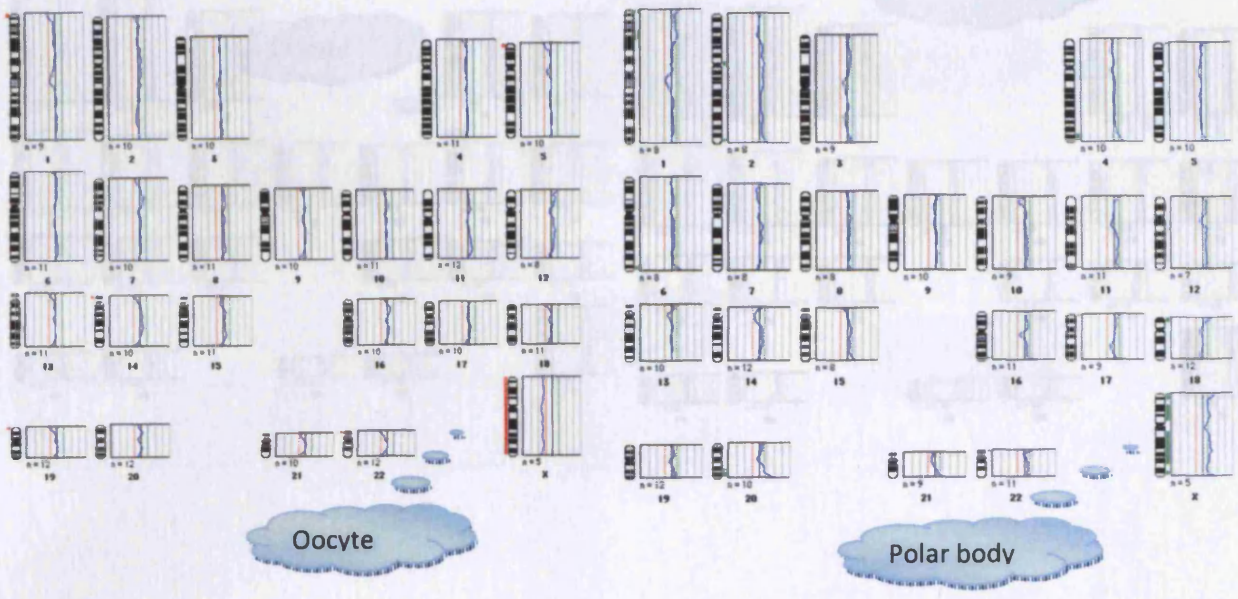


Figure 5.6. *Loss of chromosome X visible in an oocyte/polar body complexB from female S2. Reference DNA is 46, XX labelled in red in both cases. The oocyte shows loss of X while the polar shows partial loss of X.*

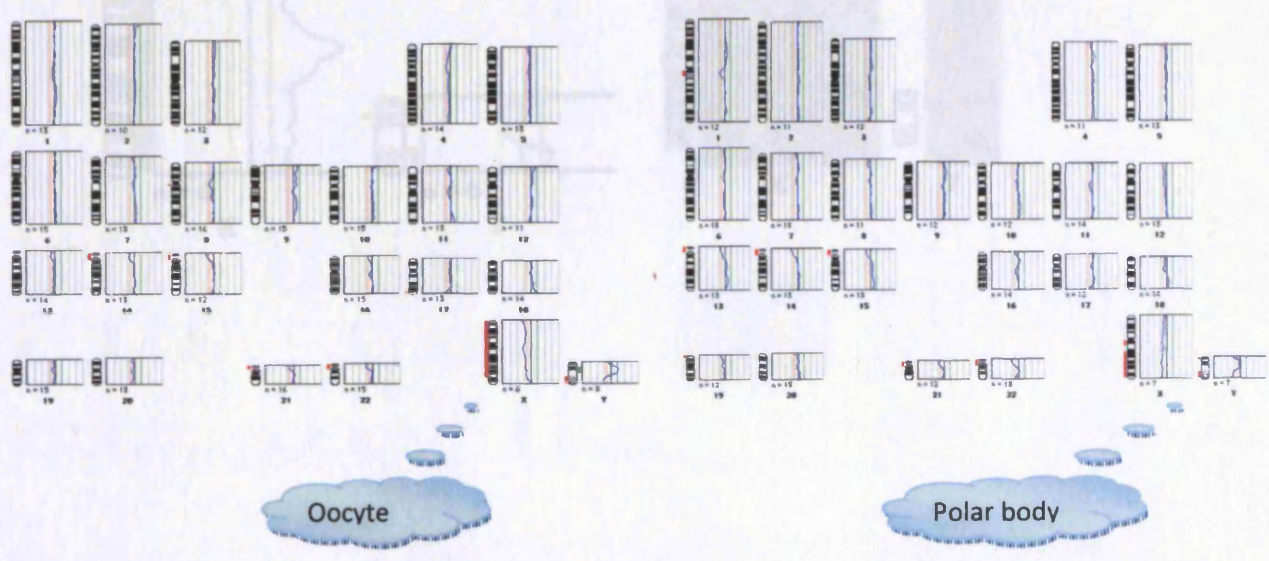


Figure 5.7. *Meiotic non-disjunction of chromosome 12 visible in an oocyte/polar body complex from female E1. Reference DNA is 46, XX labelled in red in both cases. The oocyte lost chromosome 12 and the PB has an extra chromosome 12.*

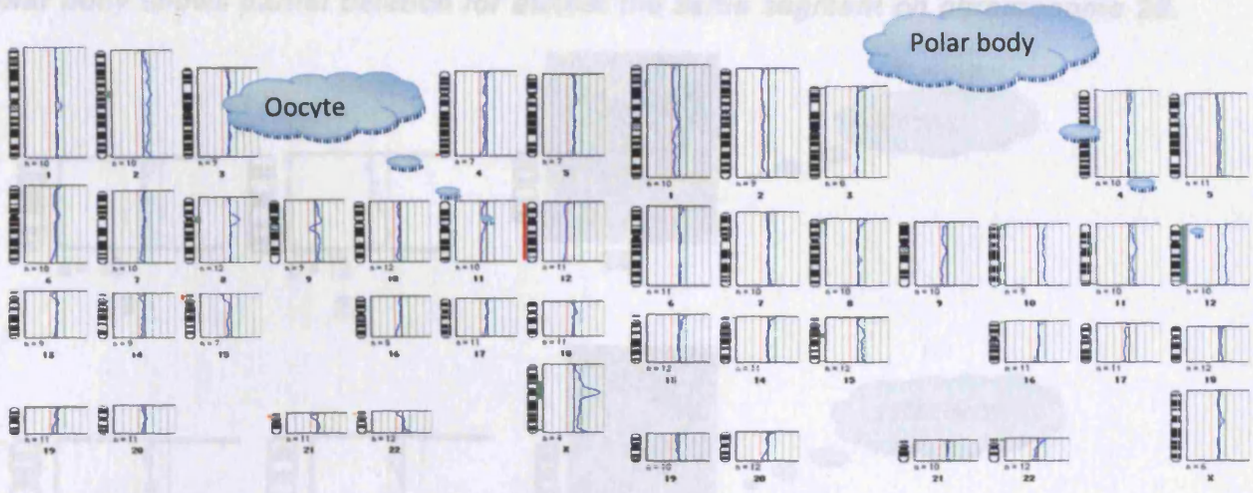


Figure 5.8. *CGH close up on the X chromosome of the abnormal 24, XX 0PN/0PB oocyte from female E2 showing disomy X. Reference DNA is female in red.*

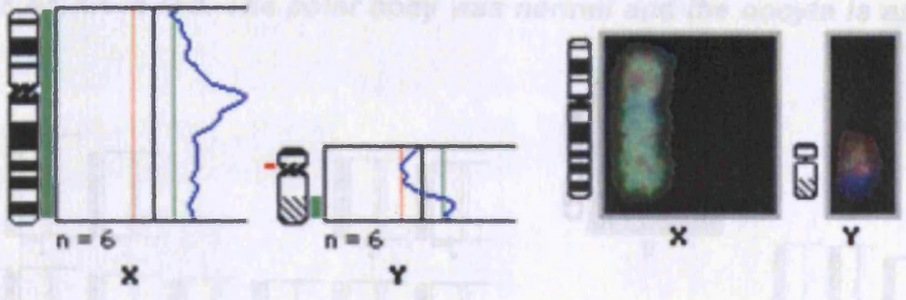


Figure 5.9. *Chromosome duplication and deletion for chromosome 20 visible in the abnormal oocyte/polar body complex from female T. Reference DNA is 46, XX labelled in red in both cases. The oocyte shows partial duplication of chromosome 20q while the polar body shows partial deletion for almost the same segment on chromosome 20.*

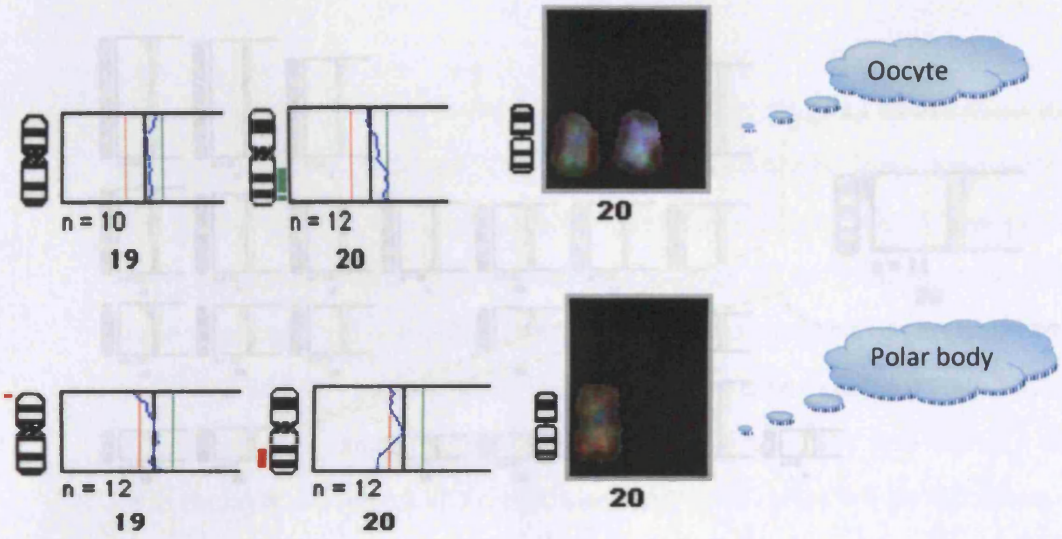


Figure 5.10. *Fertilisation with an unbalanced sperm due to paternal translocation [46,XY,t(9;15)(p12;q13)] was picked up in an oocyte from female S3. The reference DNA is 46,XX in red. The polar body was normal and the oocyte is assumed normal.*

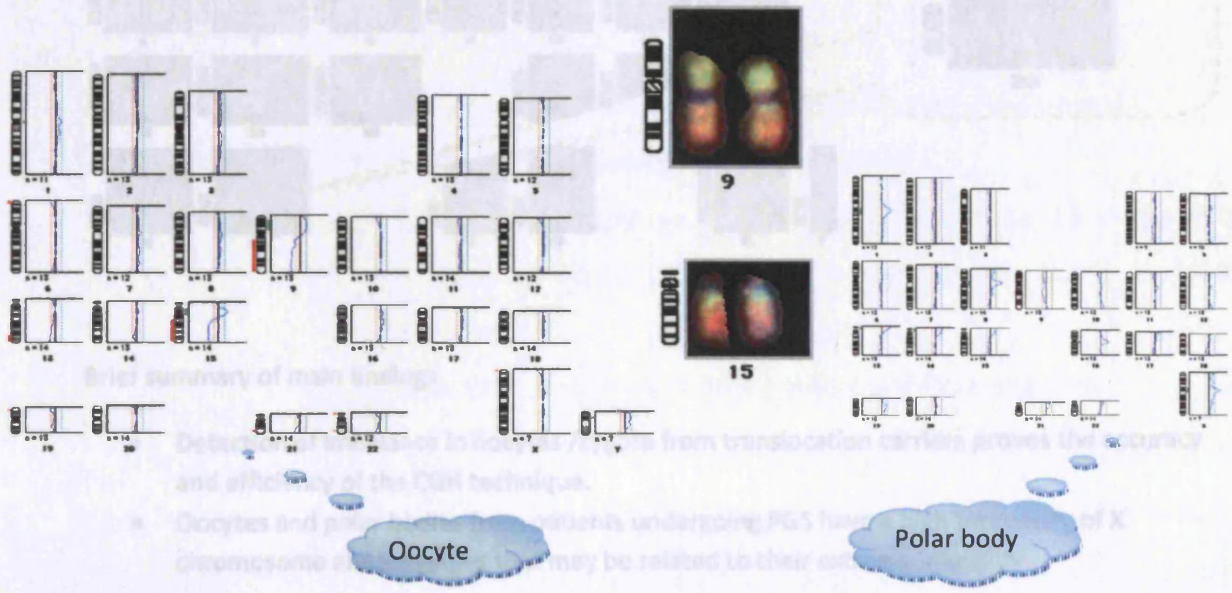
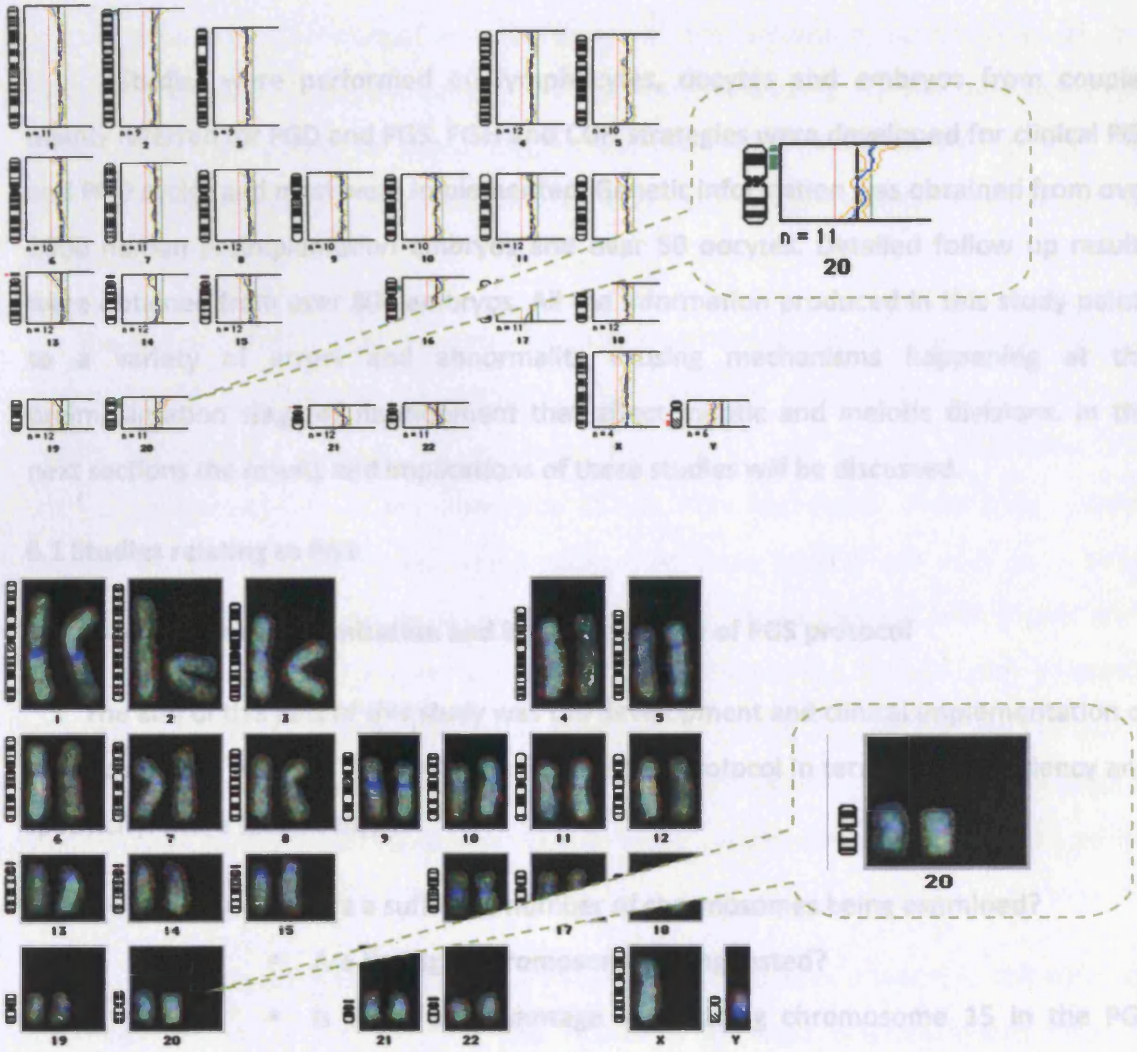


Figure 5.11. *An unbalanced oocyte identified by CGH from carrier female R of a translocation 46,XX,t(9;20)(p13;p11.2). A duplication of 20p11.2p13 can be seen. Reference DNA is 46,XX in red.*



Brief summary of main findings

- Detection of imbalance in oocytes /zygote from translocation carriers proves the accuracy and efficiency of the CGH technique.
- Oocytes and polar bodies from patients undergoing PGS have a high frequency of X chromosome abnormalities that may be related to their extreme infertility.

Chapter 6

Discussion

Studies were performed on lymphocytes, oocytes and embryos from couples mainly referred for PGD and PGS. FISH and CGH strategies were developed for clinical PGS and PGD cycles and most were implemented. Genetic information was obtained from over 1000 human preimplantation embryos and over 50 oocytes. Detailed follow up results were obtained from over 800 embryos. All the information produced in this study points to a variety of errors and abnormality causing mechanisms happening at the preimplantation stage of development that affect mitotic and meiotic divisions. In the next sections the results and implications of these studies will be discussed.

6.1 Studies relating to PGS

6.1.1 Development, optimization and implementation of PGS protocol

The aim of this part of this study was the development and clinical implementation of an efficient PGS protocol and evaluation of the PGS protocol in terms of its efficiency and specificity. More specifically;

- Are a sufficient number of chromosomes being examined?
- Are the right chromosomes being tested?
- Is there an advantage in including chromosome 15 in the PGS protocol which is not screened in some commercially available probe sets?
- Error rate; frequency of false positive and false negatives

Chromosomes and efficiency of PGS protocol

Results from 101 cycles of PGS, 935 biopsied embryos and 596 embryos with full follow up studies were obtained using FISH to screen for chromosomes 13, 15, 16, 18, 21 & 22 in two rounds of hybridisation. The PGS protocol used is unique to this centre and was established after careful consideration of the following parameters; 1) The chromosomes most frequently found in aneuploid conceptions, 2) The availability and efficiency of FISH probes or probe sets, 3) The maximum number of FISH probes and sequential hybridizations that can be used for single cell analysis without a significant loss of efficiency.

The use of different fluorochromes for each chromosome, rather than ratio labelling, increased the accuracy of the protocol. The choice of chromosomes was carefully considered in order provide the maximum benefits for the couples undergoing PGS without compromising on the efficiency of the FISH technique. Preliminary studies showed reduced FISH efficiency when more than three probes are used in a single hybridisation. Given the choice to screen for more chromosomes but with higher error rate and to screen for six chromosomes in two hybridisation rounds with increased efficiency the latter was chosen. With efficient screening for six chromosomes the proportion of embryos diagnosed as normal in this study was low (18.6%). The addition of extra probes would have probably leaded to fewer normal embryos found due to the increased error rate.

The maximum number of sequential hybridization rounds of a biopsied cell was two. After two rounds of hybridizations the structures of single biopsied nuclei begin to degenerate thus giving variable results. The combined probe efficiency of this custom made protocol ranged between 88% and 95% which was optimum for single cell applications. A small decrease in hybridization efficiency was noted by Liu *et al* (1998) after three rounds of FISH hybridizations on surplus IVF embryos. However, from this study it was observed that the effects of multiple hybridization rounds on a single blastomere were more severe than that on whole embryos on a slide. So protocols with

more than two hybridization rounds should also be tested on single blastomeres on a single slide.

There have been reports where 10- 15 chromosomes have been used either in two or three hybridization rounds (Abdelhadi *et al* 2003, Baart *et al* 2004a, Baart *et al* 2007a). Abdelhadi *et al* (2003) used three hybridization rounds to screen for 13 chromosomes in 200 embryos after follow up with an error rate of 12%. They had more errors in the LSI probes of 21 and 22 and the aqua probes 15 and 16. Baart *et al* (2004a) used two hybridization rounds to screen for 10 chromosomes and although individual probe efficiency was around 95% the combined probe efficiency for the 1st round was 86%. Additionally, the combined efficiency for both rounds was not calculated. Baart *et al* (2007a) screened for 15 chromosomes on surplus cryopreserved embryos and found that their abnormality rate went up from 67% to 81%; as before they only reported individual probe and hybridization round efficiencies. All three studies used ratio labelled probe mixtures.

The error rate in this study was 1.8% which confirms that the protocol of 6 chromosomes screen provides highly reliable results. The very low false positive rate of 1.15% showed that the abnormalities observed in the biopsied cell are a true representation of at least some of the cells found in the embryos and that the abnormality rate reported in the study is a true finding. For comparison, Daphnis *et al* (2005) found an average 5% FISH error rate in a careful study of human preimplantation embryos. A retrospective study of monosomic embryos at the biopsy stage and their follow up (Cooper *et al*, 2006), found a false positive rate of monosomy of 3.8% and concluded that monosomy in biopsy results should be taken as a true representation of the status of the embryo. False positives were mostly due unclear results on biopsy but mosaicism could not be ruled out.

The false negative rate is more difficult to establish since most embryos found normal had no follow up data because they were transferred or frozen after biopsy. There were 9 embryos found to be normal on biopsy that were reanalyzed but which were of poor

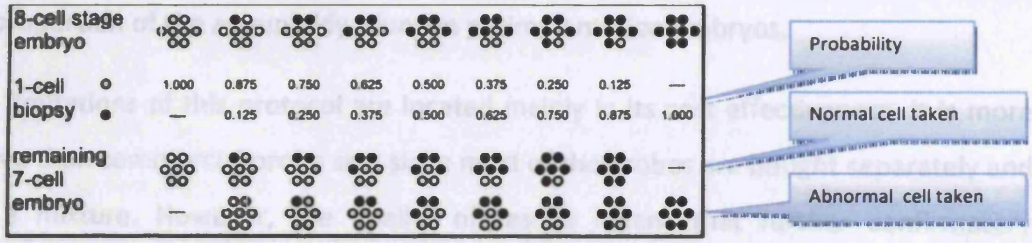
quality. Three of them were found to be abnormal, two were fully chaotic and one had meiotic trisomy 13. The latter was due to a split signal that was scored as one on biopsy making a total of two instead of three. The two chaotic embryos might have had one or more diploid cells that were removed on biopsy thus giving a false representation of the rest of the embryos. However, these embryos were not transferred or frozen due to poor morphology or to being arrested which might be indicative of disorganized cell cycles. The small number of embryos that were normal on biopsy that was followed up cannot provide a conclusive false negative rate. In addition, all 28 pregnancies after PGS in this study delivered chromosomally normal babies and there were few miscarriages.

However, even with embryo screening, a great number of transferred embryos fail to implant and this is an area that requires further investigation. It might be that screening for all chromosomes will lead to an improved pregnancy rate but the techniques for this to happen efficiently in a clinical and time limited setting at the single cell level require improvement. Mosaicism, as widespread as this study suggests (53% chaotic and 40% simple mosaics), might also contribute to the failure of implantation but since we cannot check the whole of the embryos that are found diploid on biopsy and transferred, this remains a theoretical possibility.

It appears that with an efficient FISH protocol and parental lymphocyte check to avoid polymorphic chromosomal errors (as discussed in section 1.1.2) mosaicism might be the most common source of errors in preimplantation genetic screening at least in the cases of normal/abnormal mosaicism. In this study 105 of the mosaic embryos (44%) had diploid cells (10-68% of the total cells in each embryo) and were diagnosed as abnormal on biopsy. This was also shown in a theoretical model where general mosaicism has been estimated at 70% and normal/abnormal mosaicism at around 35% (Los *et al*, 2004). In this model the biopsy of one cell and two cells from embryos with varying degrees of mosaicism on day 3 can produce a high rate of false positives and false negatives, with errors increasing in the case of single cell biopsy. The removal of an abnormal cell from an overall normal embryo might lead to a false positive result on follow up. Most

significantly, the removal of a normal cell from an overall abnormal embryo might result in the transfer of an abnormal embryo and a false negative result. Single cell abnormalities have been well documented by CGH and FISH studies (Wells & Delhanty, 2000; Daphnis *et al*, 2005). Figure 6.1 illustrates the theoretical outcome of single cell biopsy in the case of mosaicism. The repercussions of normal/abnormal mosaicism might be found in the relatively low implantation rate within the PGS group where the biopsy result might be a true one but not representative of the rest of the embryo.

Figure 6.1. ***Effects of mosaicism and biopsy in PGS*** *of normal and abnormal cells in 1-cell biopsies taken from 8-cell embryos with various levels of mosaicism and the compositions of the remaining post-biopsy embryos (From Los et al, 2004), Normal cells- white circles Abnormal cells- black circles*



However, PGS is a general screening procedure designed to increase the probability of transferring a diploid embryo. Although a normal single cell is not always indicative of the rest of the embryo it can help screen out the entirely abnormal embryos. The complete randomness of selecting the nuclei during biopsy will always produce some errors due to mosaicism. As in prenatal diagnosis, these errors have to be counterbalanced with overall effectiveness of the PGS protocol, the time available to obtain the results and the cost effectiveness in a clinical setting.

Chromosome 15 is not routinely included in most PGS programmes. However, the decision to include it in this study appears to be a valid one as errors in chromosome 15 constitute 15% of all identifiable errors of mitotic and meiotic origin. The other five chromosomes chosen also appear to have high levels of aneuploidy in embryos, a fact that gave confidence that the chromosomes this protocol was screening for were the correct

ones, as being the most significant ones in producing abnormal pregnancies. The best alternative would be the look at all the chromosomes from single cells. This can be done effectively by CGH either on blastomeres or polar bodies, however CGH is not yet routinely used clinically since it is very time consuming when single cells are involved and requires strict safety precautions to avoid contamination. Since, in this study, so few cycles produced more than two embryos suitable for transfer by testing for six chromosomes , testing the whole set is not a high priority for such high risk couples.

In general, this PGS protocol has proved highly effective in detecting abnormalities in single cells with a low false positive rate. An additional benefit to this protocol was that it allowed for flexibility in terms of different combinations of probes to be used in each round with high efficiency. Additionally, the chosen chromosomes tested appear to detect a high proportion of the aneuploidy found in preimplantation embryos.

The limitations of this protocol are located mainly in its cost effectiveness. It is more expensive than commercial probe sets since most of the probes are bought separately and not in a mixture. However, the quality of results means that further confirmatory hybridisations with the same chromosomes, in case of unclear results are not needed so, no extra individual probes need to be purchased. In contrast, when a commercial probe set is being used and there are unclear results for a particular chromosome an individual probe is used in order to confirm the result adding to the cost of each PGS cycle.

Even when only six chromosomes were being tested in this study the great majority of untransferred embryos showed varying degrees of abnormalities via several mechanisms. These are discussed in the following paragraphs.

6.1.2 Numerical chromosomal abnormalities and mechanisms of aneuploidy in preimplantation embryos

Very few reports on the outcome of PGS include full follow up studies that allow the identification of aneuploidy mechanisms in preimplantation embryos and relate them with the reproductive history. The aim here was the determination of full chromosomal outcome from follow up analysis of untransferred embryos derived from PGS cycles. Specifically, to investigate:

- The types of abnormalities
- The origin of errors either meiotic or mitotic
- The mechanisms of aneuploidy involved
- The chromosomal outcome in relation to the reproductive history of couples that have undergone PGS.

At 81.4% the rate of abnormality overall in preimplantation embryos (and 93% mosaicism rate including fully chaotic) in this study is much higher than in most previously published studies following PGS diagnoses in embryos where 50 to 70% of embryos appeared to be abnormal (Giannarolli *et al* 1997, Giannarolli *et al*, 2005, Rubio *et al*, 2005, Munne *et al* 2005, see also table 1.5). Bienlanska *et al* (2002) reported an overall mosaicism rate in untransferred embryos of 48.1% but found that mosaicism was increasing with the embryonic developmental stage and at the blastocyst stage the mosaicism rate reached 90.9% if polyploidy was included.

Additionally, Munne et al, (2007) found that the aneuploidy rate in embryos increased from 70% to 80% form women over 42 years. The high abnormality rate in this current study could be a reflection of the selection of the couples in this Centre where PGS is done as a last option and most couples chosen present with a very poor reproductive history. Baart *et al* (2006) in a study of 196 preimplantation embryos (using FISH) from women <38 years with no indication for PGS found an aneuploidy rate of 64% and 50% mosaicism. Along with other studies, these results confirm that aneuploidy and mosaicism is very frequent in preimplantation development but also that some groups of people may

be more prone of producing aneuploid and/or mosaic embryos than others, again confirming other studies (Delhanty *et al*, 1997, Voulaire *et al*, 2002).

It may also be that mosaicism is more frequent than previously thought at other developmental stages in the human life cycle. In preimplantation embryos it might be more pronounced since all the cells of a single organism can be found in the preimplantation stage. In spontaneous abortions Vorsanova *et al* (2005) found 48% mosaicism and Levedev *et al* (2004) found diploid aneuploid mosaicism for various monosomies. Cancer is well linked to aneuploidy and mosaicism with chaotic mitotic divisions and extremely heterogeneous chromosomal anomalies in different forms of tumour cells (Duesberg *et al*, 2005). Most interestingly, mosaic aneuploidy and mosaicism has been found in the human brain. In the brain tissue of fetuses (8-1wks) with normal karyotypes, 30-35% of mosaic aneuploidy has been found (Yurov *et al*, 2007) as well as in functioning adult neurons of normal and diseased individuals at around (40%) which affected all autosomes. It had also been suggested that mosaic aneuploidy of the X chromosome in male lymphocytes might play a role in autism and its high frequency in male children (Yurov and Yurov, 2007). These studies suggest that mosaicism is frequent even in the adult stages of human development and can be related to a natural occurrence in certain tissues or predisposition to disease.

Another observation in this study is the presence of a high proportion of fully chaotic embryos in all groups of patients irrespective of maternal age or reproductive history at an average rate of 53%. The formation of chaotic embryos might be partly due to environmental factors and more specifically linked to the IVF setting. Salumets *et al* (2003) found 24% chaotic embryos that were cultured after freezing, thawing and culturing compared to 6.3% in frozen/thawed non cultured embryos. Culture conditions and IVF stimulation protocols might also interfere with cell divisions in susceptible embryos (Bean *et al*, 2002 Baart *et al*, 2007).

Additionally, Delhanty *et al* (1997), studying fertile patients undergoing PGD, found the extensive generation of fully chaotic embryos to be patient specific, an observation

confirmed by Voullaire *et al* (2002). From this observation we can conclude that our cohort of couples undergoing PGS may have a predisposition to generating chaotic embryos which might or not be aggravated by the IVF procedures. In addition, levels of chaotic mosaic and mosaic embryos are lower in the embryos from carriers of reciprocal and Robertsonian translocations in this study (33% and 26% respectively) compared to the PGS cohort adding to the conclusion that for some couples genetic predisposition might play a role.

Predisposition to aneuploidy in the gametes has also been found to generate chaotic embryos in susceptible mice strains (Lightfoot *et al*, 2006). However, several postzygotic errors as in chaotic embryos hinder the identification of the original meiotic errors in the gamete. Thus, in this study very few chaotic embryos were deemed to have meiotic errors since the original, if any, meiotic errors could not be easily identified.

Maternal age has an undisputed link with embryonic aneuploidy and the risk of various trisomies in the foetus. Women who are older than 37 years present reduced fertility due to their ageing oocytes being prone to various chromosomal errors. This increased aneuploidy is observed in preimplantation embryos but to a much more severe degree than in prenatal studies (Munne, 2003, Munne *et al,* 2007). However, overall maternal age is not a major factor affecting the frequency of all abnormalities seen in preimplantation embryos and oocytes (Delhanty *et al*, 1997; Bielanska *et al*, 2002; Baart *et al,* 2006; Fragouli *et al*, 2006a-c). From this study it can be seen that uniformly abnormal, chaotic mosaic and simple mosaic embryos seem to occur irrespective of age and reproductive history in roughly the same proportion for all groups.

The frequency of meiotic abnormalities in embryos from older women was not significantly different from those in the younger age groups although there is an upward trend with increasing maternal age. This shows that although older females in general have higher rates of meiotic abnormalities, some younger females (25-35 years of age) going through this PGS programme have a high chance of a meiotic chromosomal abnormality. That is probably an explanation of why the younger women that have

resorted to PGS in this study present fertility problems beyond those that are encountered in routine IVF. As mentioned previously, it appears that the genetic makeup of individuals predetermine the extent of the meiotic aneuploidy due to recombination and other factors as well as maternal age (Warren and Gorringe, 2006).

The distribution of aneuploid mosaic (and diploid chaotic) embryos however was significantly different between the three groups. The aneuploid mosaics were more numerous in the two older groups than the younger group of females. This may be mainly due to the higher abnormality rate seen in women over 40 (84.9%) compared to those in the younger age groups (79.6 and 78% respectively) and a greater meiotic error susceptibility with increasing age. Perhaps the maternal age effect is detected in this type of mosaicism. Meiotic errors and other abnormalities can be seen to be accumulating more frequently in the embryos from older females. On the other hand, significantly more diploid/chaotic mosaic embryos were found in the younger group. This could also be due to paternal as well as maternal factors.

Of course, these meiotic abnormalities may come from either parent however it has been noted in another recent study of ovum donors that younger women can present high aneuploidy rate in their oocytes (Munne *et al*, 2006). We can conclude that maternal age alone in these couples is not enough of an indicative parameter on which to base a prognosis for genetic abnormalities in their embryos. Rather there might be other parameters either related to IVF processes and/or in their genetic makeup that predisposes them to an increased risk of aneuploidy in their gametes or embryos.

The level of uniformly abnormal embryos was very low in this study (5.7%) due to wide spread mosaicism; but was significantly different in the different referral groups. The most striking differences are in the number of embryos with meiotic abnormalities and uniformly abnormal embryos found in the AMA, RM and RIF groups. Only 8 out of 300 embryos in the RIF group were uniformly abnormal pointing to the fact that whatever the genetic makeup of the zygotes, in this group in particular, post-zygotic errors are a major factor in their subsequent demise.

Genetic predisposition might also play a role in this. Bergh *et al* (2004) found that by adding to the IVF media a naturally occurring follicular fluid sterol (FF-MAS) the rate of uniformly normal human embryos was decreased. If this is valid, then genetically determined variations of certain hormones and their levels within the follicular fluid might play a role in the formation of aneuploid and mosaic embryos. Aneuploidy via alteration of follicular environment has been seen in mice where subtle changes in oocyte growth increased the risk of non-disjunction (Hodges *et al* 2002).

In general, couples in the RIF group appear to produce embryos that are genetically susceptible to mitotic aneuploidy because of their very low level of uniformly abnormal embryos and the low identifiable meiotic error rate. However, subtle differences in susceptibility may in future differentiate this group further according to the type of mosaic embryos they produce. In general, the combination of the genetic backgrounds of both parents might be responsible for the results seen in this group.

In contrast the RM and AMA groups seem to have marked similarities in almost every aspect of the chromosomal abnormalities found in their embryos although their reproductive history is different. Most RM patients were able to conceive since most of the previous miscarriages were from natural cycles; their problem was to achieve an ongoing pregnancy. The meiotic errors within the RM group were spread across all ages; the age group 29-37 years had an average of 17% of embryos with meiotic errors and the 38-42 age group had 22% on average, not significantly different.

These results indicate an unidentified underlying common mechanism that links the infertility in these two groups (RM & AMA) and is worth investigating further. The RM group appears to be affected by an age independent predisposition to aneuploidy as detected by comprehensive studies of human oocytes (Fragouli *et al.*, 2006b). Furthermore, studies in human and mouse gametes on meiotic recombination provide some evidence of a "genetic background" effect in the causes of aneuploidy (reviewed in Lynn et al, 2004, Hunt, 2006). So, it might be that earlier susceptibility of the RM group of patients observed in this study is the result of a genetic predisposition.

As an example, a study by Sherman *et al* (2006) looked at the recombination patterns of chromosome 21 in human oocytes in relation to maternal age. They found that MI errors from younger women had an increased proportion of susceptible recombination events while in older women the incidence was near to that found in non-disjoined chromosome 21. Although altered recombination patterns have been linked to increased aneuploidy the authors concluded that while in younger women all but the most susceptible of meiotic exchanges can be resolved, in older women even non-susceptible chiasmata cannot resolve properly. For MII however the opposite was true. Oocytes with errors in 21 from younger women had fewer susceptible recombination patterns than older women. This led the authors to conclude that multiple risk factors for non-disjunction can act at different times in the meiotic processes.

Susceptibility to chromosome malsegregation in lymphocytes has been found in women who had a Down syndrome child at a young age; they had significantly higher aneuploidy frequencies for chromosome 21 and 13 compared to a control group (Migliore *et al,* 2006). Additionally, increased aneuploidy rate in embryos was observed for young women that had experienced a previous trisomic conception (Munne *et al,* 2004a). Molecular factors, therefore, not yet identified or even unknown Mendelian traits may be responsible for the manifestation of these types of aneuploidy seen in this study. In fact, several severe chromosome instability syndromes have been described. Of interest is precocious sister chromatid separation described in Cornelia De Lange syndrome which is part of an array of syndromes that are due to defects of cohesin subunits, called cohesinopathies (Kaur *et al,* 2005). Although these mentioned examples are extreme cases, it is conceivable that some less severe traits in a genome could produce such abnormalities in preimplantation embryos. Of interest will be the follow up of the fertility of the offspring of these PGS couples in a future study.

The reduced number of identifiable meiotic abnormalities in the RIF embryos is another feature that distinguishes this group from the other two. This is the first time that such a link has been made. The results of the first 60 cycles of this study first indicated

these differences (Mantzouratou *et al,* 2007). Additionally, Voullaire et al (2007) also found that the incidence of complex abnormality in healthy cleavage embryos is independent of maternal age but is increased in patients with a history of RIF. Again this fact points to post-fertilisation errors that are almost universal via a mechanism that may be independent of the outcome of parental meiosis but probably inherited by the embryos at a molecular level.

The distribution of normal embryos although not significantly different between the three groups shows that more normal embryos were found in the RIF group than in the RM and AMA groups (20.3% vs 17.2% and 15.8%). Coupled with the increased postzygotic abnormalities of normal/abnormal embryos it appears that there is a greater probability of errors arising during single cell PGS biopsy for this group due to mosaicism.

Most of the ICSI cycles in this study were performed for couples with RIF since it was our biggest referral group. Comparing the ICSI and IVF cycles within the RIF group it was found that there was no significant difference between the distribution of normal, chaotic, uniformly abnormal embryos and embryos with meiotic errors. This suggests that the pattern of embryonic abnormalities highlighted in this study for RIF couples was not solely a consequence of the insemination method or of poor sperm parameters but a characteristic of all couples in this group suggesting a predisposition to mitotic aneuploidy.

However, a significant difference between simple mosaic, aneuploid mosaic and diploid chaotic mosaic embryos was found between the ICSI-RIF and the IVF-RIF group. While aneuploid mosaics embryos were most frequent in the IVF-RIF group, more diploid/ chaotics was observed in the ICSI-RIF group compared to the IVF group (46% vs 8.8%). This also agrees with the theoretical assumption that genetic background (susceptibility to mitotic and/or meiotic errors) in combination with the error prone post-zygotic divisions will produce variations in aneuploidy and mosaicism.

Additionally, since poor sperm parameters indicated ICSI for this RIF subgroup, it might be implied that paternal factors might also be responsible for the high incidence of mosaicism and repeated implantation failure. In males with severe impairment of

spermatogenesis at least 70% of sperm might be aneuploid (reviewed by Griffin & Finch, 2005). Increased embryonic mosaicism has been reported in embryos from TESE cycles (52% mosaic, Silber *et al*, 2003). In a study by Gianaroli *et al* (2005) it was observed that the incidence of aneuploidy in embryos increased with the severity of the male factor conditions. Although no follow up of untransferred embryos was done, they noted that in RIF patients the most frequent defects were complex abnormalities like haploidy and polyploidy. Griffin *et al* (1995) found increased incidence of sex chromosome disomy in older males. Unfortunately, the sex chromosomes were not screened in the present study.

The elevated mosaicism and aneuploidy in embryos from ICSI-RIF group in this study and those mentioned indicates that the abnormal semen parameters might also predispose the embryos to mitotic aneuploidy. Errors on the Y chromosome like mutations at the molecular or the cytogenetic level have been described (Griffin and Finch, 2005). In the present study, all the couples were karyotyped previously to PGS and all the males were normal. This leaves molecular errors that could confer such embryonic abnormalities. Additionally, one study showed that males with oligosthenoteratozoospermia (OAT) and normal karyotypes showed an increased sex chromosome aneuploidy in their lymphocytes (FISH with X, Y and 12) compared to control individuals (De Palme et al, 2005), suggesting a cell division defect that can affect the germ cells as well as somatic cells.

One explanation would be that these errors lie with the male centrosome which controls the mitotic divisions after fertilization. Sperm morphological abnomalities had been linked to centrosome defects (Sathanathan 1998). If components of the centrosome are not functioning properly in some ICSI males then abnormal cell divisions in the embryo might occur. Additionally, a study by Palermo *et al* (1997) showed increased levels of mosaicism in embryos derived from oocytes injected with sperm fragments (head and tail) while not with whole sperm. This raises the possibility that the ICSI procedure might confer some of the abnormalities seen by physically damaging the centrosome in some sperm as well as genetically altered centrosomes.

The mechanisms that lead to these levels of aneuploidy and mosaicism identified by this study in preimplantation embryos are still not clear. The aberrant function of cycle checkpoint genes might provide an explanation, as has been found in cancer cells (Kops *et al*, 2005, Delhanty and Handyside, 1995). Additionally, generalized mitotic aneuploidy might be explained by the formation of tetraploid intermediates via failure of cytokinesis. Shu and King (2005) have found that an original nondisjunction error can result in tetraploid cells rather than aneuploid ones in human tissues by regression of the cleavage furrow. Although, a checkpoint exists that arrests tetraploid cells normally in mammalian cells (Margolis *et al*, 2003) in some cases this checkpoints might not stop mitosis from happening (Fujiwara *et al*, 2005, Harrison *et al*, 2000). A decrease in cleavage rate was indeed found in the 4n preimplantation mouse embryos compared to their 2n counterparts (Eakin *et al*, 2005) but several tetraploid cells progressed through mitosis.

Tetraploid cells that can re-enter mitosis (slippage), present an increased frequency of chromosomal abnormalities as has been seen in some human cell lines (review in Ganem *et al*, 2007). Additionally, Chatzimeletiou *et al* (2005) studied the spindle anomalies in preimplantation embryos and proposed that frequently seen binucleate blastomeres in human embryos, which present spindle abnormalities and result from failure of cytokinesis, can lead to the formation of tetraploid cells and widespread genome instability. In this study the ploidy mosaic embryos were only 6.7% of the total however several of the chaotic embryos could have arisen via a tetraploid or polyploid intermediate since gross abnormalities in the number of all chromosomes was regularly seen in the chaotic cell lines of diploid/chaotic, aneuploid/chaotic and fully chaotic embryos.

Nicely coupled with these conclusions is a study by Bean *et al* (2002), where mice with a mitotically but not meiotically unstable Y chromosome (WtY) were studied. The authors came to several interesting conclusions; i) the WtY is stable through meiosis but has a high frequency of non-disjunction during mitosis, ii) the non-disjunction events were concentrated mainly in the early mitotic divisions, thus indicating that the earlier post-zygotic divisions are error prone and iii) although the WtY produced errors on several

different genetic backgrounds (achieved by cross-breeding WtY mice with other strains), the rate of non-disjunction of the WtY chromosome was influenced by genetic background. Another interesting assumption that can be made here is that if susceptibility for embryonic mitotic non-disjunction exists in one parent then the genetic makeup of the other parent might increase or decrease the rate of aneuploidy in their embryos. Therefore, the combination of maternal and paternal factors might influence the outcome.

Taking the above information into account a theoretical sequence of events can be established to explain the high levels of aneuploidy, mosaicism and chaotic embryos encountered in this study; (a) couples needing PGS with a theoretical susceptibility to embryonic mitotic non-disjunction or susceptibility to produce aneuploid gametes, produce embryos (b) error occurs in the non-disjunction prone early mitotic divisions in combination with the underlying susceptibility which will result in these embryos either producing mosaic cell lines or undergoing regression of the cleavage furrow resulting in tetraploid and binucleate/multinucleated cells (c) these cells can re-enter mitosis with or without DNA replication and produce highly aneuploid embryos or cell lines. If a variety of genetic background susceptibilities play a role in this then some couples might present with differences in aneuploidy and mosaicism rates.

Few previous studies have been sufficiently detailed to provide precise information on individual meiotic and mitotic error mechanisms; Munne *et al* (2004), found that monosomy may be more common than trisomy and the chromosomes most affected overall are 22, 16, 21 and 15. However, that study was unable to distinguish between errors due to meiosis and those of post-zygotic origin. In this study, meiotic errors most frequently affected chromosomes 21, 22 and 18 and trisomy was in 1:1 ratio with monosomy. Mitotic errors affected most frequently chromosomes 13, 15 and 22 and the most common identified mechanisms of formation were mitotic non-disjunction followed by chromosome loss and chromosome gain. Overall, those most frequently affected were chromosomes 21, 22, 18, 13, 15 and 16 in that order. Differences in chromosome

susceptibilities could be seen in the different referral groups. Most notable was that chromosome 16 was most commonly involved in the mitotic errors of the RIF groups while not in the other two groups. Trisomy and monosomy was seen in all groups in 1:1 ratio which was expected since meiotic non-disjunction events will produce an equal number of aneuploid gametes of each type randomly.

Overall, mitotic non-disjunction was found to be the most frequent aneuploidy mechanism for chromosomes 13, 15, 16 and 18 but not for chromosome 21. Chromosome 21 presented most frequently due to meiotic trisomy and monosomy, while for chromosome 22 both meiotic and mitotic abnormalities existed in equal proportions. These data cannot be readily compared to other studies since differences in chromosomes tested and sample sizes can result in inadequate conclusions. The fact that very few studies exist that present detailed examination of the mechanisms of aneuploidy in preimplantation embryos is also a factor. Chromosomal abnormalities in preimplantation embryos appear in general to produce differences compared with the information produced by spontaneous abortions due to high incidence of post-zygotic abnormalities and the non-viability of most of the embryos before the clinical pregnancy stage. Thus post-zygotic errors for the chromosomes studied here are present with a very low percentage in spontaneous abortions (Hassold *et al,* 2007). Similarly, a study by Katz-Jaffe et al (2004) which found that aneuploidy for chromosome 21 arising from mitotic errors was more frequent in preimplantation embryos than more developed fetuses where only meiotic aneuploidy could be detected. Additional studies should be done in order to investigate individual chromosome aneuploidy in preimplantation embryos and to recognize patterns if any in different referral groups. More studies are also needed for the identification of the parental origin of errors so that more detailed information about their occurrence can be obtained.

The developmental potential of the untransferred embryos was also assessed in this study and from this and previous studies it is obvious that aneuploidy or varying degrees of mosaicism in embryos does not prohibit blastocyst or morale development (Magli *et al,*

2000, Ruangvutilert *et al*, 2000; Li *et al*, 2005). In this study, 3/73 blastocysts were uniformly abnormal and 52% of the mosaic blastocysts had a diploid cell line. 59% of the mosaic blastocysts in the RIF group had a diploid cell line, 45% of the RM group and 38% in the AMA group. Although the differences are not large they do show that a high proportion of blastocyst in the RIF group may be normal/abnormal mosaic and that would explain the non-existent implantation rate with routine IVF that these patients were experiencing.

Of the embryos from the RM group, 11.7% reached the blastocyst stage, a frequency higher than in the other two groups (5.5%-RIF and 9.5%-AMA) which fits in with the higher implantation potential of the embryos but the lack of progression in pregnancy due to inherited chromosomal abnormalities. Interestingly, only 5.5% of the embryos in the RIF group reached the blastocyst stage, even lower than those in the AMA group. This implies that embryo progression is significantly diminished in this group mainly because of the mitotic instability these embryos were exhibiting.

In general, this study has provided some significant evidence for the following: in a general background of error prone cell divisions and lax cell cycle checkpoint function, couples with genetic predisposition to susceptible meiotic and/or mitotic processes produce different types of abnormal embryos through different mechanisms. The mechanisms producing these errors are usually operating by a complex interplay of a variety of factors genetic and environmental. However, the genetic makeup of both parents might play a significant role in the amount of abnormality seen in preimplantation embryos.

6.1.3. Examination of the usefulness of preimplantation aneuploidy screening for couples with poor reproductive history

- To assess the validity of PGS in relation to pregnancy outcome
  - Does PGS work?

Despite the low normality rate (1.6±1.1 embryos per cycle) in this study the pregnancy rate of 33.3% per embryo transfer is above the average of the latest ESHRE data for PGS (24% per embryo transfer) (Sermon *et al*, 2007). This suggests that efficient screening for 6 chromosomes is sufficient to detect the few embryos that are suitable for transfer in this cohort of patients. All the groups presented a higher than average pregnancy rate compared with the ESHRE data. The highest pregnancy rate was for the RM group with 43.8% per ET (ESHRE 29%). The RIF group had 34.6% per ET (41% per couple) almost 10% higher than the average ESHRE rate (24%). The AMA group had the lowest pregnancy rate at 21.7% per ET but still marginally higher than the ESHRE data 19%). However, the average maternal age in the AMA group was higher in this study vs the ESHRE data. A sharp decrease in the pregnancy rates per ET could be observed with increasing maternal age from 42.9% in the youngest group to 21.6% in the oldest group. These figures indicate that maternal age is a critical parameter for the success of PGS.

A direct comparison of the current outcome of PGS cycles is not possible with routine IVF patients at the same centre since the PGS couples presented with very poor histories. However, a general comparison of the main outcomes of routine IVF and ICSI cycles with PGS cycles in this centre is shown in table 6.1. The routine cycles group had a lower average maternal age since 44% of the cycles with ET were done for women under 35 years. In the PGS group only 12% of females were under 35. Despite this, the pregnancy rates in the PGS group are acceptable considering the poor history and the increased maternal age of the PGS couples. The PGS pregnancy rate is also higher than the UK and the European average pregnancy rate for routine IVF & ICSI (29%) (EMI-ESHRE Andersen *et al*, 2006). Another observation from table 6.1 is the that implantation rate of PGS closely follows that of the routine couples. The miscarriage rate of the PGS group is

less than half of that of the routine group and the multiple birth rate is almost 10 times lower suggesting that more embryos were transferred per cycle in the routine cycles. This shows that for couples with poor reproductive history, an efficient PGS programme can improve the chances of a viable pregnancy to a level of that of routine IVF patients and decrease their chances of a miscarriage or of multiple births.

Table 6.1. Outcome of routine IVF & ICSI cycles compared with PGS cycles.

Cycles information	Routine IVF and ICSI*(%)	PGS (%)
Maternal age	34	37.8
Pregnancy rate per ET	52**	33.3
Implantation rate	26.9	24.6
Multiple birth rate per ET	10	1.4
Miscarriage rate per ET	12.8**	5.6

**For the period January 04-December 04 (HFEA published data website: http://guide.hfea.gov.uk/guide/Clinic.aspx?cliniccode=0044&tab=Clinic)*
***ACU website- http://www.conception-acu.com/subpage.cfm?level1Id=4&level2Id=0*

Studies on PGS cycles and its effectiveness on are currently non consistent in their outcome. Published reviews on PGS have concluded that more research is needed and more randomized controlled studies (Twisk *et al*, 2006, Shahine and Cedars *et al*, 2006, Donoso *et al*, 2007). Observational studies have concluded that PGS can reduce the risk of spontaneous abortions and increase live birth rate (Gianaroli et al, 2005a, Platteau *et al*, 2005, Munne *et al*, 2006a). For individual referral groups the effectiveness of PGS is questioned however since in most cases inappropriate control groups or diverse inclusion criteria have been used.

For RM, Platteau *et al* (2005a) found no beneficial effect of PGS; however, no comparison with a RM group without PGS was done. Werlin *et al* (2003) reported on a small number of patients (19) with RM and found that PGS increased the pregnancy rate for these couples, although the number of couples was very small. The same study did not find any beneficial effect for RIF couples and inconclusive results for AMA couples. The sample size was small in all couples. Platteau *et al* (2006), found that the number of

embryos available for biopsy (at least 6 embryos) is a factor in the success of the RIF group of PGS patients.

For the AMA group, two large randomized control studies have reported no beneficial effect (Staessen *et al* 2004, Mastenbroek *et al*, 2007). Staessen included 400 cycles and found no significant difference in the pregnancy rates between the control and the PGS group for women over 37 years. However, significantly more embryos per cycle were transferred in the control group (2.8±1.2 vs 2.0±0.9) which made the results inconclusive since there was higher implantation rate in the PGS group but not significantly. This study suggested that PGS is of value where the number of embryos to be transferred is limited to two.

On the other hand, Masterbroek et al (2007) studied 836 cycles for AMA and found that PGS actually reduced the pregnancy and live birth rate. However, as with most PGS studies, there were some problems. First were their inclusion criteria. They included all women over 35 as AMA and included referral for infertility for some categories unrelated to a high aneuploidy risk (tubal, endometriosis and cervical reasons). For comparison AMA-PGS patients in our centre were only referred if they were over 39 years of age and had unexplained infertility. Chromosomes 15 and 22 were not screened in the Masterbroek study although in our study errors from these chromosomes formed 33% of the total. Finally, embryos undiagnosed after PGS were transferred and counted towards the screening group when in fact they were not screened at all. The embryos that were found to be normal with PGS and transferred did have a higher implantation rate than the control group.

In this centre the patients were highly selected due to their poor reproductive history. Advanced maternal age has well known increased risk of aneuploidy however the point at which a woman is considered to be of advanced age needs to be addressed. Additionally, as seen in this study some individuals might be predisposed to extensive mosaicism and various meiotic chromosomal abnormalities. However, PGS is an invasive procedure at an early developmental stage and the author's opinion is that it should not be applied to any couples needing assisted reproduction. In order to see the benefits of PGS its application

has to be limited to the couples that need it and have not been helped with conventional IVF. This counteracts to a certain degree the damage done to the embryos by such invasive procedures. Additionally, if PGS is applied to couples that produce mainly chromosomally normal embryos, it will not confer any advantage since most of the embryos will produce a normal pregnancy without the need for screening.

This study shows that some couples will be helped with PGS when screening is done efficiently and patient selection criteria are applied. However, the very high proportion of mosaic embryos in this study means that not all abnormalities or normalities will be recognized since the single cells biopsied might not be representative of the rest of the embryo. However, the majority of the abnormal embryos will be screened out. Future studies then should probably concentrate on 1) recognizing the couples most likely to benefit from PGS, 2) understanding the mechanisms that relate to specific types of aneuploidy for infertile or subfertile couples 3) the development of less damaging biopsy protocols and 4) finding new and efficient ways of getting more information out of single cells, like the use of single cell CGH or microarrays.

As a result of this study in PGS embryos the following follow up studies were initiated: In depth study of the mechanisms of aneuploidy in RM couples using both CGH and FISH, the collection of DNA samples from all the PGS couples to screen for appropriate markers that would indicate genetic susceptibility, the investigation of embryos from couples that are not deemed at high risk of aneuploidy due to meiotic or mitotic errors in order to investigate the interaction and consequences of the IVF environment with the genetic background of individuals.

6.2. PGD and structural chromosomal abnormalities

Structural chromosomal abnormalities carriers were referred for PGD in order to increase their chances of a balanced pregnancy. The aims were:

- To produce optimum strategies for PGD to help structural chromosomal abnormality carriers achieve normal pregnancy, particularly those with rare chromosomal abnormalities that cannot be routinely investigated
- To clinically implement and evaluate these strategies
- To determine the chromosomal outcome from follow up analysis of untransferred embryos derived from PGD. To investigate the types of abnormalities and their origin and in particular the segregation patterns for the rarer chromosomal anomalies

Overall, there were 29 cycles of PGD for carriers of structural abnormalities. These included 18 cycles for reciprocal chromosomal translocations, 9 cycles Robertsonian translocations and 2 cycles for a rare ring 22 carrier. Immunofluorescence and FISH was applied to embryos of an X;autosome translocation carrier in order to investigate X-inactivation in blastomeres. A PGD strategy was developed for an interchromosomal insertion carrier.

6.2.1. Studies in embryos from reciprocal and Robertsonian translocation carriers

Translocation carriers have an increased risk of producing abnormal gametes and therefore unbalanced embryos. PGD for reciprocal translocation carriers (RCT) requires the determination of specific strategies for each individual carrier as breakpoints are usually unique. PGD for Robertsonian translocation carriers t(13;14) required a more uniform approach since the copy number of chromosomes 13 and 14 was essentially being studied in the embryos.

Strategies for translocations mainly involve the use of commercial probes combining locus specific, telomeric and centromeric probes. Numerous cases of PGD for translocations have been reported (Sermon *et al*, 2007). Although PGD for translocations

is now based on routine workup procedures several parameters need to be taken into account while a PGD strategy is developed, 1) the correct position of the breakpoints 2) a probe strategy that will produce informative combinations for both normal and derivative chromosomes 3) the size of the translocation fragments and 4) the hybridization and error rate of the proposed strategy. More efficient probes (i.e. centromeric where possible) and probe combinations in addition to a two cell biopsy strategy provide an efficient way of recognizing unbalanced embryos in this study. This strategy resulted in a very low false positive rate (0.4%) as only one error was detected, attributed to an unclear result in one nucleus and degenerate DNA content in the second nucleus.

In general, compared with the PGS group a higher percentage of normal embryos were found in embryos from reciprocal translocation carriers (22% vs 18.6%) and also in the Robertsonian type (34%). Fewer mosaic and chaotic embryos were observed in these groups compared to the PGS group. Table 6.2 shows the follow up results of the translocation carriers compared to that of the PGS group. Although direct comparisons are not appropriate since PGD couples are already predisposed to meiotic aneuploidy, the table gives an idea of the chromosomal abnormalities in embryos from clearly predisposed PGD couples compared with those having normal karyotypes but with a high risk of aneuploidy.

Table 6.2. PGD for translocations and PGS outcome information

Embryos	Reciprocal (%)	Robertsonian (%)	PGS (%)
Average maternal age	35.6	37.8	37.8
Normal	22	34	18.6
Uniformly abnormal	17.9	7.4	5.7
Mosaic	26.1	32	40.3
Chaotic	33.9	26.5	53.4
Meiotic errors	55.8	33.3	16.9
Pregnancy rate per ET	33.3	28.6	33.3
Implantation rate	22.2	21.4	24.6
Miscarriage rate per ET	14.3	0/7	5.6
ESHRE pregnancy rate per ET (Sermon *et al*, 2007)	34 (m/s:7)	31 (m/s:5.3)	24 (m/s: 7.6)

*m/s- miscarriage rate (%)

The pregnancy rate for both translocation groups is comparable to the ESHRE data. The finding that more uniformly abnormal embryos are found in reciprocal translocation carriers and to a lesser extent in Robertsonian translocation cycles than in PGS provides further evidence that the abnormalities seen in the PGS group are due to individual genetic susceptibilities to mosaicism. The miscarriage rate is higher in the RCT group however the number of cycles included in this study is still too small to allow any conclusions about pregnancy outcome.

An interchromosomal effect was not detected for either translocation group since the chromosomes not involved in the translocation were only affected in some of the chaotic and polyploid embryos. Of interest is the fact that some chaotic embryos were only exhibiting chaotic cell divisions in respect to the translocation chromosomes, in the RCT group. This could be due to each individual translocation breakpoint or the be related to acquired mitotic instability of the chromosomes after an initial meiotic error. Alternatively, other chromosomes might be affected since only a limited number was looked at. A CGH study (Mailgram *et al,* 2002) on individual blastomeres found 100% mosaicism in embryos from reciprocal translocation carriers. Future studies of more embryos with CGH might reveal that there is an intrechromosomal effect since all the chromosomes will be studied.

There does appear to be an upward trend of meiotic abnormalities with female maternal age in reciprocal translocation carriers however the sample size is small. Ogilvie and Scriven (2002) found a higher percentage of 3:1 non-disjunction (resembling aneuploidy non-disjunction) in females than in males although maternal age associations were not made. It might be possible that increased maternal age in the female translocation carriers will produce more meiotic errors in the older females than the younger carriers. Altered or reduced recombination during meiosis I for the translocation multivalent could particularly affect the older females as the aging oocytes would be more prone to non-disjunction as is the case for general aneuploidy and increased maternal age.

Chiasmata studies in human oocytes from female translocation carriers are non-existent since the access to oocytes is only through PGD. Sperm studies mainly involve the analysis of segregation patterns but some studies have mentioned the importance of the chiasmata frequencies and distribution in the outcome of meiosis in heterozygote male carriers of reciprocal translocations (Oliver-Bonet *et al*, 2004, Yahut *et al*, 2006). Oliver-Bonet *et al* (2004) studied two male reciprocal chromosomal carriers and found that the quadrivalents could only be found in closed ring configurations. They also found that for one of the carriers the frequency of chiasmata within the quadrivalent significantly increased and they were mainly localized in the interstitial region. This was not observed for the other carrier. This was also observed in a male carrier of an 11;22 translocation (Armstrong et al, 2000). This was also evident in an animal study with translocation heterozygote mice where male and female gametes were studied (Tease 1998). It appears that different translocations can produce different chiasmata patterns which might affect the outcome of meiosis I at anaphase.

There was great variability in the number of meiotic and post-zygotic outcomes in the embryos for each translocation carrier even when in the case of the Robertsonian translocations they carry the same cytogenetic abnormality. This probably is due to multiple factors like the genetic background, the frequency of recombination that will result in altered meiotic outcomes and the extent of post-zygotic errors. Variation in recombination sites between individuals as mentioned in the previous section will probably affect the recombination sites and frequency in each case. Additionally, the existence of a translocation might alter the recombination patterns by altering specific sequences at the breakpoints. The chiasmata formation will probably determine the orientation of the quandrivalent at meiosis I and consequently the mode of segregation. In the case of Robertsonian translocations, the variation in chromosome abnormalities in embryos might also be due to variation in the exact breakpoints in the centromeric sequences between chromosomes 13 and 14. A significant reduction of certain centromeric sequences might make the chromosomes more unstable during mitosis.

The size of the translocated segments in the case of reciprocal translocations appears to play a role in the meiotic outcome. In four translocations the alternate type was below 50% while there were varying degrees of the other outcomes irrespective of the sex of the carrier. Translocations where the translocated segments are of the same size appear to produce more balanced gametes while the other modes of segregation present in equal proportions. In translocations where one segment is significantly larger than the other adjacent-1 and alternate may occur in 1:1 ratio. In the case of relatively intermediate sized segments 3:1 and alternate were most common. The results were analysed according to the relative sizes of the centric and translocated fragments and compared to the expected outcomes (Albert *et al* 1980). The comparison of the sums of centric and translocated fragments ratio showed the alternate segregation being most frequent overall but adjacent-2 segregation was increased in the case of bigger translocated fragments and smaller centric as predicted by the model. The comparison between expected and observed outcome for individual translocations showed that these two parameters were in agreement in 6/9 cases, in 3/9 cases a different mode of segregation was most frequently seen. This might be a reflection of the altered recombination in the carriers of these translocations. The distribution of chaotic and mosaic embryos appears to be related to individual translocation carriers rather than the size of the abnormalities.

All the Robertsonian translocation carriers in this study were carrying the same translocation t(13;14); this would enable the study of the same structural abnormality in different individuals. Most of the embryos from each translocation couple had an alternate chromosomal complement followed by a 2:1 segregation, 3:0 was seen in some cases. Similar results were obtained in another study of spermatozoa from male Robertsonian carriers (Ogur *et al* 2006). An exception was couple 3 where more frequent 3:1 segregation could be deduced in the gametes of the female carrier. However, the distribution of mosaic chaotic and uniformly abnormal varied for each couple. This adds to the conclusion that genetic variation between individuals could be responsible for increased mitotic instability.

This study and others mentioned show that that some predictions can be made about the likely meiotic outcome of a translocation by studying the size of the translocated chromosomes and the type of the translocation. However, although more uniformity in the meiotic outcomes exists in the Robertsonian carriers, post-zygotic abnormalities and mosaicism will affect the outcome of PGD cycles for every individual carrier to a different degree.

For reciprocal translocations individual risks have to be calculated partly in relation to the size of the transolcated segments. Several general trends were observed in this study in relation to the relative sizes of translocations. Recombination and individual variability however complicate any predictions that can be made for the meiotic outcome in preimplantation embryos. For translocations between smaller fragments, increased recombination in the interstitial region might produce different proportions of normal and unbalanced gametes. Translocations between larger segments can have varied consequences depending on the position of the breakpoints and the recombination patterns. This will also affect the proportion of balanced and unbalanced embryos generated.

In this study the alternate segregation was most frequent at a rate of 44%; this is similar to another study which showed the alternate rate to be 48% (Ogilvie & Scriven, 2002). Additionally, several chance events can affect the proportion of balanced embryos according to the sex of each carrier. Male translocation carriers usually needed ICSI if they presented poor sperm parameters because of the translocation which is a random process of selecting sperm. Additionally, in male carriers all the outcomes of each meiotic division can be present at any one time since four daughter sperm are formed from an original precursor cell. Since the alternate mode is more frequent, recombination could distort or decrease the proportion of normal male gametes. On the other hand, in the female meiosis, only one oocyte is generated from each meiosis. Chromosomes or chromatids will segregate randomly to the oocyte or 1^{st} and 2^{nd} polar body. So recombination distortions might be corrected if unbalanced products are segregated in the polar body. These events

might lead to an increase of unbalanced gametes in the sperm instead of the average proportion. Additionally, the random nature of the segregation in the oocytes and polar body might produce average proportions of balanced and imbalanced gametes in female translocation carriers.

In this study, the results fit this theoretical outcome. The proportion of embryos with alternate: unbalanced ratio in the female reciprocal translocations was 1:1.1 (47.31:52.7) while for the male carriers the ratio was 1:2 (33.3:66.7). This difference was also observed in Ogilvie & Scriven (2002) where for male carriers the alternate segregation was seen in 43% of embryos and for females in 60%. It would appear that female carriers of translocations have an increased chance to produce more normal gametes and as a consequence have a higher chance of finding a balanced good quality embryo during PGD. In this study 3 out of 5 female carriers of RCTs had an ongoing pregnancy with PGD. Although the sample is still small the investigation of accumulating PGD cycles for translocations in this Centre will be required to confirm this trend. Furthermore, direct information from human oocytes from translocation carriers will enable more information to be obtained for the female carrier meiosis. This is partly addressed by the polar body analysis for translocation PGD cycles (Durban *et al* 2001, Gutierrez-Mateo *et al* 2004) although very few data have been generated so far.

PGD couples in this study presented very poor reproductive history and many of them needed IVF due to severe male factor. The pregnancy rates however were quite low. This was due to several factors. First each PGD case was depended on an adequate number of embryos being biopsied in order to ensure the finding and transfer of balanced embryos of good quality. Secondly, although mosaicism (60%) was lower than in the PGS group (>90%) it is high enough to create problems with the diagnosis and to affect the implantation potential of the embryos. Two cell biopsy in combination with an efficient FISH strategy are probably the best option to detect unbalanced embryos and mosaicim as observed in this study. The detection of normal/abnormal mosaicism in embryos at biopsy should be taken as a true finding when an efficient FISH protocol is used. Follow up of

embryos diagnosed as balanced/unbalanced proved a significant point about the nature of mosaicism for translocation carriers and the ability of two cell biopsy to detect mosaicism. Namely, that the probability of mosaicism is higher than that of FISH errors.

Additionally, although the incidence of a balanced translocation is quite common within the population, it appears that some couples present with a higher risk of infertility or spontaneous abortions and are in need of PGD. This might be due to the specific translocation and its breakpoints or an additional factor intrinsic to these couples that can confer such consequences. Despite some difficulties, PGD is a viable option for these couples as it can help them achieve a normal pregnancy by selecting balanced embryos with a high degree of accuracy.

6.2.2 PGD for a ring 22 carrier 47,XX, del(22)(p10q12), +r(22)(q10q12)

A 37 year old female carrier of a rare chromosome rearrangement was referred for PGD. She was a balanced carrier of a deleted 22 and a ring chromosome 22. This is the first report of PGD for this kind of abnormality as it is extremely rare. Two cycles of PGD were carried out using FISH with case specific probes. Unfortunately there were no embryos suitable for transfer in either cycle. All untransferred embryos were also analysed. The aim was to provide a rare glimpse of the behaviour of the derivative chromosomes 22 during preimplantation development for the first time. It would also provide an opportunity to gain our knowledge of the segregation of ring/deletion chromosomes. It also provided the opportunity to study the multiple meiotic outcomes from the carrier and to provide information regarding genetic counselling and future reproductive prospects.

The strategy devised for this couple appears to have worked well since the carriers and unbalanced embryos were identified. The position of the breakpoint within the centromere of chromosome 22 meant that by using the centromeric probe for chromosomes 14/22 in conjunction with the subtelomere probe of 14q in the second round it was possible to detect the number of centromeric signals for chromosome 22.

Considering the meiotic behaviour of the ring chromosome, almost all possible meiotic segregation patterns were seen and there does not appear to be a preferential segregation mode. Although the natural pregnancies of this couple both included partial trisomy 22 with mosaicism, any number of their IVF embryos could have produced viable unbalanced pregnancies either with partial trisomy or partial monosomy due to the small size of the chromosome involved. A ring chromosome imbalance would be 0.6% of HAL and well within the limits of viability in the monosomic or in the trisomic state in the embryos (Cohen *et al*, 1994).

The only inconsistency is the fact that the only segregation mode that would have produced a balanced non carrier embryo was completely absent. This segregation might have been found in the three oocytes that there was no available follow up analysis, out

of 13 theoretical results we would expect to see at least one oocyte with a normal chromosome 22. No conclusions can be drawn since the number of available embryos was still so small. However this pattern may also be due to the way the chromosomes pair during meiosis I. Any pairing between the normal 22, the deleted 22 and the ring 22 would be complicated and would affect the position of the chiasmata formed. The reduced size of the centromeric sequences that exist in both the del(22) and the r(22) might affect attachment to the meiotic spindle.

Postzygotic errors were also widespread in all pre-implantation embryos studied resulting in mosaicism. The two natural conceptions also showed mosaicism. The instability of ring chromosomes is well documented in other studies both in prenatal samples and liveborn offspring (Jeffries *et al,* 2005, Starke *et al* 2003, Anderlid *et al,* 2001, Friedman *et al*, 1992). The origin of this instability is mostly attributed to the nature of ring chromosomes and their difficulty in undergoing mitotic division, with a tendency to form interlocking rings, leading to anaphase lag and chromosome loss. In the preimplantation embryos and natural conceptions however a varying degree of instability is observed in 100% of the cases. Multiple cell lines can be seen in preimplantation embryos due to loss of the smaller derivative chromosomes 22 and due to chaotic cell divisions. The initial meiotic error and the instability of r(22) and del(22) in addition to the frequent the unbalanced mitotic divisions common in the case of preimplantation embryos. It is unknown if the extreme chaotic mosaicism in this case is a result of the ring chromosome only since this type of mosaicism seem to be widespread during preimplantation development (Delhanty *et al* 1997, Mantzouratou *et al* 2007). Other factors may be operating in this case since the couple was sub fertile.

In this case the ring chromosome is very stable in the mother as she is phenotypically normal and has the r(22) in all metaphases and interphases studied in her lymphocytes. The reasons for this are not well understood. One explanation is the centromeric and telomeric regions required for normal cell division are still intact in the mother while in her embryos these regions may be missing or significantly shortened and their

functionality reduced. Interestingly, the only well developed carrier embryo that appeared to have the same chromosomes as the mother lost the ring in half of the cells creating a mosaic with a normal and a partially monosomic cell line. This error must have happened very early on in development possibly during the 8 cell stage or earlier.

Although the mechanism of formation of these ring chromosomes is not yet fully understood, in this case there were two initial breaks in one maternal chromosome 22. One break within the centromere and one in the q arm creating a deleted chromosome 22 and ring 22. This mechanism is also described in a recent study (by Ledbetter *et al*-submitted) and was termed "misdivision" of the centromere.

Small Marker Chromosomes (SMCs) may interfere with the segregation of other chromosomes during meiosis and mitosis giving rise to aneuploidy (Buckton 1985). Anderlid *et al* (2001) noticed that marker chromosomes may also increase the risk of UPD, however the sample studied was small . Anneren *et al* (1984) postulated that it is very likely that marker chromosomes in healthy mothers are of pathogenetic importance for non-disjunction, resulting in trisomy 21 offspring. A finding of an extra marker chromosome in one of the parents should therefore be taken into consideration in genetic counselling. In this study no interchromosomal effect was seen since chromosome 14 was diploid for most of the cells apart from the ones where polyploidy was suspected. However, other smaller chromosomes could be affected but were not investigated.

The decision not to transfer any balanced carrier embryos appears to be a valid one since the derivative chromosomes appear to be highly unstable during mitotic divisions and could produce varying abnormal phenotypes. Unfortunately, counselling couples with similar chromosomal problems is still not very precise. The variability of the breakpoints and the rare nature of these rearrangements as well as mosaicism and the variable phenotypes that would be produced make the task almost impossible.

Considering all the above the couple presents a poor prognosis in terms of producing a normal non-carrier child when all those abnormalities in embryos and previous pregnancies are taken into account. Although PGD did not produce a pregnancy in this

case it has helped give the couple some answers about the nature of the reproductive difficulties they have encountered. PGD for this type of abnormality is a viable option as long as there are some embryos suitable for transfer. The study of such preimplantation embryos gave a rare and significant chance to study and understand these phenomena in the earliest stage of development.

6.2.3 X-inactivation studies in embryos and lymphocytes from an X;autosome translocation

The X-autosome translocation 46XX, t(X;4)(q26;p16.1) PGD becomes more complex due to the X-inactivation. If balanced female carrier embryos result in an ongoing pregnancy and the derivative X is inactivated it could mean that some of the autosomal genes are switched off and an abnormal pregnancy might occur (Glaser *et al,* 2004) or a balanced but abnormal child depending on where the breakpoints are and which X is inactivated (Waters *et al,* 2001). Male carriers might be infertile due to failed meiosis during spermatogenesis (Lee *et al,* 2003). Another problem with this translocation was that the fragments involved are very small for each chromosome and therefore there is a high risk of an abnormal viable pregnancy which can result from adjacent-1 segregation products as indeed has happened in two previous pregnancies for this couple.

The aim of this study was to try to study X-inactivation in the embryos of this carrier so the pattern of X-inactivation between the derivative and normal X could be elucidated. Such a study was not done before in human preimplantation embryos where the developmental stage when X-inactivation occurs is not yet known. The first objective was to develop an efficient fixation protocol that could allow immunofluorescence and FISH to be studied on the same sample and that could be applied with a variety of antibodies. The time for this investigation was limited and was dependent on the PGD cycles of this couple. The antibody used was for targeting histone 3 (Lys9) known to be involved with the inactive X chromosome (review in Lachner & Jenuwein, 2002).

Fixation results with the chosen protocol appeared to give good results for oocytes and some of the embryonic nuclei. The embryos that were tested in the initial step were surplus IVF embryo of poor quality and were degenerating but the oocytes gave encouraging results and thus the protocol was deemed appropriate for the antibody to be used. The main concern was the preservation of the histone structure which could be damaged with the HCL/Tween used routinely for blastomeres. In addition, a clear FISH resolution had to be achieved (Hodges and Hunt, 2002).

In addition, an X-inactivation study in the maternal lymphocytes was pointing, again not conclusively, to a slightly skewed or almost random pattern of X-inactivation probably with incomplete spread of the inactivation effect along the derivative X chromosome when that was inactivated. The inactive X was the normal X in 60% of the times. Several cases have been reported of unbalanced X;autosome inactivation where the derX was inactivated creating variable phenotypes in the individuals via incomplete autosome gene inactivation (White *et al* 1998, Sharp *et al* 2002). Balanced X;autosome carriers appear to have skewed X-inactivation patterns where the derX stays active along with the autosomal genes probably due to selective growth of these cells during embryogenesis. In the present case, if the X-inactivation is random it could be because of the small size of chromosome 4 that is translocated onto chromosome X. Incomplete inactivation of the X could allow the autosomal genes to function. In addition, as in a study by Waters *et al* (2001) the breakpoints on derX might be just outside the critical boundary of Xp26 of the Xq13-Xq26 "critical region" and therefore not disrupting the any critical genes. In that study they found that certain disomies of X were tolerated if no critical genes were disrupted. However, more normal X chromosomes were inactivated which might mean that skewed inactivation to certain extent can be occurring. More investigations are needed in order to determine fully the mechanism of X-inactivation in this case.

The untransferred embryos from cycle three of the couple were tested. The sex chromosome status of the embryos was known from the biopsy, although very poor quality embryos were generated and most arrested at the 8-10 cell stage and were fragmented some with multinucleated cells. X-inactivation was detected in 2/3 embryos with more two or more X signals on biopsy and in one embryo that was not biopsied. However, clear signals could only be seen in very few embryonic nuclei. In addition, after the FISH the nuclei positive for the antibody were either lost or too degenerate to produce any FISH signals. FISH would have identified the position of the X chromosomes and the position of the inactive X, if there was one.

Although the results from this study are inconclusive, further studies into X-inactivation in human female embryos, and those from X-autosome translocation carriers would help our understanding of early gene silencing processes and imprinting. Several mouse embryo studies revealed a complex picture about the onset of X-inactivation (reviews by Ferguson-Smith 2004, Latham 2005). Although, they show an established pattern of X-inactivation in the blastocyst stage, the time of initiation of this process is still unknown. Additionally, some of these studies indicated, by using different detection methods, that the paternal X in female mouse embryos can be inactivated at the 4 cell stage. For human embryos little information exists about this topic. Two studies detected the X-inactive specific transcript (Xist) in early preimplantation embryos and found it expressed as early as the 1-cell and 4-cell stage, however, in both studies Xist expression in the early stages was observed in both male and female embryos unlike the mouse embryos and thus was not a good indicator about the inactivation of the X-chromosomes at that early stage (Daniels *et al* 1997, Ray *et al* 1997).

In this study, it was expected that some of the embryos tested for this couple would have reached various developmental stages by day 5 as in their previous cycles, so different stages could be screened for the X-inactivation target histone3. However, arrest of all the embryos in this cycle did not permit any such study to be done in this instance. The female embryos from this patient, if they were found to be carriers of the derivative chromosome for the translocation could have indicated the parental origin of the inactivated X. The acquired results, although they showed some positive signals in some nuclei, are not enough to allow any conclusions. In addition, most of the embryos seem to have undergone abnormal post-zygotic cell divisions which were not ideal for this study.

Overall, only one of the aims of this study was achieved which was the establishment of a fixation protocol for human preimplantation embryos for the combined use of immunofluorescence and FISH. This protocol with other modifications has been applied to other studies currently under way. The detection of X-inactivation patterns in human preimplantation embryos is an area of great interest and requires more

investigation. It is also particularly important for the consequences of X-autosome translocation carriers and their reproductive risks.

6.2.4 PGD strategy for an interchromosomal insertion carrier using CGH

A carrier of an interchromosomal insertion of 46, XX, ins(14;4)(q13;q25q21.3) was referred for PGD after having affected pregnancies with 46,XX, der(4), t(14;4)(q13;q25q21.3) and 46,XY, der(4), t(14;4)(q13;q25q21.3) which were terminated. During the development of PGD protocols she also had an affected son with abnormal karyotype 46,XY, der(14), t(14;4)(q13;q25q21.3) who was trisomic for 4q21.3q25. She also had a trisomy 21 pregnancy.

The aim was to develop a PGD protocol that could be applied clinically for this couple. The main problem was the lack of any commercially available FISH probes within the translocated segment. Blastomere CGH was considered the best option for this couple. Other alternatives are described in the results section. The size of the translocation fragment is around 51.3Mb and constitutes 0.59% of HAL. The deleted or the trisomic state of the translocated segment will produce viable unbalanced pregnancies. Previous natural pregnancies, livebirth and terminations also confirmed this risk.

CGH would allow detection of all possible copy number changes of the 4q21.q25 segment, but would require cryopreservation of the embryos until the results are ready. Single cells were isolated from the unbalanced child of the carrier in order ascertain if the imbalance could be detected with CGH. Attempts to shorten the hybridization time of CGH from 72hrs to 48 or 60hrs did not produce results that could be used in clinical diagnosis mainly due to inconsistent results seen within and between experiments with the shorter hybridization times. The abnormality was clearly visible only after 72hrs hybridization.

CGH allows the identification of segments >10Mb long and this imbalance could be easily identified since it is 5 times larger than the minimum length (Wells and Delhanty, 2000). The greatest limitation of this strategy is the cryopreservation of the embryos as there had been no pregnancies in this Centre from frozen thawed biopsied embryos. However, the implementation of vitrification to biopsied embryos and was taking effect at

the same time and produced better embryo survival rates in this setting. So the CGH approach was deemed appropriate for this couple. Blastomere CGH with embryo freezing has been applied clinically and the first birth was reported in 2001 by Wilton *et al.*

PGD for an insertional translocation carrier ins(14;2) was reported in 2004 by Melotte *et al*, using a four probe FISH strategy. They reported 5/10 embryos to be unbalanced forms of the parental translocation. Reproductive risks for the interchromosomal insertion carriers mainly depend on the size of the translocated fragment and the pairing formation in meiosis I. The pair of chromosomes involved can either form bivalents in meiosis I or a quadrivalent. A review of five interchromosomal insertions in large families showed that most imbalances result from simple meiotic segregation of bivalents while larger segments would probably lead to quadrivalent formation (Van Hemel & Eussen, 2000). In the same study it was observed that the reproductive risks for carriers were 32-36%. The mean size of the inserted regions occurring only as duplications was 0.96% of HAL while for deletions it was 0.47% HAL. Both deletions and duplications were detected when the HAL of the translocated fragment was between 0.22-1.2%.

The interchromosomal insertion case in this study appears to fit in with the above criteria as the size of the insertion is 0.56% of HAL and is more likely to produce both deletions and duplications as it is evident from the reproductive history. During the course of this workup the couple was fortunate to have a balanced natural pregnancy so they did not require PGD. However, this case was used a test case to apply for an HFEA license for the use of CGH in clinical diagnosis for structural abnormalities and consequently to be used for other PGD cycles were FISH will not be appropriate.

6.3 CGH investigation of oocytes and polar bodies

In a continuing effort to recognize the aneuploidy mechanisms in oocytes from couples undergoing assisted reproduction, oocytes and their corresponding polar bodies (pbs) from females going through PGS, PGD and routine IVF were studied using CGH. The aim was to ascertain the level of abnormality in these patients and study the mechanisms of aneuploidy during meiosis. Additionally, the oocytes in the present study were added to the cumulative data of previously published data from this centre as part of an overall investigation into aneuploidy in the female gametes.

Optimisation of the CGH protocol was essential for oocyte use in order to ensure results will be obtained from the maximum number of samples. Single cell CGH requires a lot of fine handling of samples as well sterile settings. Preliminary CGH experiments were done for single diploid buccal cells, single abnormal fibroblast cells and single blastomeres with an expected abnormality. From these, it can be shown that the CGH procedure for single cells (as described in Wells and Delhanty, 2000), combined with DOP whole genome amplification could detect an array of chromosomal abnormalities very precisely. The limitation is however that CGH cannot detect ploidy errors, but for the oocytes where the polar body was isolated and tested ploidy errors would be a rare occurrence.

Results were obtained from 47 oocytes and 30 polar bodies. Of these, 27 were results for both, oocyte and the corresponding polar body, 20 from single oocytes and 3 single polar bodies. Abnormalities were seen in 8 out 47 oocytes (17%). The results were investigated according to the female infertility indication. Oocytes originating from the PGS group contained all of the abnormalities relating to general aneuploidy. The aneuploidy rate in this group was 21% with average maternal age 36.3 years. On the other hand the non-PGS group (mainly PGD for translocation carriers) did not present any general aneuploidy apart for the expected errors due to translocations and the general aneuploidy rate was effectively 0% with average maternal age 35.8 years.

The most frequent abnormality was for chromosome X was 2 out of 3 where chromosomal numerical abnormalities involved chromosome X and one for chromosome

12. Whole chromosome non-disjunction was seen in two of these cases where reciprocal abnormalities in oocytes and their corresponding polar bodies could be seen. In one oocyte chromosome X material was missing while its polar body had a partially missing X. The other polar body from this complex did not produce results after CGH. The presence of single chromatids could not be verified in this sample since a gain cannot be verified in one of the polar bodies. Four other structural abnormalities were also detected in the PGS group, three of them involving the X chromosome in a single patient. Reciprocal chromosome breakage or abnormal recombination was also detected in an oocyte and its polar body for chromosome 20. In all, 5 oocytes out of 33 with results for the PGS group had X chromosome anomalies, an extraordinary high level.

Although, the sample size is small, these results indicate a higher abnormality rate in the oocytes of some women, namely in the PGS group. This will fit in with the rest of the results generated so far in this study that indicate that some individuals have a susceptibility to generalized non-disjunction. Moreover, for the PGS group of females there was also the additional information from their lymphocyte and the follow up information of the embryos produced during the PGS cycles. Therefore a complete investigation could be done for them. Interestingly, the women that presented with structural abnormalities in their oocytes had embryos which were 100% chaotic on follow up. The women who presented the X chromosome abnormalities had 40-60% simple mosaic embryos. The female that presented with a chromosome 12 meiotic error also presented with 4% meiotic errors in the embryos and 46% chaotic mosaics. It is possible, that the genetic instability seen in the PGS embryos might be inherited from the oocytes of these women. Additional oocyte investigations in relation to embryo development may reveal patterns of the fate of embryos from couples predisposed to particular types of chromosomal abnormalities.

Two other investigations were done in this centre by Fragouli *et al* (2006a). Table 6.3 summarizes the results of these studies, as well as the current one and presents the cumulative results.

Table 6.3 Cumulative oocyte results for this Centre

Study	Cumulative data	This study	Overall
No. of oocytes	235	47	282
Aneuploidy rate (%)	22%	17 PGS	20
Average maternal age	33	36	34.5
Mechanisms identified	Unbalanced chromatid Predivision (41%) and whole chromosome errors (68%). Gonadal mosaicism	Whole chromosome errors and chromosome breakage	Chromatid errors, chromosome errors, chromosome breakage, gonadal mosaicism.
Chromosomes mostly involved	X and smaller autosomes 21 and 20	X, 12, 10 and 20	X is most common followed by the smaller autosomes

An equal risk was seen for monosomy and trisomy risk at conception. Of the autosomes the smaller chromosomes were most frequently affected, reflecting similarities with embryo and prenatal studies and increased susceptibility of these chromosomes to be involved in chromosomal errors. Age independent factors predisposing to aneuploidy were evident as errors were seen in younger as well as older females that presented errors in equal proportions. This was also observed in Fragouli *et al* (2006c) and in embryos from young egg donors (Munne *et al*, 2006). Chromosome X was most frequently involved in aneuploidy overall. This has been attributed mostly to meiosis I errors were there is aberrant or altered recombination (Hall *et al,* 2006) but as yet there no clear mechanism identified for this occurrence, although it does appear that certain individuals have a predisposition to X chromosome aneuploidy, coupled with mitotically unstable embryos.

The aneuploidy rates in oocytes ranges from 22% to as high as 62% in various studies (Table 1.4, chapter 1). The higher rate was obtained from oocytes from advanced maternal age studies. A higher rate of abnormality has been observed in *vitro* matured oocytes (Magli *et al*, 2006) which may indicate a higher error rate in oocytes that have not gone through the natural maturation process. Along those lines, a study in foetal ovaries found errors at the diplotene stage and even in premeiotic precursor cells suggesting the

beginning of the mechanism that produces increased rate of aneuploidy seen in this and other studies (Roig et al, 2005). Of interest is another suggestion by Koehler *et al* (2006) were studies of mouse oocytes showed near human levels of aneuploidy. In this study, parental mouse strains with 1% sequence divergence between them were crossed and produced normal levels of aneuploidy. However, the F1 progeny crosses, where genetically diverse homologues pair during meiosis, were exhibiting aneuploidy an order of magnitude higher than that of controls. They concluded that sequence divergence between homologues predisposes the F1 progeny to increased rates of non-disjunction. If this is true for humans then some of the abnormalities seen in this study could be due to incompatibility of the genomes of the parents of the females whose oocytes were studied here.

Finally, there are major gaps in our knowledge about the initiation of aneuploidy in human gametes and preimplantation embryos. RNA expression studies in human oocytes and embryos have opened another pathway of obtaining information (Dobson *et al*, 2004, Bermudez *et al*, 2004). These studies revealed a complex picture of various RNA transcripts found in oocytes and embryos, most of them unidentified as yet, which are down- or up regulated according to developmental stage. Another study indicated that maternal age might influence the expression of oocyte genes that function in cell cycle regulation, cytoskeletal structure, transcription control, and stress responses (Steuerwald et al, 2007). It is possible then that future studies might show similar differences in aneuploid and diploid gametes and embryos as well as indicate the causes of predisposition to aneuploidy that is evident in certain individuals.

Overall, this study presents some interesting results when comparing the oocytes and the embryo results produced from the PGS group. However, since the sample sizes are relatively small, continuing this investigation could provide a clearer picture of aneuploidy and its mechanisms in female gametes and how it may relate to the development of genetically unstable embryos. This study has fulfilled most of the main

aims of the investigation and several significant findings have been established. It has also provided a stepping stone for further studies but has also created new questions that need to be answered about the genetics of preimplantation embryos.

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A1. Appendix to Methods

A1.1 Suppliers of chemicals, reagents and nucleic acids

The chemicals used in this study were obtained from the following companies: BDH, Gibco BRL ,HT Biotechnologies, Oswel, Roche, Sigma, VWR unless stated differently.

Human COT-1 DNA (1mg/ml) was obtained from Gibco BRL, UK. Deoxunucleotide triphosphates for DOP-PCR (dNTP) were supplied from Promega, UK. For nick translation dNTPs and fluorescently labelled dNTPs were supplied from Abbott, UK. DOP oligonucleotide primer (CCGACTCGAGNNNNNNATGTGG) was supplied from Oswel, UK. 10xPCR buffer (0.1M Tris-HCL pH 9, 0.5M KCL, 15mM $MgCl_2$, 1% TritonX-100, 0.1% w/v gelatine) was supplied from HT Biotechnology, UK). 10xNick translation buffer (0.5M Tris-HCl pH 7.5, 0.5M $MgSO_4$, 1M DTT) and nick translation enzyme (DNA polymerase I) was supplied as part of the Nick Translation Kit from Abbott, UK.

A1.2 Cell culture media

RPMI 1640 enriched with 15% FBS, 0.2M L-Glutamine, 300mg/ml Penicilin, 500mg/ml Streptomycin and 20µl/ml phytoheamaglutinin (PHA) was used for peripheral blood culture. The pH was adjusted with the addition of $NaHCO_3$.

Versene solution (0.02% w/v EDTA in 1lt Hanks medium was used for fibroblast preparation and DNA extraction.

A1.3 Solutions for DNA extraction

Solution for fibroblast DNA extraction: 1.21gr Tris, 0.19gr EDTA, 0.2gr SDS, 1.17gr NaCl added to 100ml double distilled water, autoclaved and Proteinase K (10mg/100ml) added.

Solutions for lymphocyte DNA extraction: Low salt buffer (10mM Tris-HCl, 10mMKCl, 10mM KCl, 10mM $MgCl_2$, 2mM EDTA). High salt buffer (10mM Tris-HCl, 10mMKCl, 10mM KCl, 10mM $MgCl_2$, 2mM EDTA and 0.4M NaCl).

A1.4 General solutions

PBS- 10mM phosphate buffer, 2.7mM KCl, 137mM NaCl.

20xSSC- 0.15M NaCl, 15mM Sodium Citrate

TE- 10mM Tris-HCl, 0.1 mMEDTA

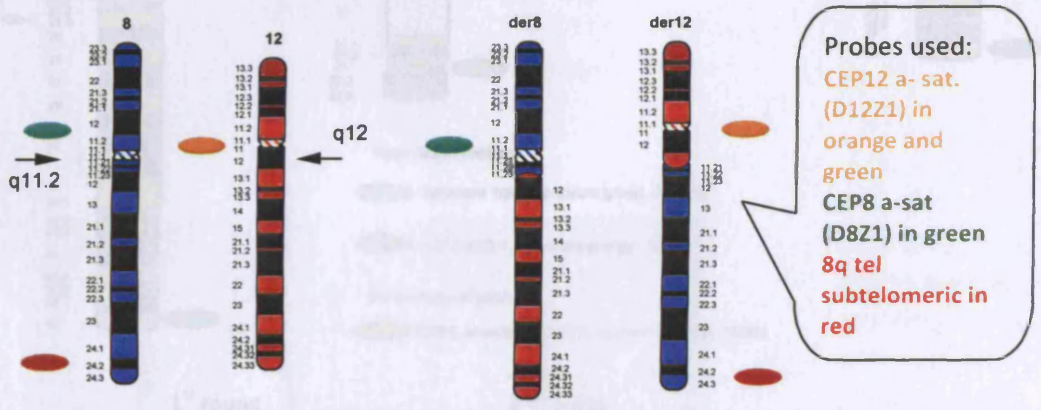
10xTBE- 90mM Tris-HCl, 2mM EDTA, 90mM Boric acid

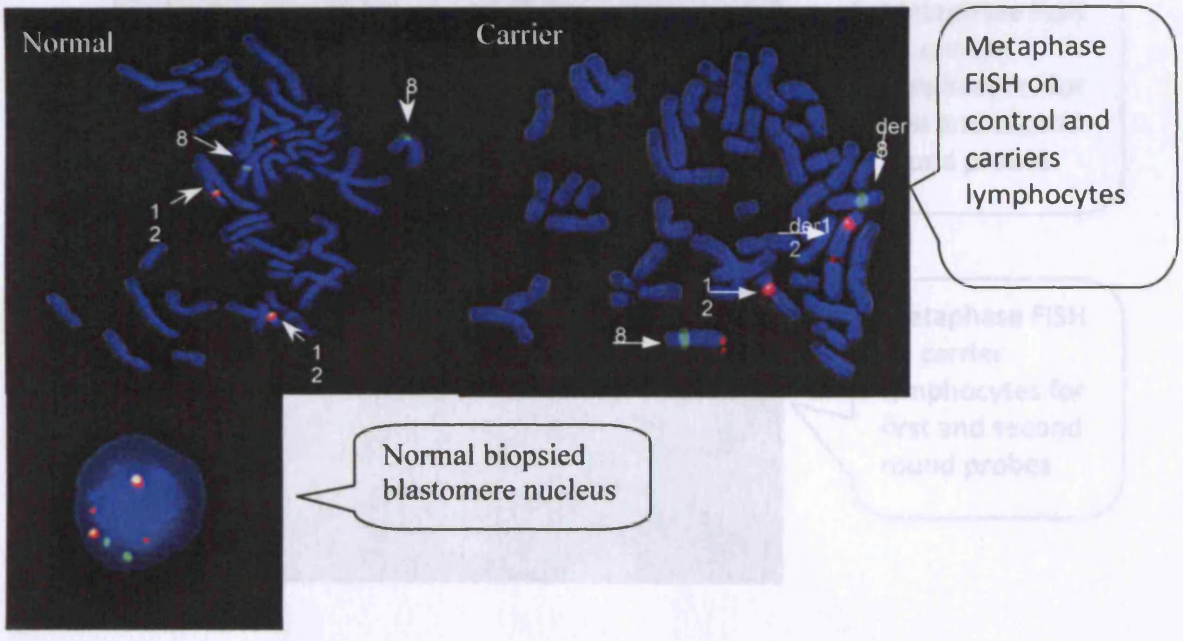
KCM- 120mM KCl, 20mM NaCl, 10mM TrisCL pH8.0, 0.5mM EDTA, 0.1% Triton-X

A2. Illustrations of PGD strategies for couples with balanced reciprocal translocations

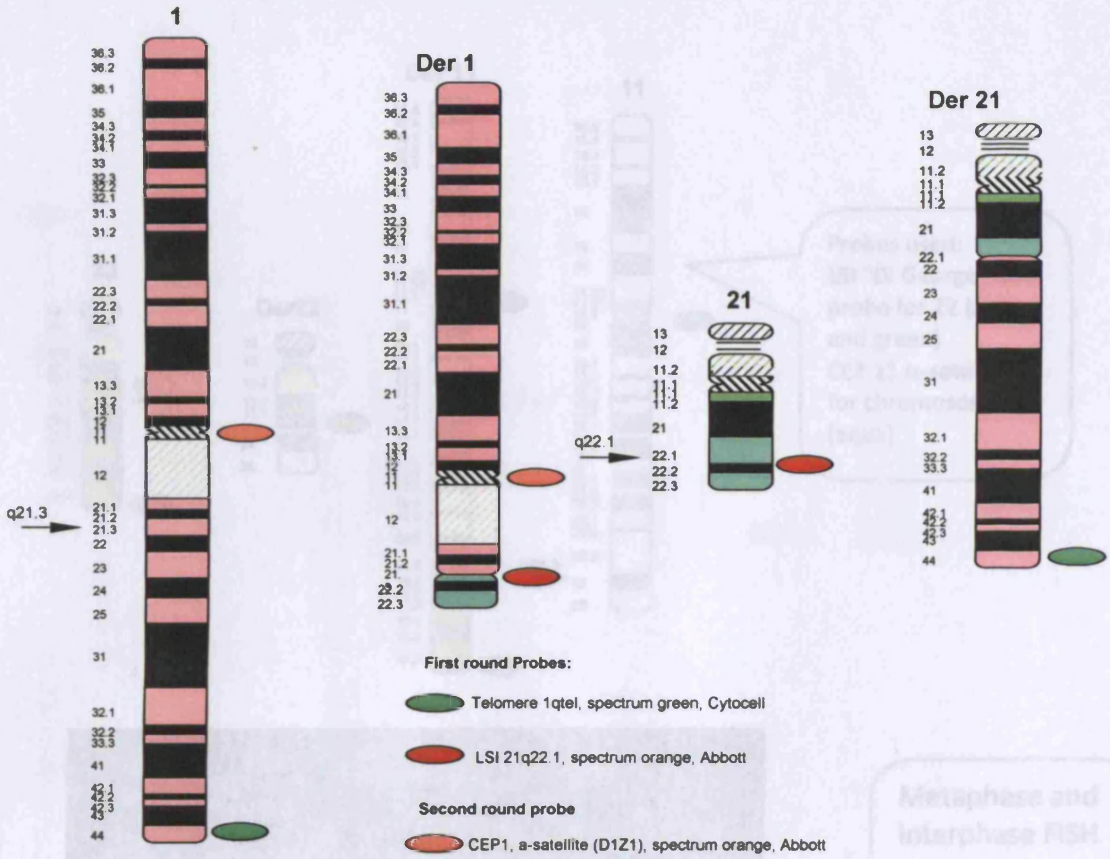
The FISH probe strategies for the balanced reciprocal translocations not shown in the main text are illustrated below with the use of ideograms for each translocation and lymphocyte results. The arrows denote the position of the breakpoints for each chromosome and the coloured shapes indicate the position and the colour of the probes used in each case. In some cases blastomere nuclei from embryos of translocation carriers are also shown.

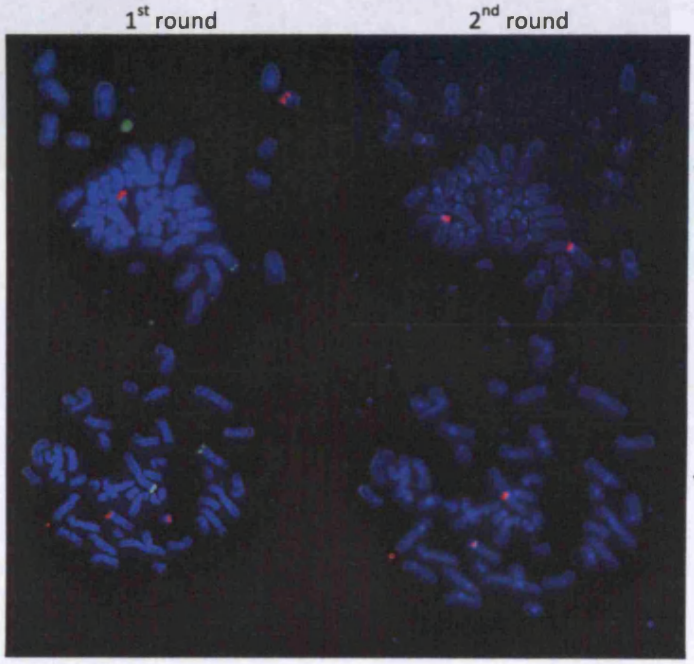
A2.1 PGD strategy for Reciprocal translocation 46,XXt(8;12)(q11.2;q12)





A2.2 PGD strategy for 46,XY,t(1;21)(q21.3;q22.1)

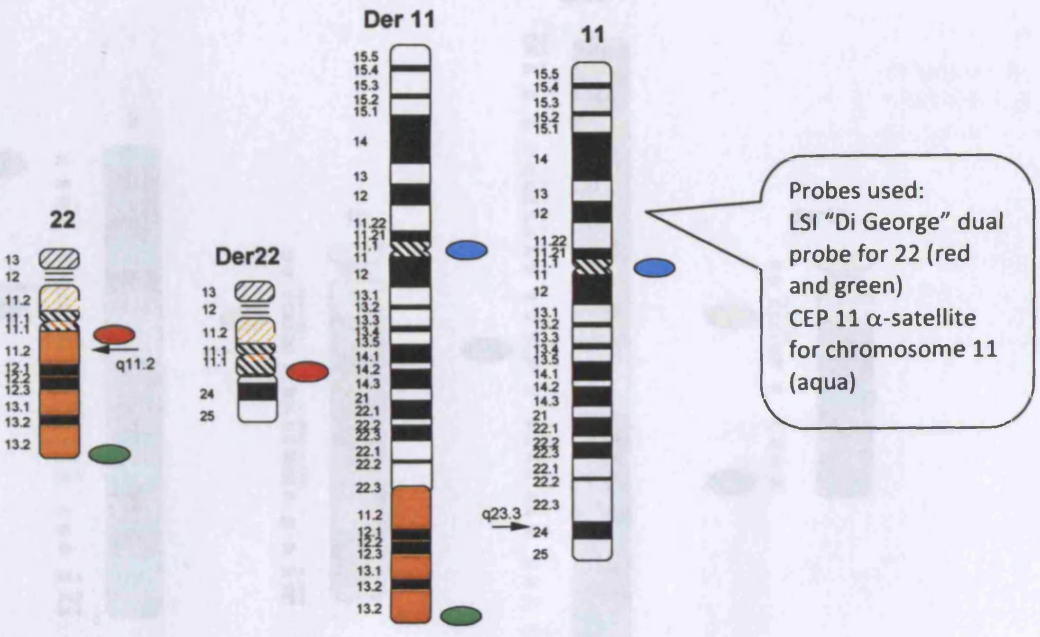


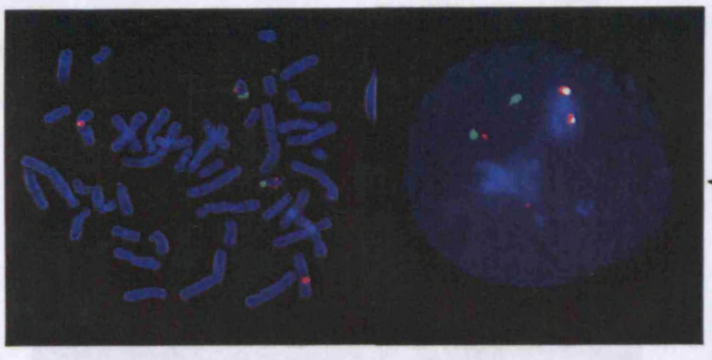


Metaphase FISH on control lymphocytes for first and second round probes

Metaphase FISH on carrier lymphocytes for first and second round probes

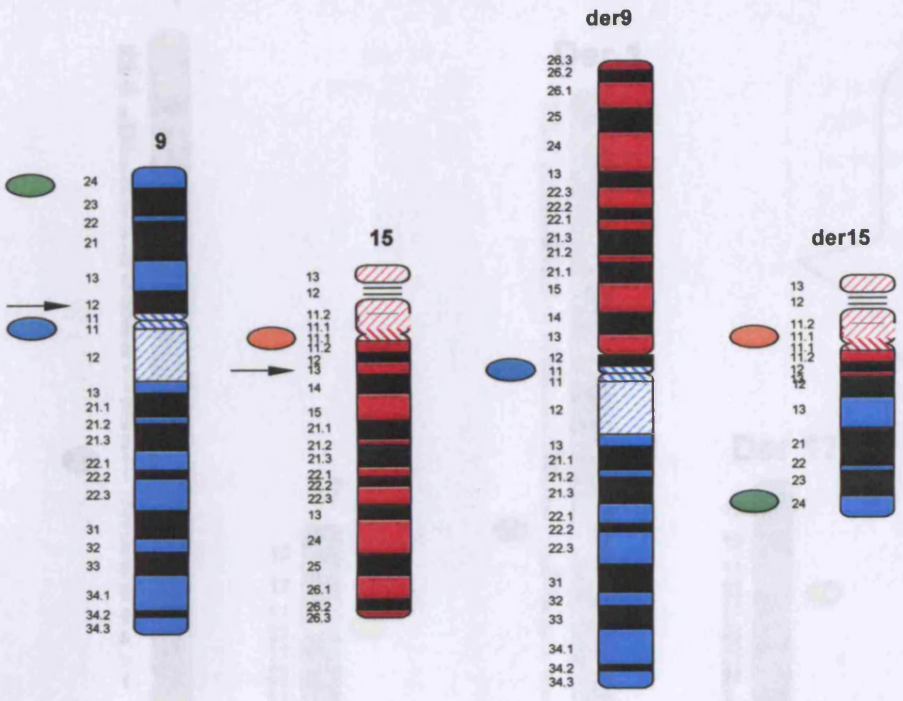
A2.3 PGD strategy for 46,XX,t(11;22)(q23.3;q11.2)





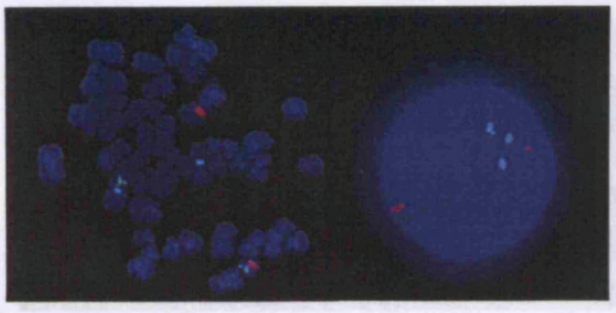
Metaphase and interphase FISH on control lymphocytes

A2.4 PGD strategy for 46,XY,t(9;15)(p12;q13)



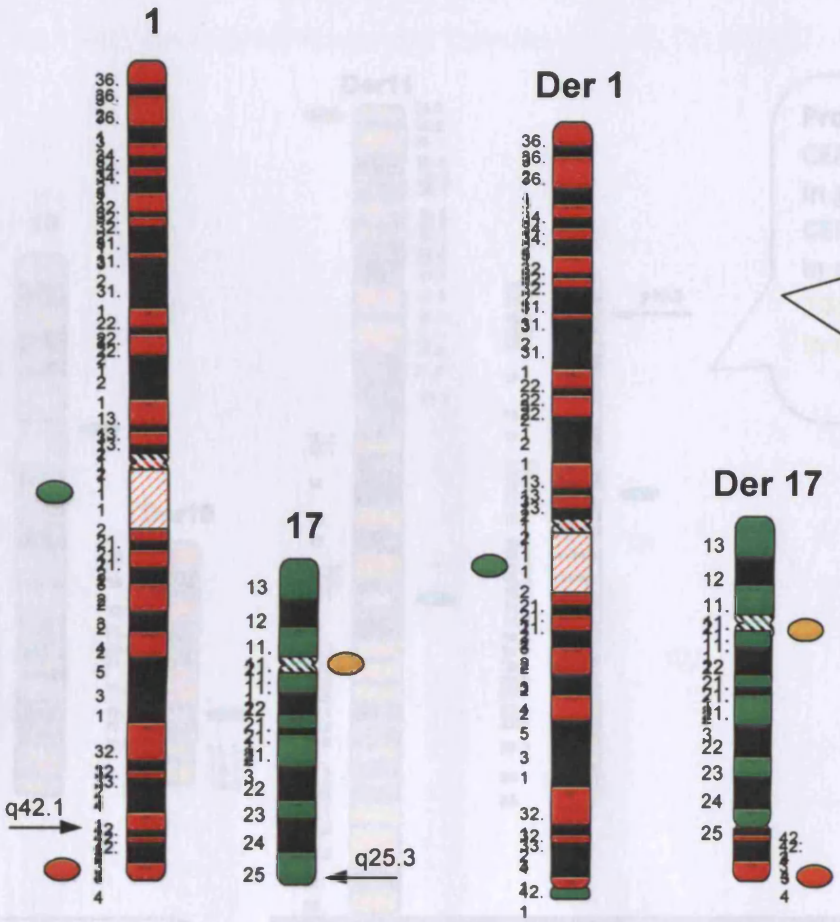
Probes used for the diagnosis

- CEP 15 (so)
- CEP 9 (sa)
- 9p Tel (sg)



Metaphase and interphase FISH on carrier lymphocytes

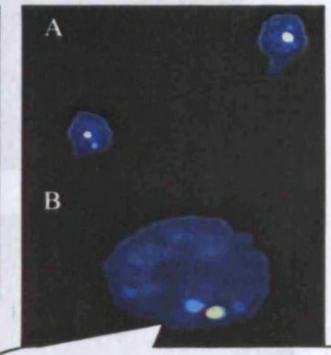
A2.5 PGD strategy for 46,XY,t(1;17)(q42.1;q25.3)



Probes used:
1 sat.II/III, lab prepared in green
CEP 17 a-sat (D17Z1) in green and orange
Subtelomeric 1qtel in orange

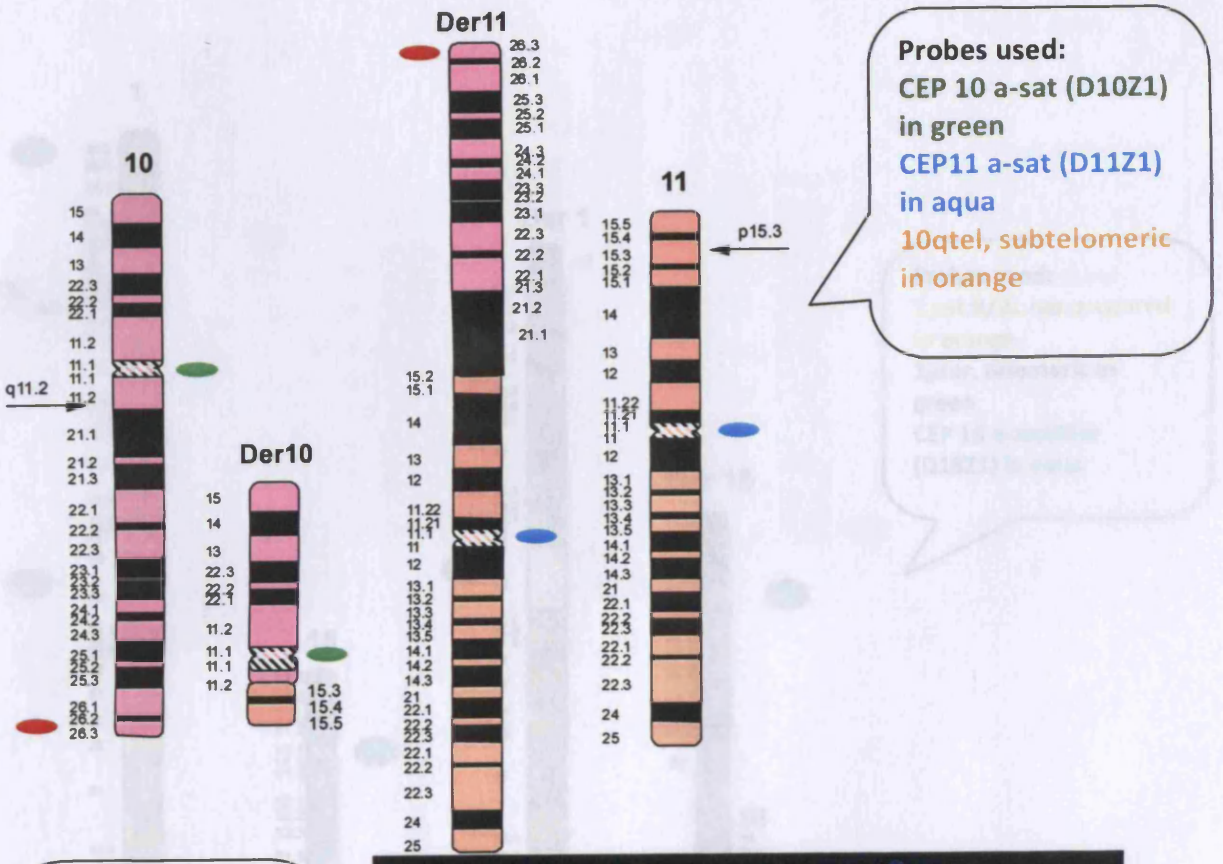
Metaphase FISH on control lymphocytes

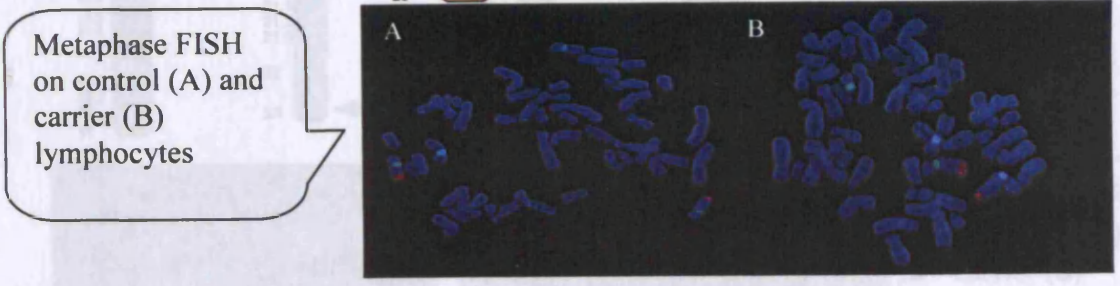
Metaphase FISH on carrier lymphocytes

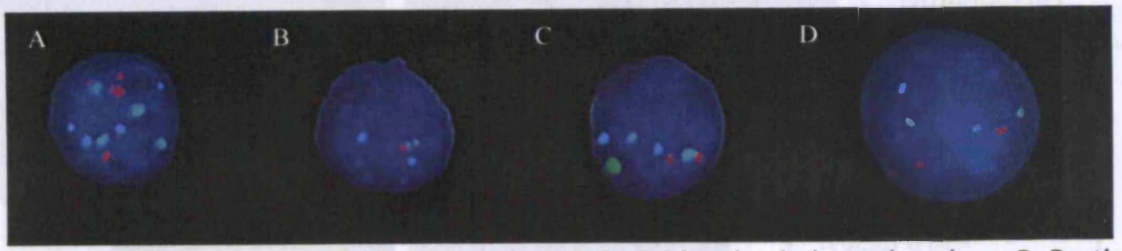


Blastomere nuclei from the same embryo showing haploidy of paternal origin with only one signal for chromosome 17 and one signal for the derivative chromosome 1. A. binucleate biopsied cell. B, mononucleate biopsied cell.

A2.6 PGD for 46,XX,t(10;11)(q11.2;p15.3)

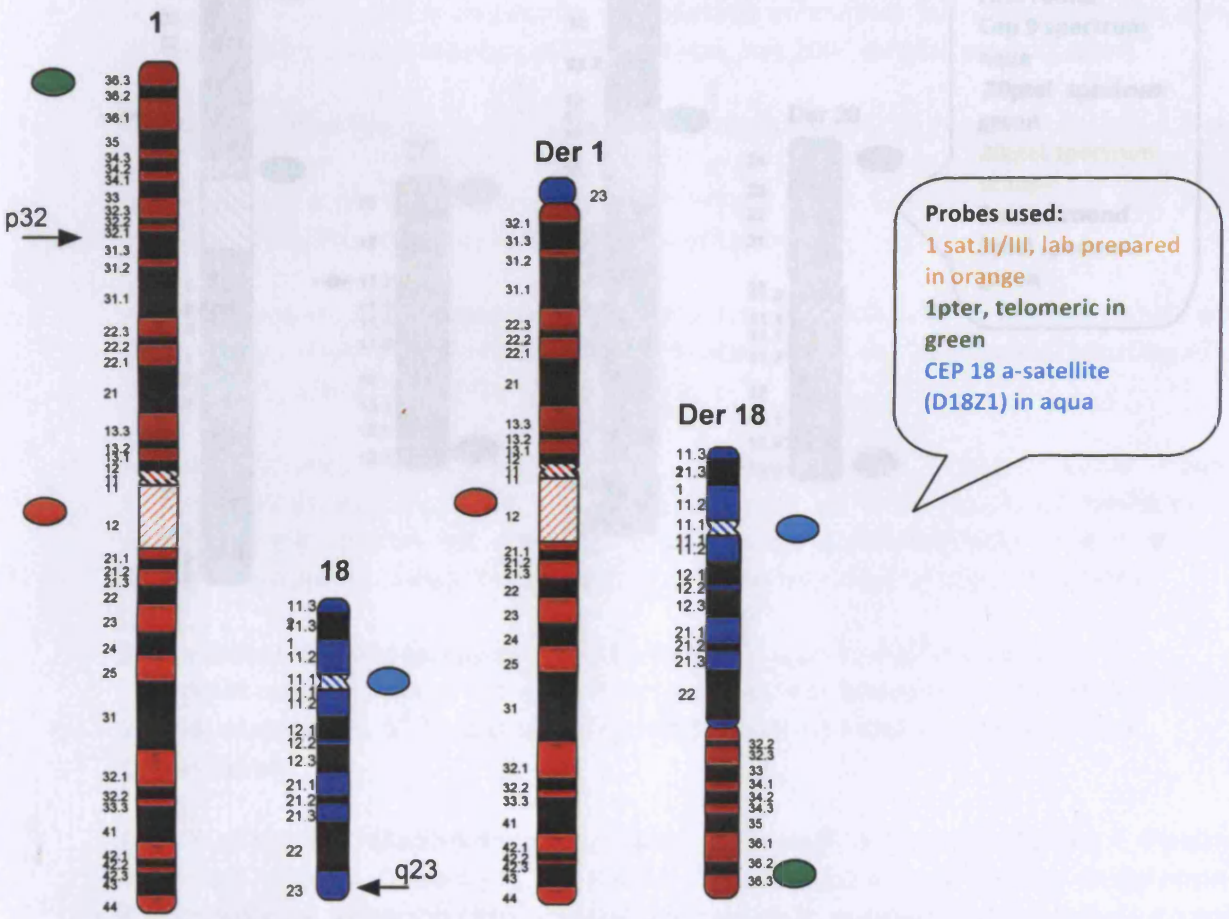


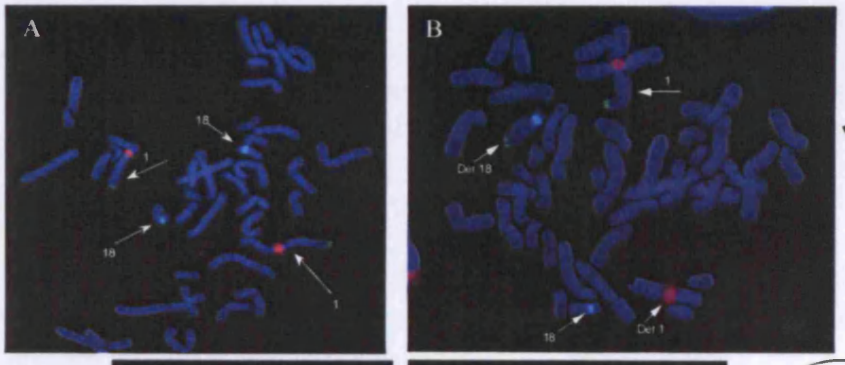


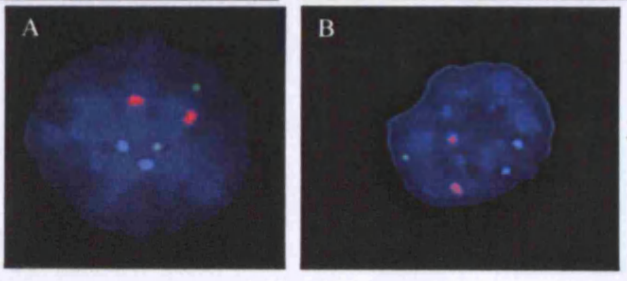


Nuclei from embryos of this PGD cycle. A.Polyploid and unbalanced nucleus, B. Partial monosomy 10q, C. Partial trisomy 10 due to extra derivative 10 present, D. Balanced nucleus.

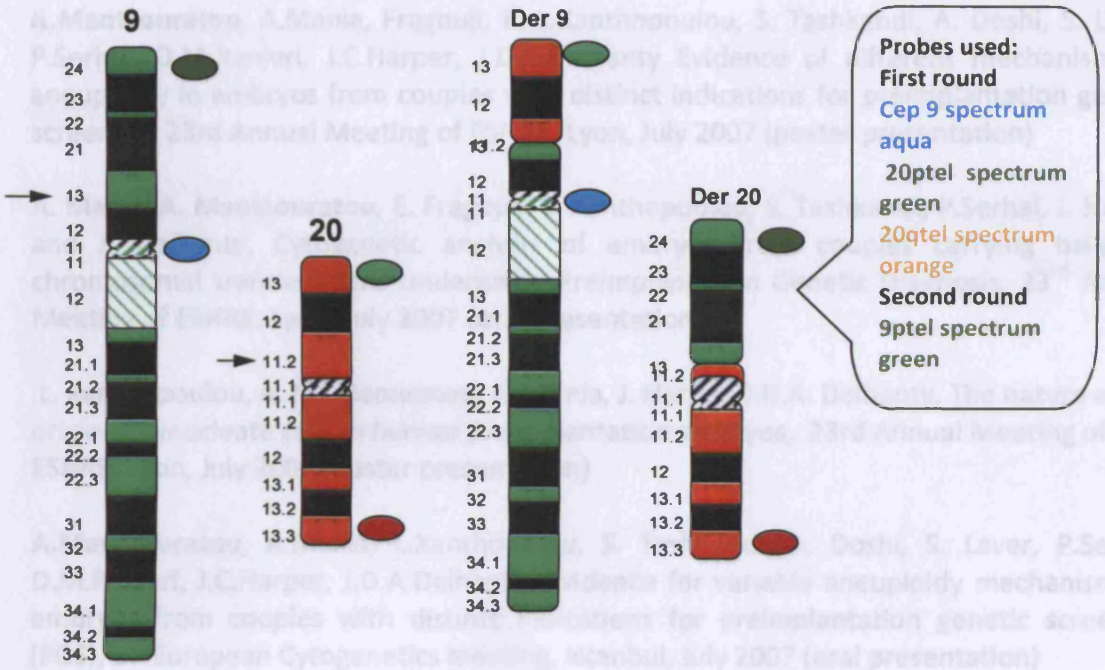
A2.7 PGD strategy for Reciprocal Translocation 46, XY, t(1;18)(p32;q23)







A2.8 PGD strategy for 46,XX,t(9;20)(p13;q11.2)



A3 Abstract presentations arising from this study

A.Mantzouratou, A.Mania, Fragouli, E. L.Xanthopoulou, S. Tashkandi, A. Doshi, S. Laver, P.Serhal, D.M.Ranieri, J.C.Harper, J.D.A.Delhanty Evidence of different mechanisms of aneuploidy in embryos from couples with distinct indications for preimplantation genetic screening, 23rd Annual Meeting of ESHRE, Lyon, July 2007 (poster presentation)

A. Mania, **A. Mantzouratou**, E. Fragouli, L. Xanthopoulou, S. Tashkandi, P.Serhal, J. Harper and J. Delhanty, Cytogenetic analysis of embryos from couples carrying balanced chromosomal translocations undergoing Preimplantation Genetic Diagnosis, 23rd Annual Meeting of ESHRE, Lyon, July 2007 (oral presentation)

L. Xanthopoulou, **A. Mantzouratou**, A. Mania, J. Harper, J.D.A. Delhanty, The nature and origin of binucleate cells in human preimplantation embryos, 23rd Annual Meeting of ESHRE, Lyon, July 2007 (poster presentation)

A.Mantzouratou, A.Mania, L.Xanthopolou, S. Tashkandi, A. Doshi, S. Laver, P.Serhal, D.M.Ranieri, J.C.Harper, J.D.A.Delhanty, Evidence for variable aneuploidy mechanisms in embryos from couples with distinct indications for preimplantation genetic screening (PGS), 6th European Cytogenetics meeting, Istanbul, July 2007 (oral presentation)

Joy Delhanty, **A. Mantzouratou** Elpida Fragouli, Dagan Wells, Paul Serhal, Comprehensive analysis of human oocytes and 1st polar bodies by comparative genomic hybridisation (CGH), 6th European Cytogenetics meeting, Istanbul, July 2007 (oral presentation)

S. A. Tashkandi, **A. Mantzouratou**, A. Mania, E. Fragouli, A. Doshi, S. Nuttall, K. Fordham, P. Serhal, J. D. A. Delhanty, J. C. Harper, Importance of the correct designation of breakpoints for structural chromosomal aberrations in preimplantation genetic diagnosis strategies, European Human Genetics Conference, Nice, June 2007 (poster presentation)

Anna Mantzouratou, Anastasia Mania, Soha Tashkandi, Paul Serhal, Alpesh Doshi, Sarah Nuttall, Joy Delhanty Insight in the causes of aneuploidy of preimplantation embryos from couples undergoing preimplantation genetic screening. Institute of Women's Health, UCL, 2nd Annual Meeting, December 2006 (oral presentation)

Anastasia Mania, **Anna Mantzouratou**, Joy Delhanty, Measuring telomere length in human blastomeres, Institute of Women's Health, UCL, 2nd Annual Meeting, December 2006 (poster presentation)

Anna Mantzouratou, Anastasia Mania, Karen Fordham, Paul Serhal, Alpesh Doshi, Sarah Nuttall, Joy Delhanty, Outcome and follow up of 60 cycles of preimplantation aneuploidy

screening for high risk couples. British Society of Human Genetics, York, 2006 (poster presentation)

Anastasia Mania, **Anna Mantzouratou**, Joy Delhanty, Measuring the length of telomeres in interphase human embryonic nuclei, British Society of human genetics, York, 2006, (Poster presentation)

Anna Mantzouratou, Anastasia Mania, Soha Tashkandi, Paul Serhal, Alpesh Doshi, Sarah Nuttall, Joy Delhanty, Outcome and follow up of 48 cycles of preimplantation aneuploidy screening, Institute of Women's Health, UCL, 1st Annual Meeting, December 2005 (poster presentation)

A4 Published papers arising from this study

Mantzouratou A, Mania A, Fragouli E, Xanthopoulou L, Tashkandi S, Fordham K, Ranieri DM, Doshi A, Nuttall S, Harper JC, Serhal P, Delhanty JD, 2007, Variable aneuploidy mechanisms in embryos from couples with poor reproductive histories undergoing preimplantation genetic screening, Hum Reprod, 22(7):1844-53

Simopoulou M, Harper JC, Fragouli E, **Mantzouratou A,** Speyer BE, Serhal P, Ranieri DM, Doshi A, Henderson J, Rodeck CH, Delhanty JD, 2003, Preimplantation genetic diagnosis of chromosome abnormalities: implications from the outcome for couples with chromosomal rearrangements, Prenat Diagn, 23(8):652-62

Copies of the published papers follow this page.