The detection of Chromosomal Abnormalities in Human Oocytes and Preimplantation Embryos by Molecular Cytogenetic Analysis

By

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Dedication

To my Mother, Father, Sister, and Grandparents

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ABSTRACT

Chromosome abnormalities are observed very frequently in humans. Several types of structural chromosome abnormalities have been identified, with chromosome translocations, both reciprocal and Robertsonian, being the most common in the population. Balanced carriers of such rearrangements could be at risk of generating abnormal offspring due to the meiotic segregation of the translocation. Preimplantation Genetic Diagnosis (PGD) has allowed the extensive cytogenetic investigation of embryos from such patients with the application of Fluorescent *in situ* hybridisation (FISH). The first part of this work involved the development of robust three-colour FISH protocols for their clinical application for the PGD for three reciprocal translocations, two different Robertsonian translocations and two cases of suspected gonadal mosaicism. Five of these patients underwent 1-2 cycles of treatment, and 21 normal/balanced embryos were detected and transferred to the maternal uterus. One clinical pregnancy was established with a subsequent live birth of a healthy male infant in a case of a female reciprocal translocation carrier. Extensive FISH examination of the non-transferred embryos showed evidence of post-zygotic mosaicism in 73.4% of them, with chaotic embryos predominating. Both meiotic and mitotic mechanisms leading to chromosome gain and/or loss were identified in this group of embryos.

Of all types of chromosome anomaly, however, aneuploidy is the most significant clinically, occurring in at least 5% of recognised pregnancies. It has been demonstrated that the errors taking place during the first meiotic division in females are the main cause of aneuploidy. The second part of the project involved the molecular cytogenetic analysis of human metaphase II oocytes and corresponding 1st polar bodies (PBs) with the application of FISH initially and then with Comparative Genomic Hybridisation (CGH). 265 unfertilised meiosis II oocytes and their corresponding PBs (when these were available) were analysed over three sequential rounds of FISH for the examination of chromosomes 1, 4, 12, 13, 17, 21, 22, and X. CGH was employed during the final part of this work for the potential analysis of 40 meiosis II oocytes and 45 first PBs, 37 of which had their corresponding oocyte also investigated. Results were obtained for 11 oocytes and 15 PBs, 6 of which were pairs. Several mechanisms leading to aneuploidy were identified: 1. Classical whole univalent non-disjunction, 2. Chromatid predivision prior to

anaphase I, leading to imbalance detected at metaphase II, 3. Germinal/gonadal mosaicism for a trisomic cell line and preferential involvement of the smaller chromosomes. The CGH investigation led to the detection of oocytes and/or PBs with missing chromosome material in a wider range of chromosomes. It was concluded that human aneuploidy is caused by a combination of meiotic factors, some of which may not be associated with maternal age.

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Abbreviations commonly found in this thesis;

ADO	Allele dropout.
***Alu*-PCR**	Alu polymerase chain reaction.
bp	Base pair.
CCD	Charged coupled device.
CGH	Comparative Genomic Hybridisation.
CPM	Confined placental mosaicism.
dATP	Deoxyadenosine triphosphate.
dCTP	Deoxycytidine triphosphate.
dGTP	Deoxyguanosine triphosphate.
dNTP	Deoxynucleoside triphosphate.
dTTP	Deoxythimidine triphosphate.
dUTP	Deoxyuridine triphosphate.
DAPI	4',6-diamidino-2-phenylindole.
der	Derivative chromosome.
DNA	Deoxyribonucleic acid.
DOP-PCR	Degenerate oligonucleotide primed- polymerase chain reaction.
ESHRE	European Society for Human Reproduction and Embryology.
ET	Embryo transfer.
FISH	Fluorescent *in situ* hybridisation.
FSH	Follicle stimulating hormone.
G-banding	Giemsa banding.
GnRH	Gonadotrophin releasing hormone.
GPS	Glutamine/Penicillin/Streptomycin.
hCG	Human chorionic gonadotrophin.
HCL	Hydrochloric acid.
HFEA	Human Fertilisation and Embryology Authority.
ICM	Inner cell mass.
ICSI	Intracytoplasmic sperm injection.

ISCN	International System for Human Cytogenetic Nomenclature.
IVF	*In vitro* fertilisation.
Kb	Kilobase pairs.
KCL	Potassium chloride.
LH	Luteinising hormone.
µg	Microgram.
µl	Microlitre.
MI	First meiotic division.
MII	Second meiotic division.
M-FISH	Multi-target/Multiplex- fluorescent *in situ* hybridisation.
mg	Milligram.
mm	Millimetre
ml	Millilitre.
MPF	Maturation/M-phase promoting factor.
ng	Nanogram.
PB	Polar body.
PBS	Phosphate-buffered saline.
PCR	Polymerase chain reaction.
pg	Picogram.
PGD	Preimplantation genetic diagnosis.
PGS	Preimplantation genetic screening.
PHA	Phytohaemagglutinin.
PK	Proteinase K.
PN	Pronucleus/ pronuclei.
RNA	Ribonucleic acid.
SA	Spectrum Aqua.
SDS	Sodium dodecyl-sulphate.
SG	Spectrum green.
SKY	Spectral karyotyping.
SO	Spectrum orange.
SR	Spectrum red.

SSC	Standard saline citrate.
SSCP	Single strand conformational polymorphism.
TE	Trophectoderm.
TOP	Termination of pregnancy.
UPD	Uniparental disomy.
v/v	Volume for volume.
WGA	Whole genome amplification.
w/v	Weight for volume.
YAC	Yeast artificial chromosome.
ZP	Zona pellucida.

Chapter 1

Introduction

1.1 The origin of human chromosomal defects

Humans as a species are not as fertile as other mammals, and that flaw becomes more pronounced with increasing age. Several studies have shown that the possibility of conception ranges between 20-25% per month for young couples with no known fertility problems (Bonde *et al.,* 1998; Edwards and Brody, 1995). The implantation rate for couples undergoing IVF procedures due to infertility of one of the two partners is also approximately 20% for each attempt (Edwards and Beard, 1999). Moreover, even if a clinical pregnancy is established, there is a possibility that it will spontaneously abort before reaching to term. Various factors have been associated with spontaneous pregnancy loss, including viral and bacterial infections of the mother such as rubella, cytomegalovirus, herpes simplex virus, syphillis (group STORCH), and infections from the *Toxoplasma* species (Spandidos *et al.,* 1998), and the lifestyle of the mother (smoking, alcohol, drug consumption). In addition, DNA alterations in the fetus are significantly associated with its rejection. Spandidos and colleagues (1998) examined seven microsatellite markers in 35 aborted fetuses and compared them with the haplotypes from the corresponding parents. They identified novel mutations in these markers in 8 of the 35 fetuses investigated, and postulated that this genetic instability could be one of the causes of spontaneous abortions, representing an increase in the embryo mutational rate (Spandidos *et al.,* 1998). However, one of the most prevalent factors negatively influencing both the establishment and/or maintenance of a clinical pregnancy is the high incidence of chromosomal abnormalities.

Studies have shown that an estimated 10-30% of fertilised oocytes tend to be abnormal in their chromosome complement (Hassold *et al.,* 1996). The effects of chromosome abnormalities are detrimental not only during the early stages of embryo development, but in later life as well. Autosomal trisomy, monosomy and triploidy account for over 50% of spontaneous abortions prior to 15 weeks gestation (Hassold *et al.,* 1980; Pellicer *et al.,* 1999). Trisomies 13, 18, and 21 may also result in liveborn pregnancies, leading in this way to large individual and socio-economic consequences. Carriers of structural abnormalities, such as balanced translocations are at increased risk of conceiving chromosomally unbalanced offspring, suffering repeated miscarriages, and have frequent fertility problems.

Fig.1.1: Mitosis and Meiosis

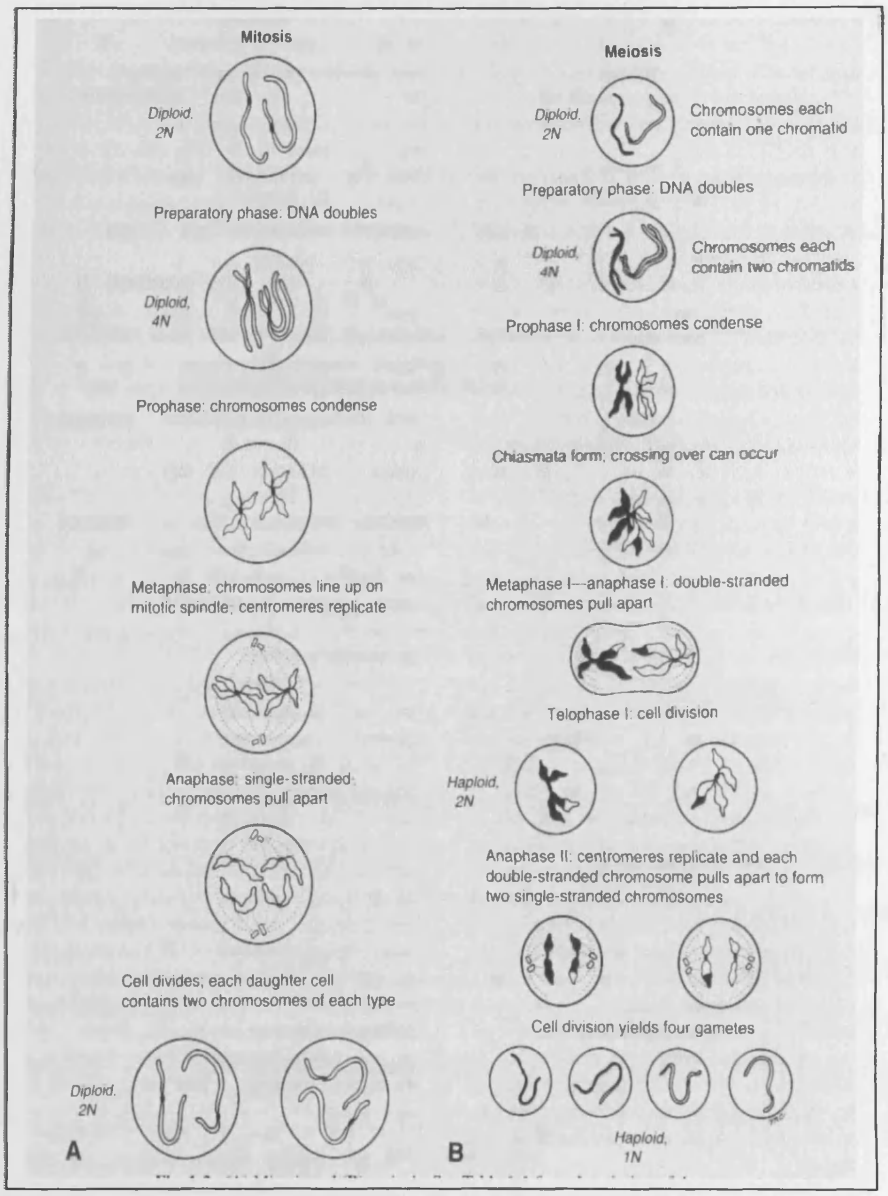


Fig.1.1: Illustration of mitotic and meiotic divisions. **A.** Mitosis occurs in somatic tissues for their maintenance and regeneration. It results in the production of identical daughter cells. **B.** Meiosis is made up of two divisions, MI and MII. During the extended prophase stage of MI the 46 chromosomes (2n) condense and form 23 homologous pairs of bivalents. These associate with each other via the formation of chiasmata. During metaphase I, these bivalents align on the metaphase plate orientated by attachment to the spindle. They then disjoin and segregate to the resulting daughter cells at anaphase I and telophase I. MI is a reduction division, whereas MII is a mitotic type division during which chromosomes align once again on the metaphase II spindle. Separation and segregation of sister chromatids to opposite poles follows at anaphase II and telophase II. Cytokinesis produces four haploid products (n). (Reproduced, by Larsen, 1997).

Chromosome defects arise as a consequence of errors in the segregation of chromosomes during both the female and male gametogenesis, but also repeatedly arise during fertilisation and the first embryonic divisions.

1.2 Meiosis in human gametogenesis

Cellular multiplication is achieved through two different types of division: mitosis and meiosis. Mitotic divisions take place for the development and maintenance of somatic tissues and organs. Such divisions result in the production of identical daughter cells required for different bodily functions and for the replacement of cells that are lost throughout an individual's lifetime (Allshire, 2004). Meiosis is a specialised cellular division that occurs only in reproductive tissue and leads to the reduction of the diploid chromosome number by half and the generation of haploid gametes. It is subdivided into two stages, meiosis I (MI) and meiosis II (MII). During the first meiotic division the sister chromatids of each pair of homologous chromosomes are held together, whilst the bivalents separate. In the second meiotic division the sister chromatids are pulled apart. The end result is the generation of four haploid cells. Both mitotic and meiotic divisions are illustrated in detail in fig. 1.1.

Subsequent union of the male and female gametes during fertilisation restores the diploid chromosome complement, ensuring in this way the propagation of the species. Meiotic recombination leading to the formation of cross-overs between the non-sister chromatids of homologous chromosomes enables their correct segregation during the different stages of this process, and enhances genetic variation.

The general mechanism underlying meiosis is the same for both males and females, but the details in the gametogenesis process are strikingly different among the two sexes (Hunt and Hassold, 2002). This variability will be analysed in the two subsequent sections.

1.2.1 Spermatogenesis

The process of human spermatogenesis is continuous and lasts 64 days. Each cell that initiates the meiotic division leads to the generation of four sperm. The latter begins when males reach puberty and continues into old age.

The precise regulation of spermatogenesis is essential for male fertility. Among the couples facing reproductive problems, 50% are affected by infertility due to male factor (Choi *et al.,* 2004). Low sperm numbers in the semen, or production of poor quality spermatozoa comprise more than 90% of infertility due to the male partner (Lilford *et al.,* 1994). Factors contributing to male factor infertility include infection, genital injury, and environmental influences. However, such cases are in their majority attributed to genetic factors (Lilford *et al.,* 1994). The long arm of the Y chromosome has been identified to contain genes that are crucial in the regulation of male fertility. It has been divided into three azoospermic factor (AZF) regions: AZFa, AZFb, and AZFc (Choi *et al.,* 2004). Deletions of these regions lead to different types of male infertility. Hence, AZFa deletions are associated with Sertoli cell-only syndrome and sometimes with oligozoospermia (Vogt *et al.,* 1996; Foresta *et al.,* 1998), while AZFb and c deletions lead to Sertoli cell-only syndrome combined with mild oligozoosopermia (Pryor *et al.,* 1997). Investigation of infertile males carrying such deletions led to the detection and characterisation of several gene families that regulate spermatogenesis. Out of these DAZ and RBM were also mapped on a specific region of the Y chromosome (Reijo *et al.,* 1995; Ma *et al.,* 1993). Other genetic factors involve chromosomal abnormalities, either numerical or structural, and CFTR mutations leading to the congenital absence of the vas deferens (Kupker *et al.,* 1999).

1.2.2 Oogenesis

Contrary to male meiosis, which is a continuous process, the human female is born with a complete set of oocytes and it is thought that there is no subsequent generation of new cells after birth. Oogenesis begins at the 6th week of fetal development with a series of mitotic divisions that lead to the formation of primordial follicles. The latter contain the diploid primary oocytes. These enter MI by 12 weeks of development and arrest at the diplotene stage of prophase I. The nuclear size of these arrested oocytes enlarges at this stage and becomes watery, leading to the formation of cellular structures known as germinal vesicles (GVs) (Larsen, 1997).

The germinal vesicles remain in a dormant state until puberty and the initiation of the menstrual cycle, which releases one female gamete each month and prepares the uterus for embryo implantation. During the fifth day of this cycle, an increase in the gonadotrophin releasing hormone (GnRH) that is secreted by the hypothalamus, affects the anterior pituitary which in turn secretes the follicle stimulating hormone (FSH) initially, and then the LH. Independently of the secretion of these hormones, the coronal cells that surround the oocytes change in their morphology and from flattened, they become cuboidal and are called primary follicles. The latter is combined with an increase in oocyte diameter and both these events mark the beginning of the preantral period (Erikson, 1986) The primary follicles along with the oocyte release a thin layer of acellular material that surrounds the oocyte and forms what is known as the zona pellucida. Each month, several oocytes are recruited and enter this process of maturation, but only one further proceeds, whereas the remaining become atretic and are subsequently lost via apoptosis. Completion of MI leads to the extrusion of the first polar body (PB), whilst the primary oocyte proceeds to MII and arrests again, at the metaphase stage of this second division. This cell is now called a secondary oocyte (Wassarman and Albertini, 1993). Ovulation occurs when the mature follicle ruptures and releases the MII oocyte surrounded by a layer of cumulus cells, the corona radiata, into the uterine tube. The secretion of LH regulates the formation of the corpus luteum from the remaining follicle. This structure enables the preparation of the uterine cavity for implantation by secreting progesterone. If fertilisation does not take place, the levels of oestrogen and

progesterone decrease and menstruation starts approximately fourteen days later (Moore, 1988).

All the above stages involving the gradual maturation of the oocyte are controlled by several factors. It has been shown that cAMP produced by the granulosa cells and transported to the oocyte via gap junctions is responsible for the arrest at prophase I (Heikinheimo and Gibbons, 1998). In a study carried out by Downs (1995) it was observed that the *in vitro* decline in cAMP levels led to resumption of meiotic division. This decline is caused by the LH surge (Granot and Dekel, 1998) It is thought that cAMP kinases phosporylate another protein, called maturation promoting factor (MPF) sustaining it in this way inactive. The decline in their concentration results in the activation of MPF. The latter has three different functions during oocyte maturation (Jones, 2004). Dephosphorylation and activation of the MPF has as an effect the breakdown of the nuclear envelope also known as GV breakdown, the resumption of MI and progression to MII and the arrest at metaphase II (Mattioli *et al.,* 1991; Furuno *et al.,* 1994). The asymmetric cell division that leads to the generation and extrusion of the 1st PB and the condensation of chromatin in the oocyte are regulated by a MAP-kinase, called c-mos (Verlhac *et al.,* 2000). This kinase is considered to be acting in combination with MPF in maintaining the metaphase II arrest, as oocytes produced by c-mos knockout mice were capable of completing MI, progressing to MII and arresting at metaphase II (Clarke and Masui, 1983, reviewed in Jones, 2004).

From the several million oogonia with which the female is born, only a few hundred mature, while the rest are lost after birth. Menopause is reached when the ovarian reserve is depleted, which renders the woman infertile.

1.3 Fertilisation and preimplantation development

In humans the meeting of oocyte and sperm and subsequent fertilisation occur in the oviduct ampulla. After deposition into and migration up the female reproductive tract, the sperm undergo a process known as "capacitation". This process removes inhibitory factors and remodels sperm to prepare them for the acrosome reaction (Wassarman, 1999). Having passed through the cumulus mass of the oocyte the spermatozoon reaches the hard zona pellucida, and binds to it (sperm-oocyte interaction). This interaction is mediated by the terminal α- and β-galactose of the ZP3 glycoprotein receptor (Litscher *et al.,* 1995). The latter along with ZP1 and ZP2 glycoproteins are the main components of the zona pellucida. Once this binding is complete, the sperm acrosome releases its degradative enzymes, enabling zona perforation. Fusion of the gamete cell membranes results in the release of the small cortical granules ingredients into the perivitelline space, located between the oocyte and the zona (Edwards and Beard, 1997).

During syngamy the oocyte resumes and completes the second meiotic division, by producing the second polar body, whilst the male and female pronuclei start moving towards each other. Chromosome condensation, DNA replication and disintegration of pronuclear envelopes follow, and the male and female chromosomes mix with subsequent formation of the first mitotic spindle. The fertilised oocyte is now called a zygote. The sperm centrosome provides the aster that organizes the first and all following mitotic spindles. Hence, the embryo's potential to divide is of paternal origin (Sathananthan, 1998).

An important factor throughout both fertilisation and syngamy is calcium (Ca^{2+}). Penetration of the sperm causes the first Ca^{2+} oscillations in the oocyte, inducing the release of the hydrolytic enzymes by the cortical granules (Heikinheimo and Gibbons, 1998). These oscillations continue throughout embryogenesis with a spatiotemporal manner, possibly crucial in the regulation of embryonic development (Edwards and Beard, 1997). Intracellularly, two types of channels are seen as responsible for Ca^{2+} release. The first is called the inositol 1,4,5-triphosphate receptor (IP_3R) operated channel, while the second is known as the ryanodine receptor (RyR) one (Berridge, 1993, Coronado *et al.,* 1994). Goud and colleagues (1999) have shown that the distribution of

the IP_3R operated channel is different during oocyte maturation, zygote formation and early embryonic development (6-8 cell stage).

It seems that the IP_3R and RyR operated channels have different sensitivities to Ca^{2+} release, and distinct locations within the oocyte. This was concluded *in vitro*, as Ca^{2+} oscillations after IVF begin at the sperm attachment site, proceed in the periphery of the oocyte, and reach its central regions (Nakano *et al.*, 1997). After ICSI however, these oscillations are delayed, and when they start they are observed all over the oolemma (Nakano *et al.*, 1997).

One day after syngamy the zygote commits itself to a succession of mitotic divisions, known as cleavage. These divisions separate the zygote into smaller blastomeres, without changing its overall size. During the first few mitotic divisions, the blastomeres are spherical and totipotent. This totipotency is progressively lost, whilst the cell divisions are asynchronous (Heikinheimo and Gibbons, 1998).

The newly formed blastomeres communicate with each other via two types of intercellular junctions: gap junctions and desmosomes. In a study carried out by Hardy *et al.* (1996) it was demonstrated that human preimplantation embryos at the 4-cell stage consisted of gap junctions containing the protein connexin-43 that enables the transfer of ions and small molecules between blastomeres. As development proceeds, these junctions increase in size and organisation. The presence of an IP_3-sensitive receptor at the gap junction sites indicates a possible relationship between Ca^{2+} and the formation of these junctions, as it is believed that the increase of intracellular free Ca^{2+} establishes a pattern of cell-cell communication. Desmosomes are observed at the 16-cell embryos and are zonular tight junctions (Edwards and Beard, 1997). They implement the formation of the inner cell mass (ICM) and trophectoderm (TE) by attaching blastomeres together. At the blastocyst stage they are found exclusively in the TE cells (Hardy *et al.*, 1996).

Genes involved in the regulation of the above processes include *Oct-4* and *6*. The OCT-4 protein controls the initiation of transcription, while OCT-6 is important for the cellular differentiation of the blastomeres (Woodward *et al.*, 1993). Liu and colleagues (2004) examined Oct-4 expression during mouse preimplantation development. They first detected transcripts of this gene in the nuclei of 8-16 cell stage morulas, followed by an

increase in expression in early blastocyts, with subsequent decline in late blastocysts, in which most of the OCT-4 protein was confined in the ICM (Liu *et al.*, 2004). The protein calmodulin is located in the blastomeres and zona environment only and regulates the first embryonic cleavages (Woodward *et al.*, 1993).

All the events that have been reported so far take place under the control of the maternal genome, as the embryonic genome is not globally activated until the 4-8 cell stage. This transition relies on cAMP-dependent protein kinase-mediated mechanisms and coincides with the degradation of the remaining maternal mRNA stores due to the shortening of their poly-A tails (Heikinheimo *et al.*, 1995). However, some Y chromosome paternal transcripts have been detected during very early stages of embryonic development, including the Y-linked genes ZFY, SRY and the myotonic dystrophy associated protein kinase DK (Taylor *et al.*, 1997). The paternal effect during the initial cleavages was also evident in a more recent study carried out by Tesarik and colleagues (2002) on the quality of preimplantation embryos resulting from the fertilisation of sibling oocytes with sperm from different donors. It was established that paternally derived problems become obvious even before the initial cleavage division, and that the quality of the fertilising sperm is crucial for the further development of the embryo (Tesarik *et al.*, 2002).

The morula is formed by the 32-cell stage and the individual blastomeres can no longer be visualised due to compaction. This process begins at the 8-cell stage and leads to the flattening, polarisation and increase of blastomere adherence, enabling in this way the maximisation of cellular communication. Near the end of compaction and due to polarisation, two different cell types develop: the ICM consisting of apolar and totipotent cells, and the TE consisting of polar ones. The cellular polarisation is dependent on Ca^{2+}, the intercellular contacts via the desmosomes, and the apical accumulation of the cytoskeleton, clathrin and actin (Houliston and Maro, 1989, reviewed in Edwards and Beard, 1997). An embryonic clock regulates compaction, which is independent of cleavage times and DNA synthesis (Edwards and Beard, 1997). The ICM and TE differentiation is regulated by the leptin and STAT3 oocyte proteins (Antczack and VanBlerkom, 1997). Leptin is a cytokine able to activate the STAT3 signal transducer (Baumann *et al.*, 1996; Vaissee *et al.*, 1996). The spatial localisation of these proteins is polarised in the mature oocyte (Antczack and VanBlerkom, 1997). At the morula stage,

inner blastomeres are poor in their content of leptin/STAT3, while outer ones are both reach and poor. By the blastocyst stage, these two proteins are only located in the TE (Antczack and VanBlerkom, 1997).

Morula cavitation leads to the formation of the blastocyst. This takes place on day 5 post-fertilisation, when the embryo arrives at the uterus. Cavitation involves the liquid accumulation among ICM cells, which leads to the formation of the blastocoel cavity. The TE cells construct a thin epithelial layer surrounding this cavity. Two models have been proposed to explain the development of the blastocyst. According to the inside-outside hypothesis the blastomeres located in the morula are destined to form the ICM cells, while the exposed ones will form the TE cells (Tarkowski and Wroblewska, 1967, reviewed in Edwards and Beard, 1997). According to the polarisation hypothesis, the ICM cells are to be formed by apolar blastomeres, while the TE cells from polar ones, depending on whether the division will be parallel or perpendicular to the axis of polarity. Due to the increase of cell-cell contact, the apolar cells are internalized and give rise to the ICM, while the polar ones stay exposed and form the TE (Mottla *et al.,* 1995). Evidence for this hypothesis was obtained in a study by Mottla and co-workers (1995). By injecting a fluorescent dye into blastomeres of 2-8 cell stage human embryos and then culturing them to the blastocyst stage, it was demonstrated that the dye was distributed evenly prior to differentiation, while afterwards it was visible in both the ICM and TE (Mottla *et al.,* 1995).

The final stage prior to implantation is the hatching of the blastocyst by the release of enzymes that penetrate and open a hole in the zona. The blastocyst is now ready to initiate communication with the maternal tissues and implant in the endometrium.

Fig.1.2 : The G-banded Karyotype

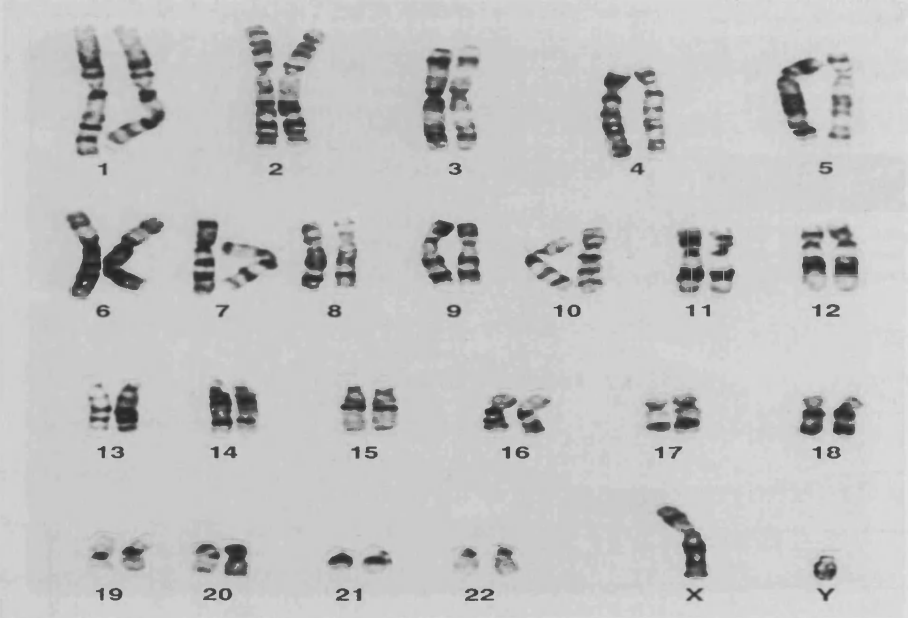


Fig.1.2: G-banded metaphase chromosomes from male peripheral lymphocytes, which were treated with trypsin prior to Giemsa staining. The normal diploid complement is comprised by 46 chromosomes. Out of these, 22 are pairs of autosomes and one is the pair of sex chromosomes (XY in males, XX in females). Each chromosome is divided by the centromere into a short (p) and a long (q) arm, each of which terminates to a telomere. Chromosome classification is standardised through the International System for Human Cytogenetic Nomenclature (ISCN, 1995) according to size, centromere position and banding pattern. Normal variable regions have been identified near the centromeres of chromosomes 1, 9, and 16, the p arm and satellites of the acrocentric chromosomes and Yq.

1.4 Chromosome abnormalities

Initial estimates on the frequency of chromosome anomaly came from several cytogenetic studies of consecutive series of livebirths carried out during the 1960s and 1970s. Results were obtained on approximately 60,000 newborns (Hassold and Jacobs, 1984). Out of these 0.3% of liveborns were characterised to be chromosomally abnormal, with trisomy of chromosome 21 or trisomy for a sex chromosome being the most common (Hassold and Jacobs, 1984). The advent of IVF and the rapid development of cytogenetic methods to investigate chromosomes of embryos in the context of preimplantation genetic diagnosis (PGD) resulted in the accumulation of data about such abnormalities prior to implantation. Chromosome abnormalities can be classified into three groups and are shown in Table 1.1 below. The G-banded karyotype of a normal male, 46,XY, is shown in fig.1.2.

Table 1.1: Different types of chromosomal abnormalities

Type of chromosome abnormality	*Category*
Numerical	Aneuploidy: monosomy, trisomy, and tetrasomy. Polyploidy: triploidy and tetraploidy
Structural	Translocations: reciprocal and Robertsonian Deletions Insertions Inversions: paracentric and pericentric Rings Isochromosomes
Different cell lines (mixoploidy)	Mosaicism Chimaerism

1.4.1 Numerical chromosome abnormalities

Aneuploidy is the commonest of all chromosome abnormalities and the most significant clinically (Hassold *et al.,* 1996). It can be defined as the loss or gain of one or more chromosomes, and its mainly caused by an error during chromosome segregation, called "non-disjunction". During non-disjunction, homologous chromosomes and/or sister chromatids do not segregate against one another in a balanced manner, but in a way that leads to unequal chromosome numbers in daughter cells (Griffin, 1996). This error can occur during meiosis I, II, and mitosis. Trisomy is one of the most frequent types of aneuploidy, being observed in 0.3% of all newborns (Bond and Chandley, 1983), and 25% of spontaneously aborted fetuses (Hassold *et al.,* 1980). Trisomies that could lead to viable pregnancies include those for chromosomes 13, 18 and 21. Of the monosomies, the one for chromosome X (45 X, Turner's syndrome) with a fetal survival rate of 1 in 20,000 is the only one to survive implantation to any degree (Griffin, 1996).

The incidence of aneuploidy is estimated by studies carried out on live births, stillbirths, spontaneous abortions, human preimplantation embryos, human oocytes and sperm (Griffin, 1996). Such studies indicate that in our species aneuploidy is observed to be an order of magnitude higher, compared with that of other mammals (Bond and Chandley, 1983). Hassold and colleagues (1996) estimated that at least 5% of all human conceptions are aneuploid. These studies also attempted to establish the parental origin of aneuploidy. It has been demonstrated that the majority of autosomal trisomies are due to maternal meiotic errors (Hassold *et al.,* 1996), while most sex chromosome abnormalities are paternally derived (Griffin, 1996). Fig. 1.3 demonstrates the aneuploidy incidence at different developmental stages.

Non-disjunction can be characterised as a *de novo* event, whose frequency is associated with two major factors: advancing parental age and aberrant genetic recombination. Several models attempted to describe the mechanisms of this process, but two are generally accepted:

(i) The classical model which involves the segregation of homologous chromosomes to the same pole, instead of segregating to opposite poles during meiosis I. This produces disomic and nullisomic daughter cells (Griffin, 1996).

(ii) This model was proposed by Angell and co-workers (1991; 1994) who in their analyses of 179 meiosis II oocytes, observed either cells with 22 or 23 chromosomes and an extra chromatid, or others with 22 chromosomes and two extra chromatids. This observation led them to suggest that non-disjunction is caused by premature division (predivision) of the chromosome centromere, instead of the wrong segregation of two homologous chromosomes to the same pole, as proposed by the classical model (Angell *et al.,* 1991; 1994). Fig.1.4 illustrates the two different models of non-disjunction.

Subsequent studies supported both the above models. One such example is a report on the analysis of 383 oocytes that failed to fertilise with the application of FISH carried out by Dailey and co-workers (1996). The authors examined chromosomes 13, 18, 21, and X and identified cells containing both extra chromosomes, but also extra single chromatids. The results obtained also indicated that non-disjunction of whole chromosomes increased with advancing maternal age. (Dailey *et al.,* 1996). In a more recent study, Mahmood and colleagues (2000) investigated 127 oocytes that had failed to fertilise and 57 corresponding 1st polar bodies (PBs) using three sequential rounds of FISH, targeting a wider range of chromosomes, including 1, 9, 13, 16, 18, 21, and X. They too identified six oocytes and three PBs with additional signals, with half the anomalies involving extra single chromatids and the remaining extra chromosomes (Mahmood *et al.,* 2000). These observations provided evidence that both the above mentioned mechanisms lead to maternal aneuploidy.

The various types of trisomy originate from errors that take place in meiosis I or II. Thus, 75% of trisomy 21 cases and 100% of trisomy 16 cases arise from maternal meiosis I errors (Antonarakis *et al.,* 1993; Hassold *et al.,* 1995), while maternal meiosis II errors account for most trisomy 18 cases (Fisher *et al.,* 1995).

Fig. 1.3: The incidence of aneuploidy at various stages of development

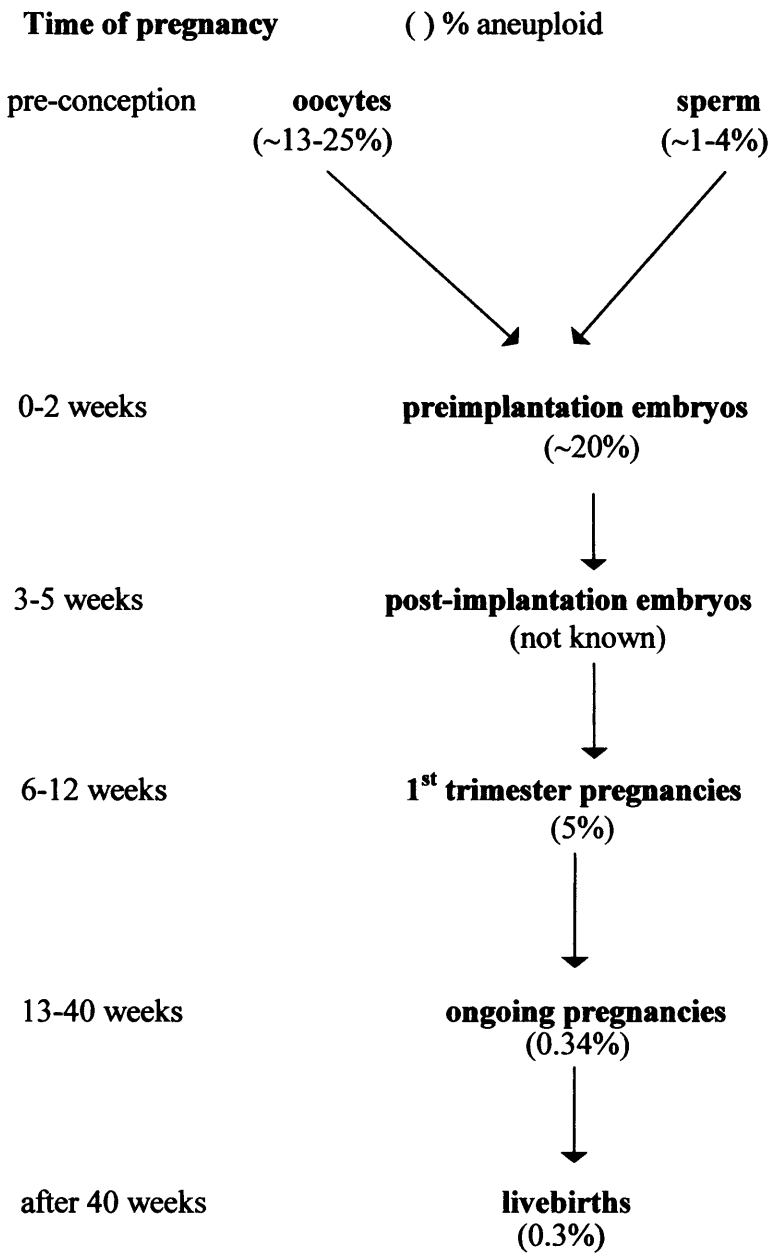
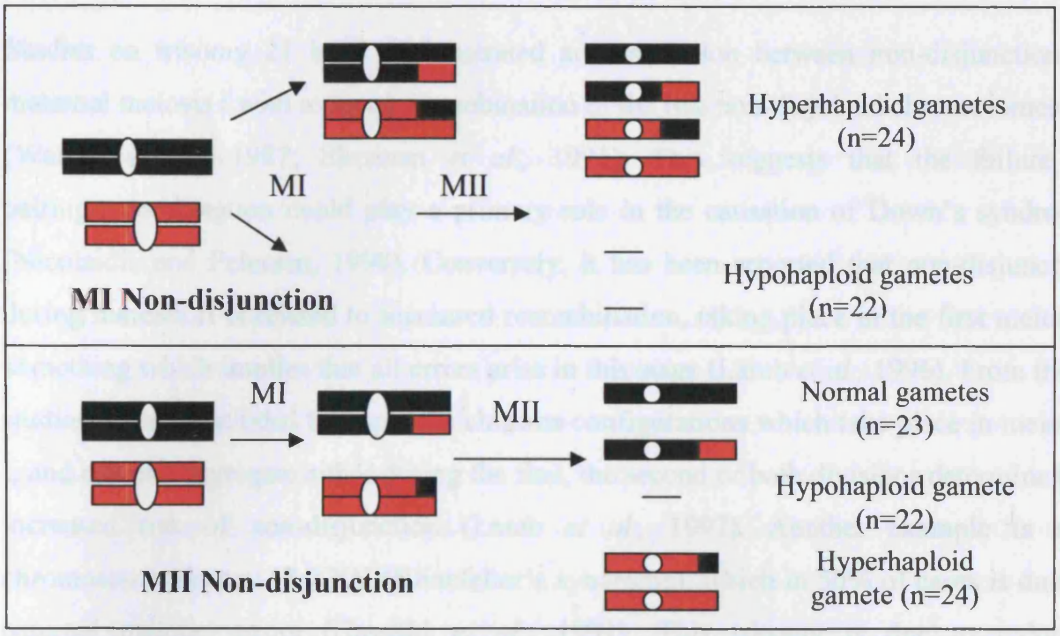
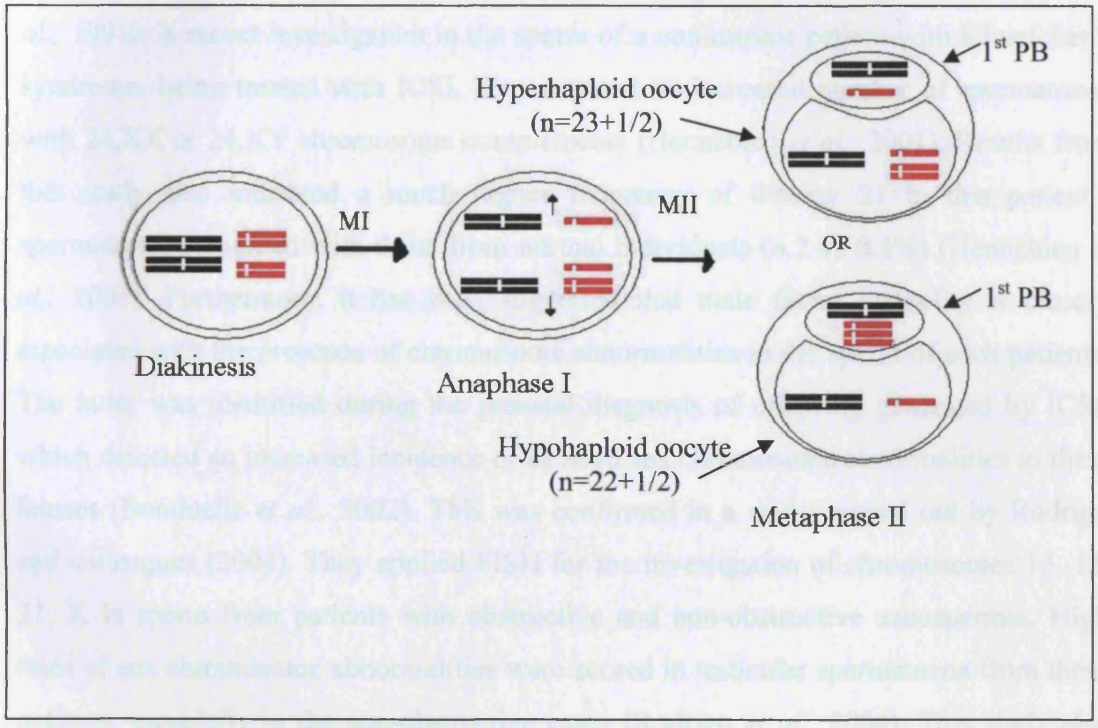


Fig.1.3: Numerical chromosome abnormalities take place during gametogenesis, fertilisation, and postzygotically with different frequencies. (From Griffin, 1996)

Fig.1.4: Models of Non-disjunction



Model 1: Classical non-disjunction during Meiosis I and II



Model 2: Premature division of a chromosome into its sister chromatids and random segregation during Anaphase I

Fig.1.4: Two models of meiotic chromosome malsegregation have been proposed. Model 1 involves the segregation of homologous chromosomes (MI) or chromatids (MII) to the same pole, which leads to the formation of disomic and nullisomic gametes. Model 2 involves the premature separation of a chromosome into its sister chromatids. These are subsequently distributed at random during Anaphase I. This predivision has been observed in MII oocytes, and could have as an effect the formation of gametes that either have an extra or a missing chromatid.

Studies on trisomy 21 have demonstrated an association between non-disjunction in maternal meiosis I with reduced recombination of the two non-disjoined chromosomes 21 (Warren *et al.,* 1987; Sherman *et al.,* 1991). This suggests that the failure of pairing/recombination could play a primary role in the causation of Down's syndrome (Nicolaidis and Petersen, 1998). Conversely, it has been reported that non-disjunction during meiosis II is related to increased recombination, taking place in the first meiosis, something which implies that all errors arise in this stage (Lamb *et al.,* 1996). From these studies it was concluded that specific chiasma configurations which take place in meiosis I, and can mal-segregate either during the first, the second or both divisions determine the increased risk of non-disjunction (Lamb *et al.,* 1997). Another example is sex chromosome trisomy 47 XXY (Klinefelter's syndrome), which in 50% of cases is due to paternal meiotic errors (Hassold *et al.,* 1991). This trisomy is due to reduced recombination leading to the prevention of the formation of the single obligatory chiasma between the X and Y bivalent, with subsequent non-disjunction at anaphase I (Hassold *et al.,* 1991). A recent investigation in the sperm of a non-mosaic patient with Klinefelter's syndrome, being treated with ICSI, demonstrated an increased number of spermatozoa with 24,XX or 24,XY chromosome complements (Hennebicq *et al.,* 2001). Results from this study also indicated a much higher frequency of disomy 21 in this patient's spermatozoa compared with those from normal individuals (6.2 vs 0.4%) (Hennebicq *et al.,* 2001). Furthermore, it has been suggested that male factor infertility is closely associated with the presence of chromosome abnormalities in the sperm of such patients. The latter was identified during the prenatal diagnosis of offspring generated by ICSI, which detected an increased incidence of *de novo* sex chromosome abnormalities in these fetuses (Bonduelle *et al.,* 2002). This was confirmed in a study carried out by Rodrigo and colleagues (2004). They applied FISH for the investigation of chromosomes 13, 18, 21, X in sperm from patients with obstructive and non-obstructive azoospermia. High rates of sex chromosome abnormalities were scored in testicular spermatozoa from these patients, especially in the non-obstructive cases (Rodrigo *et al.,* 2004). This study also provided evidence for the paternal origin of sex chromosome aneuploidy.

Polyploidy involves the generation of cells that consist of multiples of the haploid chromosome number, i.e., 69- triploidy or 92- tetraploidy. Triploidy is observed in about 16% of chromosomally abnormal spontaneous abortions (Tharapel *et al.,* 1985). In the majority of cases triploidy arises due dispermy or due to failure of the first or second meiotic division of oocytes or spermatocytes, or less commonly through participation of the second polar body in fertilisation, or by defective segregation of one haploid set of chromosomes during the first zygotic division (Niebuhr, 1974; Niikawa and Kajii, 1974; Kaji and Nikawa, 1977). The risk of triploidy is not increased with advancing maternal age, and usually such conceptions are not recurrent. A case, however, of recurrent triploid conceptions was described by Pergament and co-workers (2000). The origin of the extra set of chromosomes was established to be maternal, and was the result of an error during the second meiotic division leading to the generation of diploid oocytes (Pergament *et al.,* 2000). Tetraploidy originates from first cleavage division suppression of a zygote after the duplication of chromosomes has taken place (Jacobs and Hassold, 1980). The fusion of two diploid gametes is also possible. It has been identified in approximately 6% of chromosomally abnormal fetuses (Tharapel *et al.,* 1985).

Mitotic errors taking place during the first embryonic cleavage divisions are another cause of non-disjunction, having as an effect the formation of embryos consisting of mosaic or even chaotic chromosome complements (Delhanty *et al.,* 1997). These errors, their effects and the underlying mechanisms will be described in a subsequent section.

1.4.2 Structural chromosome abnormalities

Structural chromosome abnormalities are formed due to chromosome breakage followed by reunion in a different configuration. The latter could have as an effect the development of numerically or structurally abnormal gametes and embryos. There are different types of such abnormalities and these are the following:

- Translocations, grouped as reciprocal and Robertsonian
- Deletions
- Insertions
- Inversions, grouped as pericentric and paracentric
- Ring chromosomes
- Isochromosomes

These abnormalities can either be balanced, i.e. with no loss or gain of genetic material, or unbalanced, where there is a loss or gain of genetic material. Carriers of such abnormalities are phenotypically normal. However, they may produce abnormal offspring, due to the way the normal and derivative chromosomes segregate during meiosis. In addition, they frequently experience problems of reproductive fitness, such as infertility, subfertility, or continuous unexplained pregnancy losses (Tharapel *et al.*, 1985).

The most commonly met structural chromosomal abnormalities in the general population are reciprocal and Robertsonian translocations. Both types of translocations, their patterns of segregation and their genetic risks for carriers and embryos, will be described below.

Fig.1.5: Configuration of a reciprocal translocation during meiosis

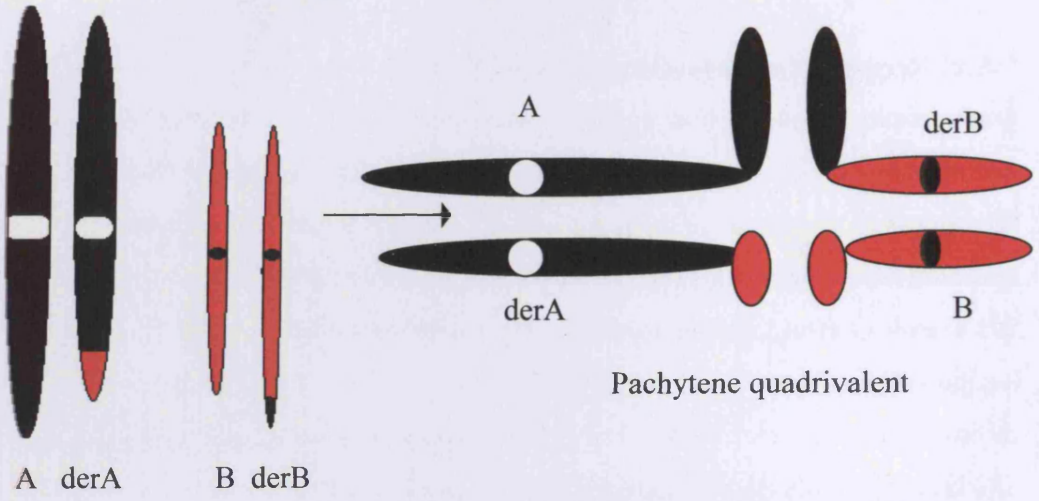


Fig.1.5: Meiosis in a balanced reciprocal translocation carrier. The process of meiotic pairing is different, compared to karyotypically normal individuals. The two normal and two derivative chromosomes align with homologous material. The latter results in the formation of a structure called the pachytene quadrivalent. One of five possible modes of segregation follows, to resolve this structure. These modes of segregation and the resulting gametes are analysed in Table 1.2 below.

Table 1.2: Patterns of segregation of a reciprocal translocation and resulting gametes

Pattern of segregation	*Segregating chromosomes*	*Gamete chromosomes*
Alternate	A,B derA,derB	Normal Balanced
Adjacent-1	A,derB B,derA	Partial disomy and partial nullisomy for translocated segments
Adjacent-2	A,derA B,derB	Partial disomy and partial nullisomy for centric segments
3:1 Interchange	derA,derB,A B	Interchange disomy for A Nullisomy for A
3:1 Interchange	derA,derB,B A	Interchange disomy for B Nullisomy B
3:1 Tertiary	A,B,derA derB	Tertiary disomy for transl. B and centric A Nullisomy for transl.B and centricA
3:1 Tertiary	A,B,derB derA	Tertiary disomy for transl.A and centric B Nullisomy for transl.A and centric B
4:0	A,B,derA,derB, 0	Disomy A,B, nullisomy A,B

1.4.2.1 Reciprocal translocations

A reciprocal translocation is generated when a break occurs in each of two chromosomes with the segments being exchanged to form two new derivative chromosomes. These can be either both monocentric, or one of them dicentric and the other acentric. This type of structural abnormality is very common in humans, being present in approximately 1 in 500 live births (Hook and Hamerton, 1977).

Reciprocal translocations may involve any combination of two non-homologous chromosomes. An exception to this rule is the reciprocal translocation t(11;22)(q23;q11), which is repeatedly occurring in the general population (Armstrong *et al.,* 2000), and has been identified in many unrelated families (Estop *et al.,* 1999).

The process of homologous chromosome pairing and recombination during meiosis I is somewhat different for balanced carriers of reciprocal translocations, compared to normal individuals. Thus, the two normal and two derivative chromosomes align with homologous material and form a structure termed the pachytene quadrivalent. One of five possible modes of segregation follows. These include the alternate mode, adjacent-1, adjacent-2, 3:1 or 4:0 and result in gametes that are either balanced or unbalanced. Fertilisation of these gametes leads to the generation of 32 possible zygotes, out of which only two are genetically balanced, one carrying a normal chromosome complement whilst the other carries the reciprocal translocation in the balanced form. The pachytene quadrivalent, the possible modes of segregation and the gametes that result from those are shown in fig.1.5 and Table 1.2.

Data obtained from the study of both balanced and unbalanced carriers of reciprocal translocations suggest that only one mode of segregation leading to the generation of unbalanced gametes is likely to result in the attainment of a viable pregnancy. This segregation mode is dependent on the reciprocal translocation and the sex of the carrier (Gardner and Sutherland, 1996). Jalbert and colleagues (1980) described several pachytene shape algorithms, produced by comparing the length of the translocated and non-translocated segments, and suggested this as a way to predict the most likely segregation pattern leading to imbalance for any translocation. Ogilvie and Scriven (2002) analyzed the meiotic outcomes of a total of 16 different reciprocal translocations,

12 of which were identified in females and 4 in males, by examining the embryos generated from these patients with the application of FISH. They identified that the alternate and adjacent-1 segregation modes were occurring with the same frequency for both males and females, whilst the 3:1 mode was observed more often in females, rather than in males. The authors attributed the latter, to the generation of a large spectrum of gametes during spermatogenesis and the possible negative selection of highly abnormal ones. This negative selection is not feasible in the female meiosis, which could predispose to specific segregation modes depending on the size of the translocated and centric segments of the chromosomes that participate in the translocation (Ogilvie and Scriven, 2002).

Balanced carriers of reciprocal translocations are usually phenotypically normal, as there is no loss of genetic material. However, there have been cases of such carriers suffering from mental retardation (Funderburck *et al.,* 1977), or some types of cancer when the transfer of chromosome segments is accompanied with gene disruption (Therman and Susman, 1993). This type of structural abnormality is often identified in cases where it affects the reproductive fitness of the carrier, leading to subfertility, infertility, unexplained spontaneous abortions, or the birth of an abnormal child. The risk for such births ranges between 10-15% (Midro *et al.,* 1992) and is associated with the translocation involved. Viable offspring that are carriers of unbalanced reciprocal translocations run a high risk of being mentally retarded, have serious congenital abnormalities or a combination of both. This is dependent on how large the duplications and deletions are on the two derivative chromosomes. The latter can be predicted by constructing a pachytene diagram and measuring the sizes of the centric and translocated segments, as mentioned above. In most cases, however, fetuses with unbalanced reciprocal translocations fail to proceed past mid-pregnancy.

Fig.1.6: Configuration of a Robertsonian translocation during meiosis

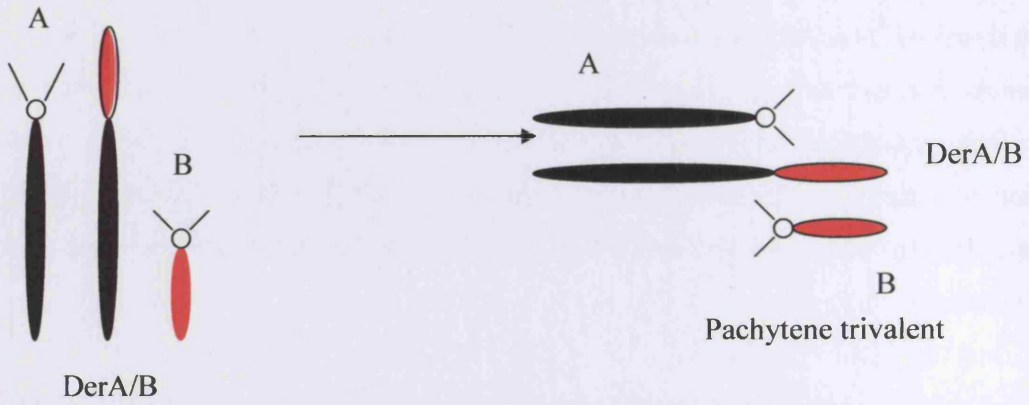


Fig.1.6: Meiosis in a balanced Robertsonian translocation carrier. As with reciprocal translocations, the process of meiotic pairing is different, than that observed in normal individuals. The two normal and the derivative chromosomes align with homologous material. The latter, in this case, results in the formation of a structure called the pachytene trivalent. One of four possible modes of segregation follows. These modes of segregation and the resulting gametes are analysed in Table 1.3 below.

Table 1.3: Patterns of segregation of a Robertsonian translocation and resulting gametes

Pattern of segregation	*Segregating chromosomes*	*Gamete chromosomes*
Alternate	A, B	Normal
	DerA/B	Balanced
Adjacent	DerA/B, A	Disomy for A
	B	Nullisomy for A
Adjacent	DerA/B, B	Disomy for B
	A	Nullisomy for B
3:0	A, B, DerA/B	Disomy A, B
	0	Nullisomy A, B

1.4.2.2 Robertsonian translocations

Robertsonian translocations are formed when two acrocentric chromosomes (13, 14, 15, 21, and 22) break at a position on or close to their centromeres with subsequent fusion of their long arms. In this way, a single metacentric derivative chromosome is produced and the diploid number is reduced by one. These translocations are identified in humans with a frequency of approximately 1 in 1000 livebirths. Of all the possible chromosome combinations, two Robertsonian translocations are observed more often in the general population. These are 45,XX or XY, t(13;14)(q10;q10) and 45,XX or XY, t(14;21)(q10;q10), leading to 73% and 8% of all newborn Robertsonian translocation carriers (Therman and Susman, 1993).

As with reciprocal translocations, the meiotic segregation patterns of the chromosomes involved in a Robertsonian translocation are crucial, as far as the production of aneuploid gametes and embryos is concerned. During the first meiotic division the normal homologues and the derivative chromosome synapse together and behave as a trivalent (Munne *et al.,* 2000a; Morel *et al.,* 2001). The subsequent segregation modes are not as complicated as is the case for the reciprocal translocations. Hence, the alternate mode is most commonly observed and results in normal or balanced gametes, while the adjacent mode is also sometimes identified leading to the generation of two disomic and two nullisomic gametes (Munne *et al.,* 2000a). This trivalent association of chromosomes, patterns of segregation and resulting gametes can be seen in fig.1.6, and Table 1.3.

Studies that were carried out on spermatozoa of balanced (13;14) Robertsonian translocation carriers identified the alternate mode of segregation as being the most prevalent one, found in 73.5% out of 117 spermatozoa (Martin, 1988). A similar observation was made in a much larger study on the sperm of two (13;14) Robertsonian translocation carriers by the application of FISH (Escudero *et al.,* 2000). Out of an average of 1000 gametes scored for each of these carriers, approximately 77% had either a normal or balanced complement, as far as the chromosomes that were involved in the rearrangement were concerned (Escudero *et al.,* 2000). Investigation of embryos generated from Robertsonian translocation carriers confirmed the above. Scriven and colleagues (2001) applied FISH for the PGD of embryos coming from five couples in

which one of the partners was a carrier of such a translocation. The data obtained led to the conclusion that the segregation of a Robertsonian translocation predisposes mostly to the production of normal or balanced gametes, and subsequent generation of embryos consisting of the expected chromosome complement.

The phenotype of balanced carriers of Robertsonian translocations is usually normal, but this rearrangement may again affect their fertility. Many male Robertsonian translocation carriers have been shown to be infertile, whilst the reproductive capacity of others is not affected at all. In a research letter Daniel (2002) attempts to explain this phenomenon by describing a model proposed by Henikoff and colleagues (2001). In this model, the infertility of the male carriers of such rearrangements is connected to the dicentric derivative chromosome, which leads to the formation of more spindle attachment sites. This in turn affects the normal chromosome segregation during meiosis, and thus male fertility (Henikoff *et al.,* 2001). Female carriers of such translocations may experience recurrent spontaneous abortions. Moreover, the offspring of Robertsonian translocation carriers have a high risk of being born genetically handicapped, suffering from Down's syndrome (4% of all trisomies 21), rarely Patau's syndrome (trisomy 13) and sometimes of uniparental disomy for chromosome 14 (Boue and Galiano, 1984; Mutton *et al.,* 1996; Tomkins *et al.,* 1996).

Carriers of both reciprocal and Robertsonian translocations that have experienced infertility or repeated miscarriages and are at a high reproductive risk are becoming increasingly interested in PGD in order to improve their chances of a viable healthy pregnancy.

1.5 Preimplantation Genetic Diagnosis

Preimplantation genetic diagnosis (PGD) was developed in 1989 and it was initially utilised in sexing and identifying healthy embryos for couples that were at risk of transmitting X-linked recessive disorders with the application of the polymerase chain reaction (PCR) (Handyside *et al.,* 1990). In brief, the procedure involves the generation of embryos by IVF, the biopsy of either one or two blastomeres on day 3 post fertilisation (cleavage stage of development), their genetic analysis either by PCR or by FISH, and the transfer of healthy embryos on day 4 of preimplantation development (Handyside and Delhanty, 1997). At present, PGD can be considered as an alternative to prenatal diagnosis of certain single gene disorders and chromosome abnormalities (Harper and Delhanty, 2000). This identification of genetic anomalies in the embryo prior to implantation is advantageous as it enables parents who have had affected children or have suffered repeated miscarriages to initiate an unaffected pregnancy, avoid considering termination, and possibly even eradicate a disorder from the family.

Patients that turn to PGD to achieve a healthy pregnancy can be divided in the following groups:

(i) Patients at risk of transmitting an X-linked disorder for which the development of a specific molecular diagnosis is not feasible.

(ii) Carriers of single gene disorders, dominant and recessive, autosomal or X-linked. PGD enables them to have a healthy child, but also to remove the inherited disease from the family, in the case of dominant disorders.

(iii) Carriers of structural chromosome abnormalities. These chromosome abnormalities could be affecting their fertility, as mentioned above, or they could have undergone recurrent spontaneous abortions as a result of these abnormalities.

(iv) Women over the age of 35 that undergo IVF could use PGD to screen for age-related aneuploidy to improve their chance of an embryo implanting.

(v) Male patients that are infertile and require ICSI. In such cases PGD can be applied for the detection of cystic fibrosis, Y chromosome deletions and/or chromosome imbalance where appropriate.

(vi) Couples who are against the termination of an affected pregnancy due to moral or religious reasons. PGD offers the ability to select for embryos prior to implantation and reduce or even eliminate the necessity for a termination.

From the above one could conclude that PGD is the ideal solution for patients belonging to one of the listed categories. However, this procedure is not as widespread as prenatal diagnosis with only a small number of centres providing it worldwide, and less than 2000 cycles being carried out in fifteen years (Harper and Bui, 2002). This could be attributed to the fact that the process of selecting and transferring healthy embryos whereas the remaining are discarded still remains controversial. There have been quite a few studies and surveys about the attitudes towards PGD. Possible advantages offered by PGD include the following (Viville and Pergament, 1998):

(i) Avoiding the elective termination of pregnancy, especially for couples that have had to undergo this procedure repeatedly or are against it due to moral or religious reasons.

(ii) Preventing severe and disabling inherited disorders prior to embryonic implantation.

There have been however, some ethical considerations. More specifically, people against PGD have expressed worries about eugenics and the creation of embryos whose characteristics such as sex, height, and intelligence are selected by the parents (Harper and Delhanty, 2000). However, such characteristics are multifactorial, and their diagnosis would be very complicated, if not impossible (Harper and Delhanty, 2000). In the UK all centres that offer PGD are under the control of the Human Fertilisation and Embryology Authority (HFEA), and the diagnosis of every new disease must first be approved before application. Other problems challenging the efficiency of PGD include the possibility of misdiagnosis either by PCR due to allele dropout (ADO) or contamination, and by FISH due to embryo mosaicism (Handyside, 1998). For this reason the ESHRE PGD Consortium was established in 1997. This Consortium collects prospective and retrospective data on the accuracy, reliability, and effectiveness of PGD and has the following aims (ESHRE PGD Consortium Steering Committee, 2000):

(i) Perform surveys on the availability of PGD for the different disorders

(ii) Begin follow-up studies of pregnancies achieved and children born

(iii) Develop guidelines and recommended PGD protocols to assist the best practice possible

(iv) Formulate a consensus on the use of PGD

The various stages of PGD will be discussed in the sections to follow.

1.5.1 Biopsy methods

There are two stages involved in the PGD of a genetic disorder, the biopsy and the diagnosis (Harper and Delhanty, 2000). Biopsy can be performed on three types of cells: first and second PBs (Verlinsky *et al.,* 1992), blastomeres from cleavage stage embryos (Tarin and Handyside, 1993; Inzunza *et al.,* 1998), and trophectoderm cells from blastocysts (Veiga *et al.,* 1997).

1.5.1.1 Polar body biopsy

Polar body biopsy involves the examination of oocytes prior to fertilisation by investigating the first and/or second PBs. Neither of these cells are required during fertilisation and subsequent embryonic development, and hence their removal should not have any adverse effects on the embryo.

Before the application of this type of biopsy on human oocytes, the method was evaluated on those from the mouse (Gordon and Talansky, 1986). The PBs were removed through an opening on the zona pellucida that was achieved with the use of Acid Tyrode's solution. Mouse oocytes that were treated this way resulted in the production of live offspring after their subsequent fertilisation (Gordon and Talansky, 1986). This was not the case, however, with human oocytes when they were treated with acidified Tyrode's prior to their fertilisation. This solution posed an inhibitory effect on embryonic development, even though a normal fertilisation was observed (Malter and Cohen, 1989). Now mechanical means or a laser are used in order to create an opening in the zona of human oocytes to (Montag *et al.,* 1998).

The actual process is as follows: the aspiration of the first PB usually takes place within six hours after oocyte retrieval to avoid its degeneration in culture, whilst the second one is removed after formation of the zygote (Kuliev *et al.,* 1998; Strom *et al.,* 1997). It is also possible to biopsy both the first and second PBs at the same time from the zygote (Verlinsky *et al.,* 1995; 1996).

PB biopsy was developed by two groups in the United States (Munne *et al.,* 1995; Verlinsky *et al.,* 1996) and has been applied mostly for the detection of chromosome abnormalities with the application of FISH, in the context of aneuploidy screening. The rationale behind this approach is that chromosome loss or gain taking place in the oocyte has the reciprocal effect of gain or loss in the PB (Munne and Wells, 2002). PBs have also been aspirated for the PGD of both reciprocal and Robertsonian translocations (Munne *et al.,* 1998d; Durban *et al.,* 2001), and single gene disorders (Strom *et al.,* 1997; Kuliev *et al.,* 1998).

There are several advantages in this PGD approach, including the fact that there is no intervention with the actual embryo, the origin of aneuploidy is predominantly maternal and thus detectable in PBs, and the fact that no abnormal embryos are being discarded (Munne and Wells, 2002). However disadvantages do exist and involve the decrease in the accuracy of the FISH procedure due to the chromatid predivision that takes place if PBs are cultured for prolonged periods, and the inability to score for paternally-derived abnormalities (Munne and Wells, 2002). For these reasons, this approach is not as widespread as cleavage stage biopsy.

Fig.1.7: Cleavage-stage Embryo Biopsy for PGD

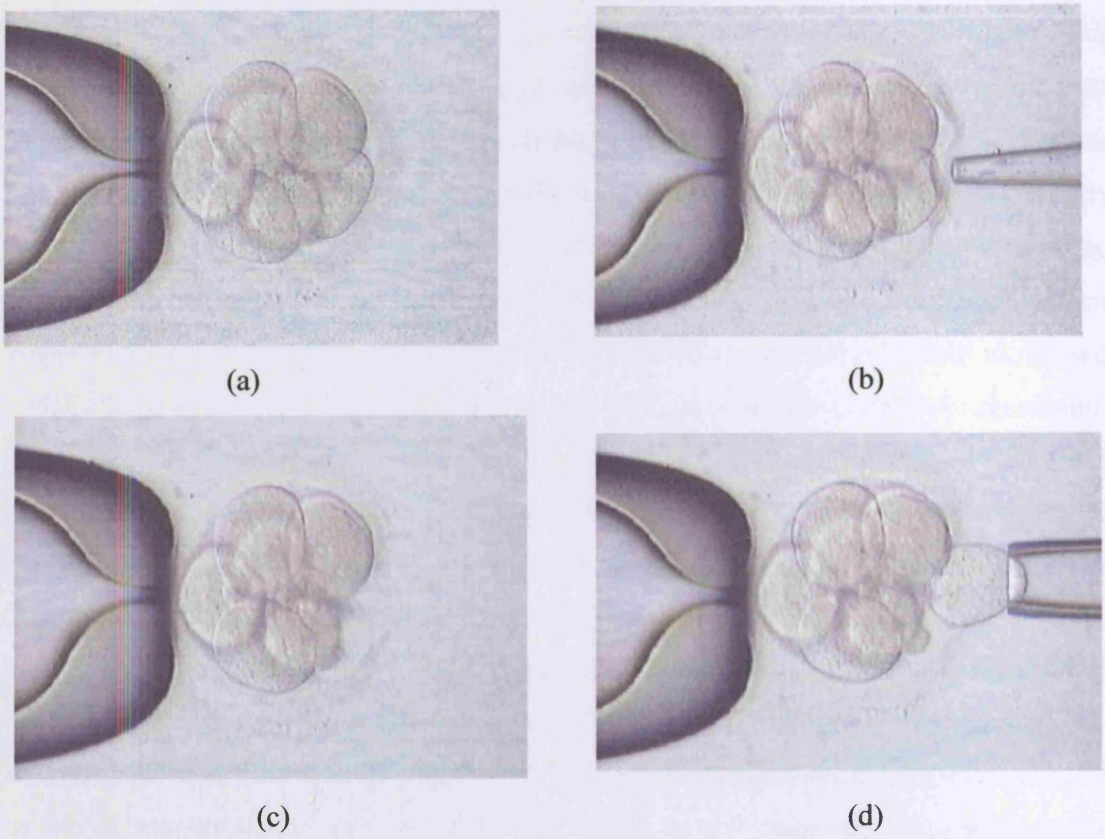


Fig.1.7: Illustration of a cleavage-stage embryo biopsy. (a) The day 3 post-insemination embryo is immobilised by gentle suction, imposed through a holding pipette. (b) A localised stream of acidified Tyrode's solution is directed at the zona pellucida through a tapered micropipette (diameter 5-7 μm). (c) The stream of Acid Tyrode's solution has as an effect the opening of a hole in the zona pellucida. (d) Blastomere aspiration follows with the use of a second sampling pipette (diameter 30-40 μm). The aspiration of embryonic cells requires great care to avoid their lysis. Once the blastomere is removed from the embryo, it is placed and washed in handling medium for several times. It is then further processed for genetic analysis. In the meantime the remainder of the embryo is returned to normal culture conditions to await the diagnosis results. (Courtesy of W. Piyamongkol).

1.5.1.2 Cleavage stage biopsy

Cleavage stage biopsy is the method of preference for the majority of the 50 centres currently offering PGD worldwide (J. Haprer, personal communication). It is based on the fact that all the blastomeres of an embryo are totipotent at this early stage of development (Mottla *et al.,* 1995). As with PB removal, this method of biopsy was initially applied to the mouse to evaluate its feasibility, and embryonic survival rates after blastomere removal (Nijs *et al.,* 1988; Wilton *et al.,* 1989; Krzyminska *et al.,* 1990; Kola and Wilton, 1991). Its subsequent evaluation on human embryos demonstrated that two cells could be aspirated from 8-cell embryos at day 3 after fertilisation, without significantly affecting further development and blastulation (Hardy *et al.,* 1990)

Hence, cleavage stage biopsy takes place on day 3 post insemination when the embryo consists of 6-8 blastomeres, whereas the actual procedure has practically remained unmodified, compared to the original (Handyside *et al.,* 1989; 1990; Van de Velde, 2000). The biopsy begins by making a hole in the zona pellucida that still surrounds the embryo with localized application of acidified Tyrode's solution (Hardy *et al.,* 1990). This procedure is illustrated in fig.1.7. Partial zona dissection achieving a hole on the zona by mechanical means has been reported for PB biopsy, but also for cleavage stage biopsy (Malter and Cohen, 1989). Cieslak and colleague (1999) have improved this method and achieved a three-dimensional partial zona dissection for the same purpose. In addition, more recently there have been reports on the application of a laser for the drilling of the zona pellucida (Veiga *et al.,* 1997; Boada *et al.,* 1998; Montag *et al.,* 1998)

Blastomere aspiration usually takes place with a 30μm pipette that is inserted into the hole. Alternatives to this approach include the use of direct mechanical pressure (Anver *et al.,* 1996) or a flow of medium to displace cells (Pierce *et al.,* 1997). The removal of blastomeres is directly dependent on how compact are the embryos at the time of biopsy. Medium without calcium and magnesium has been employed by several groups to overcome the problem of compaction. The latter disrupts intracellular contacts, enabling the easier aspiration of blastomeres and the prevention of lysis during biopsy (Dumoulin *et al.,* 1998).

Soussis and colleagues (1996a,b) assessed the safety and efficacy of embryo biopsy by examining established pregnancies and subsequent births from biopsied embryos. Biochemical and ultrasound measurements of these fetuses did not demonstrate any significant developmental differences compared to pregnancies that were achieved after routine IVF. Deliveries, including birth weight and apghar scores were considered normal in both categories (Soussis *et al.*, 1996a,b).

Blastomere biopsy has the advantage that errors that are maternally-, paternally-, and postzygotically-derived can be detected and eliminated. However, especially in the cases of chromosomal abnormalities, embryo mosaicism poses a significant misdiagnosis risk. In a recent report, Munne and colleagues (2002a) detected a 7.2% misdiagnosis rate, of which 5.6% was attributed to mosaicism. Therefore, it has become routine practice to remove two cells from embryos consisting of six blastomeres or more in cases of chromosome abnormalities such as reciprocal translocations and dominant disorders (Delhanty, 1994; Delhanty and Handyside, 1995; Kuo *et al.*, 1998; Van de Velde *et al.*, 2000). One cell is usually taken for aneuploidy screening (PGS), where the aim is to maximise implantation rates.

1.5.1.3 Blastocyst biopsy

This type of biopsy was developed as an alternative to cleavage stage biopsy, and involves the removal and examination of TE cells from the blastocyst, taking place on days 5-6 post-insemination. This approach has the advantage that it enables the sampling of a larger number of cells, reducing in this way the risk of misdiagnosis and providing more data about the embryonic DNA constitution (Wells and Delhanty, 2001). Moreover, blastocyst biopsy does not interfere with the embryonic ICM. As before, the procedure involves making a hole in the zona pellucida and then replacing the blastocyst in culture. TE cells herniate from this opening and are removed for further investigation (Muggleton-Harris *et al.*, 1993).

The drawback of this method is the possibility that the TE cells are genetically and chromosomally diverged from the ICM cells, which could pose a risk of misdiagnosis if the former were use for PGD. The phenomenon of different chromosome complements of

the embryo proper and the placental tissues is known as confined placental mosaicism (CPM), and is observed in approximately 1-2% of conceptions (Kalousek and Dill, 1983). Moreover, the TE cells are required for embryo implantation, and their reduction could adversely affect the latter. Another disadvantage, is that only about 40% of embryos reach the blastocyst stage *in vitro*, while the remainder tend to arrest beforehand (Harper and Delhanty, 2000). This has the effect of testing only a few embryos. Even though embryo survival to the blastocyst stage has improved in recent years, currently the most widespread method of acquiring embryonic cells is the cleavage stage biopsy (ESHRE PGD Consortium, 2000, 2002).

1.5.2 Diagnosis of single gene defects

PCR has the ability to increase the quantity of a specific fragment in a DNA sample to a level that it can undergo further genetic testing. It was the first technique to be applied for the PGD of X-linked disorders (Handyside *et al.,* 1990). This method was used for sexing embryos by amplifying a repeat sequence on the long arm of chromosome Y. However, after a misdiagnosis, FISH has largely replaced PCR for this purpose (Griffin *et al.,* 1994). Since then, PCR has been used almost solely for the diagnosis of single gene disorders that can be dominant, recessive or X-linked.

The first single gene disorder to be diagnosed at the preimplantation stage with the application of PCR was cystic fibrosis, caused by the ΔF508 mutation. The diagnosis took place with the use of heteroduplex analysis to detect unaffected homozygous normal and heterozygous embryos for transfer (Handyside *et al.,* 1992). As time progressed, the PCR strategies became more sophisticated and this was accompanied by an increase in the number of diseases for which PGD could be applied and a subsequent increase in patient demand (Wells and Sherlock, 1998). The diseases currently diagnosed in this way, are more than 20 and some of them can be seen in Table 1.4 below.

Table 1.4: Some examples of the clinical application of PGD for single gene disorders to illustrate different methodologies (from Wells and Delhanty, 2001).

Disease diagnosed	***Method used for mutation analysis***
Cystic fibrosis	Heteroduplex formation; analysis of allelic size differences; restriction enzyme digestion (Handyside *et al.*, 1992; Goossens *et al..*, 2000)
β-thalassaemia	Restriction enzyme digestion and use of linked marker; denaturing gradient gel electrophoresis (Kuliev *et al.*, 1998; Kanavakis *et al.*, 1999)
Sickle cell anaemia	Restriction enzyme digestion and use of linked marker (Xu *et al.*, 1999)
Lesch-Nyhan syndrome	Restriction enzyme digestion (Ray *et al.*, 1999)
Tay-Sachs disease	Heteroduplex formation (Gibbons *et al.*, 1995)
RhD blood typing	Allele specific amplification (Anver *et al.*, 1996)
Medium chain acyl CoA dehydrogenase deficiency	SSCP and use of linked marker (Ioulianos *et al.*, 2000)
Spinal muscular atrophy	Mutant allele is refractory to PCR (Dreesen *et al.*, 1998)
Marfan syndrome	Use of linked markers; restriction enzyme digestion (Blaszyk *et al.*, 1998; Sermon *et al.*, 1999)
Myotonic dystrophy	Mutant allele is refractory to PCR (fluorescent PCR) (Sermon *et al.*, 1998)
Familial adenomatous polyposis coli	SSCP, heteroduplex formation and use of linked marker (Ao *et al.*, 1998)
Retinitis pigmentosum	Site specific mutagenesis and allele dependent length polymorphism (Strom *et al.*, 1998)
Huntington's chorea	Analysis of allelic differences (fluorescent PCR) (Sermon *et al.*, 1998)
Fragile X	Mutant allele is refractory to PCR (Sermon et al., 1999)
Congenital adrenal hyperplasia	Fluorescent PCR and restriction enzyme digestion (Van de Velde *et al.*, 1999)
Duchenne muscular dystrophy	Mutant allele is refractory to PCR (priming sites deleted); use of linked markers (Lee *et al.*, 1998)

Charcot-Marie-Tooth disease	Duplication detected by a linked marker (De Vos *et al.*, 1998)
Osteogenesis imperfecta	Fluorescent PCR and restriction enzyme digestion (De Vos *et al.*, 2000)
Herlitz junctional epidermolysis bullosa	Restriction enzyme digestion (Cserhalmi-Friedman *et al.*, 2000)

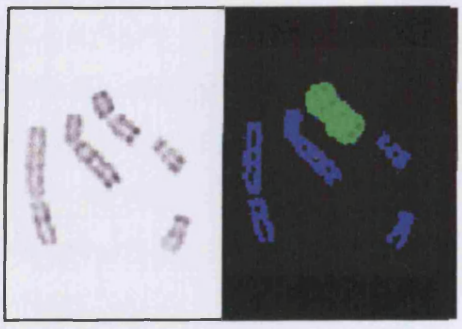
The development of PGD protocols for the detection of single gene disorders is technically very demanding as there is only of 5-10pg of DNA in a single cell, and hence requires quite a few cycles of amplification in order for the possible mutation to be visualised. Moreover, the evaluation of the PCR protocols prior to clinical application can turn out to take longer than expected and be very labour intensive. Additional problems could be caused due to failure of amplification, contamination and a phenomenon known as allele dropout (ADO), all of which increase the risk of misdiagnosis (Navidii and Arnheim, 1991). ADO can be defined as the amplification of only one of the parental alleles present in the single cell (Handyside and Delhanty, 1997). The latter is thought to be due to suboptimal PCR conditions and rapid degradation of the target DNA during thermocycling (Ray and Handyside, 1996). Thus, ADO could lead to a misdiagnosis of a heterozygous cell as homozygous. This would pose a problem especially in the case of a dominant disorder, in which the heterozygous embryo would be affected. In order to avoid this potential risk of misdiagnosis, Ao and co-workers (1998) suggested the inclusion of a polymorphic marker, which is located on the same chromosome and near the disease-causing gene (multiplex PCR). As far as the possibility of contamination is concerned, it can be caused by an extra sperm, cumulus cells, culture media, or the build up of PCR products in the laboratory (Wells and Sherlock, 1998). These can be overcome by using ICSI for the fertilisation procedure, washing the blastomere after the biopsy, and applying nested PCR, which involves two separate sets of cycles using two different sets of primers.

As can be seen in Table 1.4 a variety of mutation analysis methods are currently being applied in PGD protocols. These include heteroduplex analysis, restriction endonuclease digestion, and single-strand conformational polymorphism (SSCP). Incorporation of the highly accurate fluorescent-PCR is also widely used, and preferred to the more conventional methods (Wells and Sherlock, 1998). The best PCR PGD protocols employ multiplex PCR so as for the amplification of the DNA fragment encompassing the mutation to be achieved, another fragment onto which a linked polymorphism is located to avoid ADO, and at least one more highly polymorphic marker to detect possible contamination (Wells and Delhanty, 2001). Ideally the primers to be used are fluorescently tagged, as this type of PCR is much more sensitive and requires a lower number of amplification cycles, making the whole procedure much quicker and more robust.

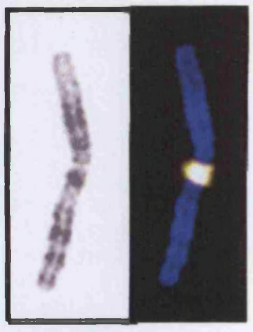
1.5.3 Diagnosis of chromosome abnormalities

Cytogenetic analysis of chromosome abnormalities involves the visualisation of chromosomes at the metaphase stage. Embryonic blastomeres, however, do not in most cases contain analysable metaphases, and even if a metaphase is identified, the spreading of the blastomere would be very difficult, due to potential chromosome loss (Handyside and Delhanty, 1997). FISH therefore is an ideal method for the investigation of the chromosome complement of blastomeres, as it enables the analysis of interphase nuclei by using fluorescently tagged DNA probes, specific for the chromosomes in question (Harper *et al.,* 1995). The principles of FISH along with its applications for the PGD of chromosome abnormalities will be discussed in the subsequent sections.

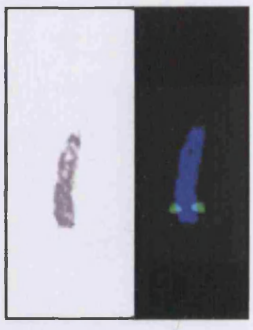
Fig.1.8 : Types of DNA Probes employed in FISH



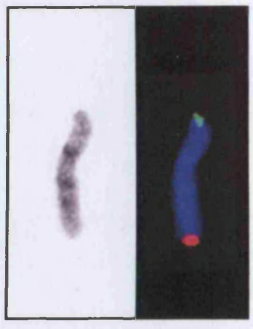
(a)Whole chromosome paint probe



(b) α-satellite probe



(c) Locus-specific probe



(d) Sub-telomeric probe

Fig.1.8: Illustration of four different types of FISH probes hybridised onto lymphocyte chromosomes that are counterstained with DAPI (blue), giving a G-band pattern when inverted. (a) Whole chromosome paint probe hybridised onto chromosome 16 (green). Such probes are only employed for the analysis of metaphase chromosomes, due to their large signal domains. (b) Repeat-sequence alpha-satellite probe hybridised onto the centromeric region of chromosome 1(orange). Such probes give large signals, and can be used for both metaphase and interphase analysis. (c) Locus-specific probe hybridised onto the long arm of chromosome 13 (green, position 13q33). Such probes give smaller signals, compared to repeat-sequence probes, which sometimes can be split. They can be employed for the analysis of both interphase and metaphase nuclei. (d) Sub-telomeric probes hybridised onto the short (green) and long (red) arm telomeres of chromosome 6. Similarly with the locus-specific probes, they give small signals, which sometimes appear as split. They too are employed for both metaphase and interphase examination.

1.6 Fluorescent in situ hybridisation

The basis of the FISH method is the ability of DNA single strands to bind onto complementary target sequences and form a stable DNA hybrid complex. In other words, this technique employs fluorescently tagged DNA probes that are specific for different chromosomal regions. Three types of probes are used in FISH and these are the following:

(i) Repeat sequence or centromeric probes- These hybridise to the repeat alpha-satellite sequences of the centromeres of all chromosomes, apart from 1, 9, 16 and Y. The centromeric heterochromatin for these four chromosomes includes alpha-satellite repeats, but also consists of repeats of different nature, such as beta-satellites. The required hybridisation time for probes of this type is approximately 1-2 hours, while visualisation is feasible both on metaphase and interphase nuclei.

(ii) Locus-specific probes- These bind to specific regions located either on the short or long arms of chromosomes, corresponding to genes and surrounding sequences. The required hybridisation time ranges between 6-16 hours, and these probes can be visualised both on interphase and metaphase nuclei.

(iii) Subtelomere probes- These hybridise to the short and long arm telomeric regions of chromosomes, and are a relatively recent addition to the various FISH probe categories. The required hybridisation time is 16 hours, and they can be applied for the analysis of both interphase and metaphase nuclei.

(iv) Whole chromosome paints- These bind onto the whole of the chromosome and they are visualised after approximately 16 hours of hybridisation. They can be employed only for metaphase nuclei. The different types of probes are shown in fig.1.8.

During the first applications of FISH the probes used were obtained from cloned fragments of cDNA or genomic DNA. These fragments were usually inserted into vectors, such as plasmids, cosmids, P1s, P1 derived artificial chromosomes (PACs), bacterial artificial chromosomes (BACs), and yeast artificial chromosomes (YACs). Probe preparation took place in the laboratory and involved the amplification of the DNA either directly with the application of the Alu-PCR or by growing bacterial or yeast

cultures that were carrying these vectors. Extraction and fluorescent labelling of the DNA followed. This procedure was very time consuming and labour intensive and meant that the FISH could not be as widely applied as required according to patient demand. The extended commercial availability of directly labelled probes belonging to all categories, and especially the development of subtelomere probes complementary for telomeric regions of the p and q arms of almost all chromosomes (Knight and Flint, 2000), led to the adaptation of this method for several diverse biological investigations. (Heng *et al.*, 1997). These range from clinical genetic diagnosis to purely research purposes.

The speed and accuracy of FISH has made it the ideal technique for the screening of aneuploidy in the context of prenatal diagnosis to complement standard karyotyping. Ward and colleagues (1993) employed this method for the chromosomal analysis of 4,500 uncultured amniotic fluid samples, and reported an overall detection rate of aneuploidies of 73.3%, with an accuracy as high as 93.9%. FISH has the advantage that it can be applied for the examination of interphase nuclei and hence enables a more rapid result, since it omits the culturing of chorionic villi or amniotic fluid samples (Klinger *et al.*, 1992). Moreover, FISH has been used in the mapping of single or clusters of genes or even whole chromosomal regions, in the context of positional cloning. This data is crucial in the integration of physical and genetic mapping information and the construction of high resolution cytogenetic chromosome maps (Heng *et al.*, 1997).
FISH was also the basis for the development of three similar methods that enable the labelling of all 24 chromosomes. These include a method called spectral karyotyping (SKY) (Schrock *et al.*, 1996), and another called multifluorochrome karyotyping (M-FISH) (Speicher et al., 1996). These two methods employ 24 chromosome specific paint probes labelled with different combinations of fluorochromes, something which allows the simultaneous analysis of all chromosomes in the human complement (Harper and Wells, 1999). Comparative genomic hybridisation (CGH) is also associated with FISH, but its principles and applications will be analysed in a following section.

At the single cell level, FISH has been used in the detection of individual chromosomes coming from samples for which good metaphases are difficult or impossible to obtain,

such as oocytes (Dailey *et al.,* 1996; Mahmood *et al.,* 2000; Cupisti *et al.,* 2003), embryonic nuclei (Munne *et al.,* 1993; Harper *et al.,* 1994;) and sperm nuclei (Spriggs *et al.,* 1996; Van Hummelen *et al.,* 1997).

As with PCR, the first application of FISH for PGD was for the sexing of embryos to avoid X-linked disorders. The latter became the preferred method after a diagnostic error had taken place in one of the pregnancies that were achieved with the use of PCR to identify embryos free of cystic fibrosis (Griffin *et al.* 1994). At the same time, several groups were attempting to evaluate the FISH procedure with the aim of possibly employing it for the PGD of chromosome abnormalities. These studies revealed the high efficiency and reliability of this technique. An example of such a study was that carried out by Griffin and colleagues (1994) who reported on seven clinical pregnancies achieved after selecting embryos for sex with the application of dual-colour FISH, in a 2-year period. Hence FISH became the method of choice for the detection of chromosome associated abnormalities.

As far as the sexing of embryos is concerned, initially two repetitive sequence probes were used to detect chromosomes X and Y. These probes were labelled with digoxygenin and biotin, in a way that a detection step was required so as for the fluorescent tag to be acquired. This process was also known as indirect labelling (Griffin *et al.,* 1994). Later apart from the probes for the sex chromosomes, one or more autosome probes were added, to also establish ploidy of the embryo for these specific chromosomes (Staessen *et al.,* 1999). Embryos identified to be normal female for the examined chromosomes, i.e. showing two signals for X and two signals for the autosome under investigation are transferred in sexing cases. All male embryos are excluded due to the possibility of being affected, even though 50% of them should be normal unaffected. The distinction between affected and unaffected male embryos can be achieved with the application of a PCR strategy enabling the identification of the exact mutation. Such tests, however, are not feasible for all X-linked diseases, as mentioned previously.

At present, different FISH strategies have been devised for the PGD of a variety of chromosome abnormalities, both numerical and structural, as will be discussed below.

Fig.1.9 : Diagnosis of Biopsied Blastomeres for PGD

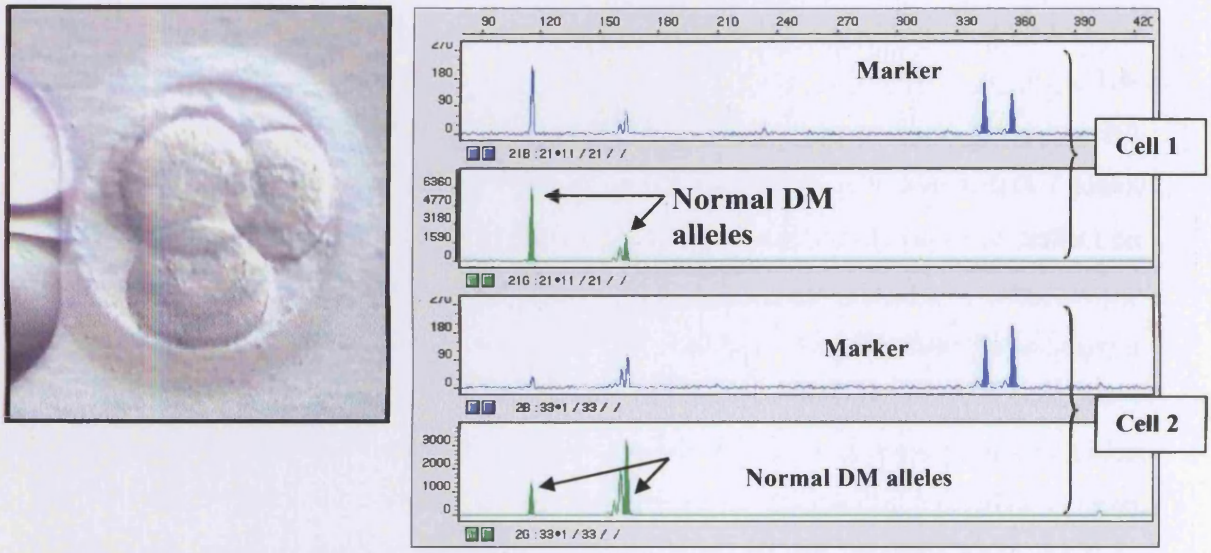


Fig.1.9a: Mutation analysis using a PCR-based method. Diagnosis of single gene disorders occurs by initially transferring the biopsied blastomere to a PCR tube containing lysis buffer, with subsequent amplification of its DNA, using specific oligonucleotide primers. The figure illustrates the results obtained from multiplex PCR using fluorescent primers for the identification of the DM alleles (PGD for myotonic dystrophy) and a contamination marker (D21S11). Analysis in such cases takes place with electrophoretic fragment separation achieved with a laser analysis system. Lanes 1 and 3 show the peaks resulting from the amplification of the contamination marker. Lanes 2 and 4 demonstrate the peaks resulting from the amplification of the DM alleles from two cells. According to these results, the embryo was characterised as not being affected with DM and recommended for transfer. (Courtesy of W. Piyiamongkol)

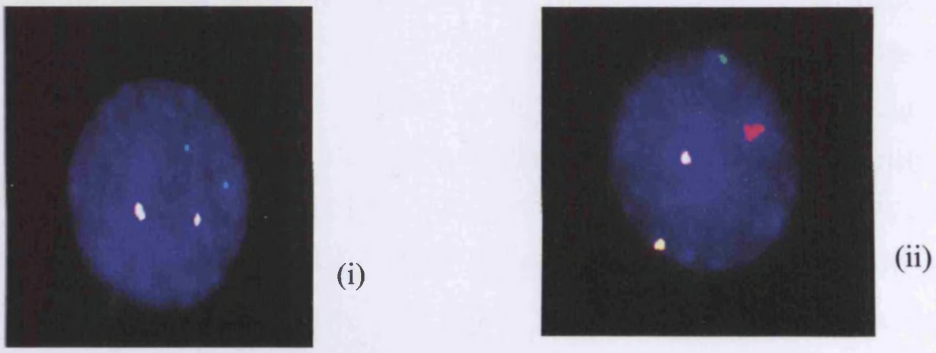


Fig.1.9b: Chromosome analysis using a FISH-based method. Diagnosis of chromosome defects occurs by spreading the biopsied blastomeres onto microscope slides using one of three methods (see 1.6.4). Such methods remove most of the cell cytoplasm, making in this way the nucleus accessible to the FISH probes. Both the above nuclei were analysed with three repetitive sequence probes for chromosomes X (green), Y (red) and 1 (orange). Such probes are employed during the PGD for embryo sexing to avoid X-linked disease. Nucleus (i) was diagnosed as female (XX; 1,1). Nucleus (ii) was diagnosed as male (XY; 1,1). Both embryos were recommended for transfer.

Fig.1.9 illustrates examples for the PGD of myotonic dystrophy with the application of PCR and the diagnosis of sex in two embryos, with the application of FISH.

1.6.1 Aneuploidy screening

In recent years FISH has been applied for the screening of aneuploidy in embryos from women of advanced age that are undergoing IVF, due to fertility problems (Handyside and Delhanty, 1997). This procedure is termed PGD for aneuploidy screening or PGS (J. Harper, personal communication). Studies have shown that the aneuploidy frequency in oocytes and embryos of women aged 35 years or older is relatively high (30-40%) (Strom *et al.,* 2000). It has also been shown that there is a correlation between aneuploidy and a decline in implantation rates (Bahce *et al.,* 2000). For this reason, it was assumed that by applying PGD and FISH to negatively select chromosomally abnormal embryos, and transfer only the normal ones, the implantation rates for older women with such problems would improve (Munne *et al.,* 1993).

Most PGD centres offer this type of diagnosis to couples with the following indications: female age 35 years or more, 3 or more previous unsuccessful embryo transfers with regular IVF procedures, or repeated spontaneous loss of pregnancies when parents have a normal karyotype.

Currently there is at least one FISH probe commercially available for every human chromosome, but a limited number can be hybridised to embryonic nuclei at any one time, to avoid the occurrence of overlapping signals, that could pose a misdiagnosis risk. The hybridisation efficiency is also reduced with each extra probe added. Two different approaches have been employed for PGS. In the first one, FISH analysis takes place on the first and/or second PBs. Verlinsky and colleagues (1999) applied this strategy for the examination of 1st and 2nd PBs of women with an average age of more than 34 years. The DNA probes used for this analysis were specific for chromosomes 13, 18, and 21. Out of the 3943 oocytes tested, 43% were identified as abnormal (Verlinsky *et al.,* 1999). This aneuploidy rate was relatively high, as the group was examining only 3 chromosomes. Out of the abnormalities detected, 35.7% were attributed to errors taking place during the

1st meiotic division and most of them were due to chromatid malsegregation. The resulting pregnancy rate after transfer of embryos for which the oocytes were considered to be normal was approximately 22% (Verlinsky *et al.,* 1999). However, no comparison with control data took place during this study, to ascertain the efficacy of PB analysis for PGS (Wilton, 2002). PB investigation for the identification of aneuploidy is hampered by two critical problems. First of all, only maternally derived aneuploidies can be detected, and the possibility of fertilisation of an oocyte with an aneuploid sperm cannot be investigated. Second, identification of post-zygotic chromosome abnormalities is not feasible (Wilton, 2002).

The second approach involves the direct FISH testing of biopsied blastomeres and this is more widely applied. The most extensive FISH screen to date has been employed by the group at St Barnabas, USA, and involved the examination of blastomeres for chromosomes X, Y, 13, 14, 15, 16, 18, 21, and 22 in two sequential rounds (Munne *et al.,* 1998b). This probe set would detect 70% of the aneuploidies identified in spontaneous abortions. Out of a total of 25 cycles carried out and analysis of 247 embryos, 42% of them were classified as karyotypically normal with the remaining being trisomic, monosomic or carrying complex abnormalities for the chromosomes tested. Ten pregnancies were achieved after embryo transfer, while the FISH misdiagnosis risk was reported to be about 15% (Munne *et al.,* 1998b). A different set of probes was applied for the analysis of 194 embryos in a similar study carried out by Bahce *et al.,* (1999). Chromosomes 1, 4, 6, 7, 14, 15, 17, 18, and 22 were investigated in an attempt to establish whether chromosome abnormalities that are associated with implantation failure were distinct from those observed in spontaneous abortions. The chromosomes most frequently affected by aneuploidy events were identified to be 22, 15, 1, and 17. The authors concluded that chromosomes X, Y, 13, 18, and 21 should be examined in case of repeated trisomic conceptions, whereas chromosomes 1, 15, 16, 17, and 22 in cases of recurrent miscarriages and implantation failure (Bahce *et al.,* 1999).

Gianaroli and colleagues (1997a,b) compared the implantation rate between two groups of poor-prognosis patients. The first group had their embryos examined for chromosomes

X., Y, 13, 18, and 21, whilst the second acted as a control and the generated embryos were transferred after having the zona pellucida opened, but without the subsequent removal of blastomeres. The implantation rate for the first group of patients was estimated to be 28%, which was much higher compared to the 12% identified for the second group. A similar implantation rate of 30% was reported in a more recent study by Kahraman and co-workers (2000) after transfer of embryos that were investigated for the same set of chromosomes. These rates seemed to be encouraging, especially in cases of patients with IVF implantation failure as reviewed by Wilton (2002). A similar conclusion was drawn by Munne and colleagues (2002a), who advocated an increase in implantation rates and a decrease in recurrent miscarriages and trisomic offspring for women over the age of 37 with at least six good quality embryos. Hence, from the data obtained so far, PGS appears to be fulfilling its purpose.

1.6.2 FISH diagnosis of structural abnormalities

The reproductive history of couples in which one of the partners is a carrier of a structural chromosome abnormality is often complex. The incidence of subfertility or complete infertility, multiple spontaneous miscarriages, or the birth of abnormal children may be frequent in this group. Such couples can either attempt to conceive naturally or via IVF procedures if necessary and then have prenatal diagnosis and a possible termination of an affected pregnancy, or can choose to have their embryos investigated via a PGD FISH protocol specific for their abnormality and ensure that if a pregnancy ensues, it will be normal. Increasing numbers of couples who are experiencing difficulties due to structural rearrangements opt for the PGD choice, now.

Various FISH strategies have been devised for the diagnosis of reciprocal and Robertsonian translocations and for other structural abnormalities such as inversions. These strategies will be outlined in the paragraphs that follow.

PGD FISH protocols for reciprocal translocations are not straightforward to develop, due to the fact that the breakpoints can arise at any position of any chromosome. Thus, each reciprocal translocation case is normally unique to a carrier or a family. Both PB and

cleavage stage analysis strategies have been devised for the identification and transfer of normal/balanced embryos.

PB analysis strategies for the identification of reciprocal translocations of maternal origin were developed by Munne and colleagues (1998d). The FISH approach involved the use of whole chromosome painting probes for the analysis of 1st PBs, biopsied just after oocyte retrieval. In this way, the detection of normal, balanced and unbalanced oocytes was feasible (Munne *et al.,* 1998d). The first application of this strategy was during the PGD for a female carrier of a reciprocal translocation involving chromosomes 4 and 14. Identification of three unbalanced and two normal PBs (oocytes balanced) was feasible with subsequent transfer of two balanced embryos. A clinical pregnancy was established, but spontaneously aborted after 7 weeks. Cytogenetic analysis of the fetus confirmed the balanced karyotype (Munne *et al.,* 1998d). Similar PGD strategies have been used by several centres (Verlinsky and Evsikov, 1999; Durban *et al.,* 2001; Pujol *et al.,* 2003b). PB analysis has the advantage that the actual detection of both normal and balanced embryos is attainable. However, the possibility of crossing-over taking place within the translocated segments, chromatid predivision occurring in the oocyte, and poor chromosome morphology of the PB, could lead to a potential misdiagnosis. Therefore this approach should be followed by analysis of the 2nd PB and/or of embryonic blastomeres (Munne, 2002).

Two different FISH schemes have been used for blastomere analysis. The first involves the design and application of locus-specific probes that span the breakpoints of the translocation (Munne *et al.,* 1998a), while the second one uses FISH probes that flank the breakpoints of a translocation (Conn *et al.,* 1999). The first type of probes are designed to span the breakpoints of a translocation, leading in this way to the visualisation of two distinct signals on the normal chromosomes and two signals that are the result of the combination of the 2 probes on the derivative chromosomes. The latter has as an effect the distinction between normal and balanced embryos, as different signal patterns are obtained in each case (Munne *et al.,* 1998a), which is also the advantage of this strategy. The drawback in this case, is that such probes could require a longer period for their optimisation, and as they are entirely patient-specific, the whole PGD procedure may not be as cost-effective. Hence, this approach is not as widely applied.

The second strategy ideally involves the application of four probes that flank the breakpoints on each of the two chromosomes that form the translocation. As however there is a limited availability of fluorochromes with which the DNA probes could be tagged, a three-colour FISH strategy is used instead (Conn *et al.,* 1999). Hence, a pachytene diagram is drawn and the probes that are selected in this way will enable the detection of all possible segregations during meiosis (Delhanty, 1998). Distinction between normal and balanced embryos is not feasible with this strategy, as the signal pattern is identical for both types. This FISH strategy is much simpler compared to the first one, and the wide range of commercially available probes, both centromeric and locus specific, means that the diagnosis of practically any translocation is possible. This strategy was first developed by Conn and colleagues in 1995 at the UCL centre for PGD and it has been used ever since. Its relative simplicity made it the main strategy for the preimplantation diagnosis of reciprocal translocations and currently most PGD centres (Scriven *et al.,* 1998; Coonen *et al.,* 2000; Iwarsson *et al.,* 2000; Fridstrom *et al.,* 2001; Simopoulou *et al.,* 2003) use either the original or slightly modified protocols, applying the newly available subtelomere probes (Munne *et al.,* 2000c), for the detection of such structural rearrangements.

Analysis of first PBs and embryonic blastomeres has also taken place for the PGD of Robertsonian translocations. The FISH strategies applied are not as complicated as the ones for reciprocal translocations. However, in cases of Robertsonian translocations, centromeric probes cannot be used due to the sequence homology of the satellite regions between chromosomes 13 and 21 and 14 and 22. The general PGD FISH strategy devised for these translocations involves the application of two probes, each one hybridising on one of the two chromosomes that form this rearrangement. In cases where one of the chromosomes involved in the rearrangement could result to the generation of a viable trisomic pregnancy, such as chromosome 21, then two probes are ideally applied for that chromosome, to ensure its visualisation during diagnosis (Conn *et al.,* 1998). PGD for Robertsonian translocations was initially carried out with the application of YAC probes, due to the lack of commercial locus specific probes for the acrocentric chromosomes. However, more recently locus-specific probes for these chromosomes have become

commercially obtainable, and thus the development of PGD FISH protocols for the detection of these rearrangements in blastomeres is relatively easy (Harper and Delhanty, 2000). As Robertsonian translocation breakpoints are the same for each carrier, once a FISH protocol is optimised it can be applied for any similar case without any further modifications.

First PB analysis of female Robertsonian translocation carriers has also been employed (Munne *et al.,* 2000b; Durban *et al.,* 2001). In a study carried out by Munne and colleagues (2000b) on female Robertsonian translocation carriers for both the t(13;14) and t(14;21), whole chromosome paint probes for chromosomes 13, 14, and 21, plus a locus-specific probe for either 13 or 21 were used to screen their 1st PBs for chromosomal imbalance due to these translocations. In both cases, the oocytes that were normal outnumbered the abnormal ones (Munne *et al.,* 2000b).

FISH strategies have been developed for other less frequently occurring chromosome abnormalities, such as pericentric inversions (Iwarsson *et al.,* 1998b; Escudero *et al.,* 2001), deletion of part of chromosome 22 leading to Di George syndrome (Iwarsson *et al.,* 1998a) and for patients that are gonadal mosaic for a trisomic cell line (Conn *et al.,* 1999). In all, the wider availability of commercial probes increased the number of chromosome cases feasible, and hence the number of patients that could be treated.

A limitation to the PGD of chromosome abnormalities is the presence of mosaicism in the resulting embryos (Conn *et al.,* 1998; Conn *et al.,* 1999; Malmgrem *et al.,* 2002). There have been reports demonstrating the presence of highly abnormal chromosome complements in 70-100% of embryos generated from some patients with poor histories, and could result in the reduction in the success rates of PGD (Harper and Bui, 2002). However, as such couples may be unable to establish or maintain a pregnancy naturally, PGD still is the most attractive option.

1.6.3 FISH limitations

FISH is in general a very efficient technique that enables the chromosomal constitution to be analysed in all the nuclei in an embryo. Problems, however, may arise in the application of this method in the PGD of chromosome abnormalities, leading to a potential misdiagnosis. These include the reduction of FISH efficiency with the addition

of every extra probe, the loss of micronuclei or even whole nuclei during fixation, signal overlaps and, most importantly, mosaicism (Magli *et al.,* 2001b).

The decrease in multicolour FISH efficiency with the addition of every extra probe, especially on interphases was demonstrated in a study carried out by Ruangvutilert and colleagues (2000a). In their investigation on metaphase and interphase nuclei from non-mosaic trisomic fibroblast cultures it was revealed that multicolour FISH was 100% efficient on metaphases, whereas the efficiency dropped to as low as 80% for interphases. Probe combinations for autosomes and sex chromosomes were used in this study, and both centromeric and locus-specific probes were tested (Ruangvutilert *et al.,* 2000a). Thus, it is essential to assess the efficiency of probe combinations for each PGD case on lymphocytes and spare embryos (Harper and Wells, 1999).

The method with which the embryonic nucleus is fixed on the microscope slide is crucial and it must ensure both the minimal loss of material and the best nuclear morphology. Three methods are currently being applied. The first involves the use of a combination of HCL and Tween 20 solution (Coonen *et al.,* 1994), which leads to nuclei that are compact and with a small surface area. The latter could have as an effect the higher incidence of signal overlap if more than three probes are used at a time (Munne *et al.,* 1996). The second one is based on the method suggested by Tarkowski and colleagues (1966) and uses the Carnoy solution (methanol/acetic acid) to ensure fixation of the cells on slides. The resulting nuclei have a much larger surface area due to the hypotonic pretreatment, but the FISH error rate for this method is still relatively high (10-15%) (Munne and Weier, 1996). The third fixation method is a combination of the two above (Dozortsev and McGinnis, 2001). Evaluation of all three fixation methods took place in a study carried out by Velilla (2002). It was reported that the first fixation method resulted in inferior nuclear quality and had a higher rate of FISH signal overlaps compared to the other two (Velilla, 2002). In practice, however, the decision of which fixation method to be used for the spreading of cells depends on the actual handler, while the FISH protocol should be adjusted accordingly.

The greatest risk of misdiagnosis, however, during the application of FISH for the PGD of chromosome abnormalities is attributed to the presence of embryonic mosaicism (presence of two different cell lines). This phenomenon has been observed in almost all

studies of human preimplantation embryos (covered in section 1.8) (e.g. Delhanty *et al.,* 1997; Iwarsson *et al.,* 2000). These were carried out on both normal and abnormal embryos, and all of them revealed a high rate of complex abnormalities in their chromosome complement. The misdiagnosis risk in PGD involves examining a blastomere that could be scored as normal or balanced, whilst the majority of cells of the embryo are abnormal. This risk increases when diagnosis is carried out on only one blastomere (Iwarsson *et al.,* 2000). Therefore, in most PGD centres it is considered routine practice to obtain two blastomeres from embryos consisting of 6 cells or more in cases of structural chromosome abnormalities. Biopsy of one blastomere only takes place for aneuploidy screening cases, as for these the aim is to optimise implantation rates.

1.7 Comparative Genomic Hybridisation

Comparative genomic hybridisation (CGH) is another method closely related to FISH, which allows the copy number of every chromosome to be assessed in a single hybridisation by reference to a normal DNA sample. In principle, a green fluorescent molecule is incorporated in the "test" DNA, while a DNA sample coming from a karyotypically normal individual (46,XY, or 46,XX) is labelled in red, and serves as reference. The two are then mixed together and hybridized to normal male (46,XY) metaphase spreads on a microscope slide. Test and reference DNAs compete for hybridisation sites on each of the 23 chromosomes. In the case that the test DNA is karyotypically normal, i.e., as the reference DNA, no difference in fluorescence intensities would be observed and the chromosomes would have a yellow/orange colouration. If however, the test DNA carried a trisomy for a specific chromosome, then this chromosome would appear to be greener. The opposite would happen if the test DNA was monosomic for a chromosome, which would then appear more red rather than green. Such differences in the intensities of the two fluorochromes are identified with the aid of specialised computer software, which is able to identify chromosome areas that are either over- (gain) or under- (loss) represented in the test DNA sample. CGH sensitivity involves the detection of gains and/or losses in the range of 3-5 Mb (Ghaffari *et al.,* 1998; Kirchoff *et al.,* 1999; 2001). Hence, this method is very accurate and robust in detecting

Fig.1.10 : Comparative Genomic Hybridization

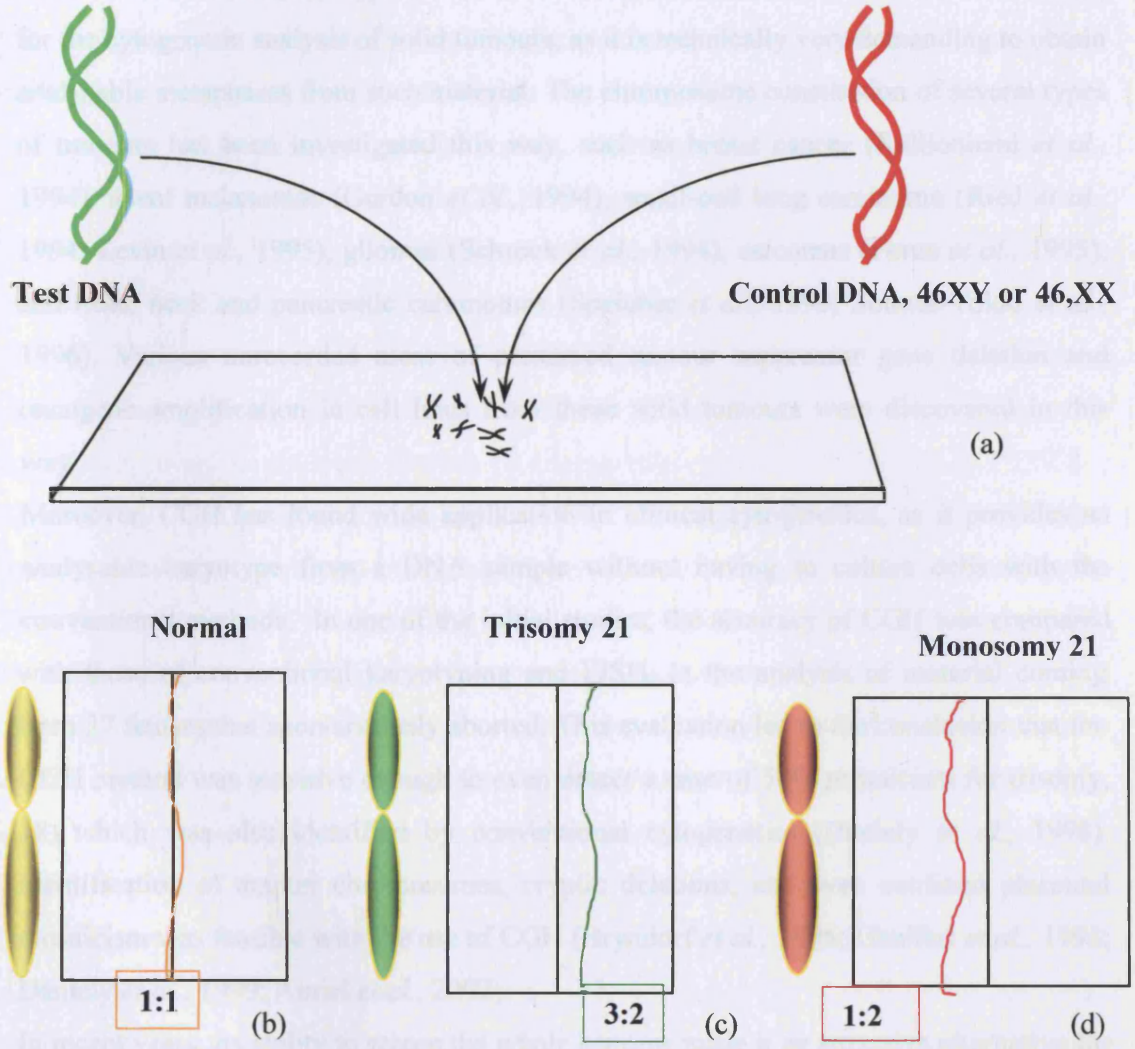


Fig.1.10: The principle of CGH. (a) The DNA of unknown karyotype (test) is labelled with a green fluorescent molecule and is mixed with DNA from a karyotipically normal individual (46,XX or 46,XY). The latter is labelled with a red fluorescent molecule and serves as the reference, with which the test is compared. The mixture is hybridised onto normal male (46,XY) metaphase chromosomes on a microscope slide. (b) If the test DNA is normal, then there is no difference in fluorescence intensities and the chromosomes have an even orange colouration. (c) If the test DNA is trisomic for a specific chromosome, then the latter would be more green rather than red. (d) In the case that the test DNA is monosomic for a specific chromosome, then the latter would be redder. The differences in fluorescence intensities between the red and the green are detected by employing specialised computer software. This software has the ability to identify chromosome areas that are either over- (gain) or under- (loss) represented in the test DNA sample.

whole chromosome aneuploidy, but also duplications or deletions of noticeable size (Wells and Levy, 2003). The principle of this technique is illustrated in fig.1.10.

CGH was initially developed by Kallioniemi and colleagues (1992) as a means of identifying previously unknown amplification regions in the DNA from primary bladder tumours. Since this initial application, CGH became an essential tool in cancer research, for the cytogenetic analysis of solid tumours, as it is technically very demanding to obtain analysable metaphases from such material. The chromosome constitution of several types of tumours has been investigated this way, such as breast cancer (Kallioniemi *et al.,* 1994), uveal melanomas (Gordon *et al.,* 1994), small-cell lung carcinoma (Ried *et al.,* 1994; Levin *et al.,* 1995), gliomas (Schrock *et al.,* 1994), sarcomas (Forus *et al.,* 1995), and head, neck and pancreatic carcinomas (Speicher *et al.,* 1995; Solinas-Toldo *et al.,* 1996). Various unrecorded areas of presumed tumour suppressor gene deletion and oncogene amplification in cell lines from these solid tumours were discovered in this way.

Moreover, CGH has found wide application in clinical cytogenetics, as it provides an analysable karyotype from a DNA sample without having to culture cells with the conventional methods. In one of the initial studies, the accuracy of CGH was compared with those of conventional karyotyping and FISH, in the analysis of material coming from 27 fetuses that spontaneously aborted. This evaluation led to the conclusion that the CGH method was sensitive enough to even detect a case of 50% mosaicism for trisomy, 18, which was also identified by conventional cytogenetics (Daniely *et al.,* 1998). Identification of marker chromosomes, cryptic deletions, and even confined placental mosaicism was feasible with the use of CGH (Bryndorf *et al.,* 1995; Ghaffari *et al.,* 1998; Daniely *et al.,* 1999; Amiel *et al.,* 2002).

In recent years, its ability to screen the whole genome made it an attractive alternative for the chromosomal analysis of gametes and embryos, in the context of PGD.

1.7.1 CGH application in PGD

The ability of CGH to provide information for all 23 chromosomes led several different research groups to attempt to modify this method so that it can be applied in PGD for the analysis of embryonic blastomeres. The difficulty in this case was that the DNA content of a single cell was in the range of 5-10 pg (Vendrely *et al.,* 1955, reviewed in Wells and Levy, 2003), and thus required amplification, prior to its use. Wells and colleagues (1999) were the first to identify an efficient way of amplifying the DNA of a single cell with a type of PCR that uses a primer that anneals at many sites throughout the genome and results to an approximately 40,000 fold increase in the DNA concentration. This whole genome amplification (WGA) method was called degenerate oligonucleotide primed (DOP) PCR. It was used for the amplification of the DNA from normal and trisomic single cells, which were then further analysed with CGH. In all cases the expected karyotype was confirmed (Wells *et al.,* 1999). A similar study was carried out by another group in Australia leading to comparable results (Voullaire *et al.,* 1999). Subsequently, both these groups used the DOP-PCR method followed by CGH analysis for the examination of blastomeres from 12 cleavage-stage embryos (Wells and Delhanty, 2000; Voullaire *et al.,* 2000). In both investigations, the findings confirmed and extended the data obtained from FISH studies into the chromosome constitution of embryos at this stage of development. Specifically, mosaicism and chaotic chromosome complements were determined, but also some extreme abnormalities, including monosomies of the larger chromosomes and also nullisomy (Wells and Delhanty, 2000; Voullaire *et al.,* 2000).

The first clinical application of CGH in the context of PGD, came from the Australian group (Wilton *et al.,* 2001). They used this method in a case of PGS for a 38-year old patient with seven years of unexplained infertility. Out of the eleven generated embryos, a single blastomere from six was analysed with FISH using probes for chromosomes 13, 16, 18, 21, and 22, while a single cell from the remaining five was examined with CGH, after the DNA was amplified with the use of DOP-PCR. Since, however, the CGH required a hybridisation period of about 72 hours which was longer than the embryos could survive in culture, they had to be cryopreserved. Analysis of these embryos revealed several chromosome abnormalities including a blastomere with monosomy 14, a

second one with monosomy 4 and another with trisomy 16. One blastomere was characterised as normal female (46,XX), the corresponding embryo was thawed and transferred, resulting in the establishment of a pregnancy and the birth of a healthy female baby (Wilton *et al.,* 2001). One year later, CGH was applied by another group (Wells *et al.,* 2002) in the context of PGS for the analysis of eleven 1st PBs biopsied from the oocytes of a 40-year old woman, just after their retrieval. Nine of these PBs were characterised as aneuploid, and abnormalities affecting the larger chromosomes 2 and 5 were detected among them. These results were confirmed by FISH analysis of blastomeres that were biopsied on day 3. The chromosomes analysed by FISH included X, Y, 13, 15, 16, 17, 18, 21, and 22, and also those that were highlighted as potentially abnormal after the PB CGH analysis. One embryo was characterised as normal, but its transfer did not result in a clinical pregnancy. The extremely high aneuploidy rate found, is interesting in view of the history of repetitive IVF failure of this patient (Wells *et al.,* 2002).

The Australian group continues to employ CGH for the cytogenetic analysis of embryos generated from women undergoing PGS (Voullaire *et al.,* 2002; Wilton *et al.,* 2003). In one of their more recent reports, they have used a combination of FISH for chromosomes 13, 16, 18, 21, and 22 and CGH for the analysis of single biopsied cells of 110 out of 198 embryos from 20 patients, in an attempt to compare the two techniques (Wilton *et al.,* 2003). From the obtained data they concluded that if these blastomeres were examined solely with FISH, 38% of the aneuploidies would have been missed, and if the chromosomes X, Y, 14, 15, and 19 were also investigated, then 25% of abnormalities would not have been scored. Furthermore, they observed that the clinical pregnancy rate was higher in the group of patients who had had their embryos analysed with CGH, though the difference was not statistically significant.

Hence, CGH seems to be a very promising method, as far as PGD is concerned, as it is capable of providing data for all 23 chromosomes, even at the single cell level. As with FISH though, there are some limitations involved.

1.7.2 CGH limitations in clinical PGD application

The CGH protocol used by Wilton and colleagues in all of their reports (Wilton *et al.*, 2001; Voullaire et *al.*, 2002; Wilton *et al.*, 2003), involves the cryopreservation of embryos, and their transfer in subsequent cycles, as this procedure requires 5 days to yield results. In general, embryo cryopreservation does not adversely affect viability to a great extent, but this not the case for biopsied embryos (Wells and Levy, 2003). One of the aims, however, of PGS is to improve implantation rates, and this may not be best achieved by freezing biopsied embryos (Munne and Wells, 2003). First PB analysis may be a more acceptable alternative for the application of CGH in the context of PGS for women in the older age group. However, further FISH analysis of blastomeres is advisable since chromatid anomalies detected in meiosis I have only a 50% chance of leading to an aneuploid embryo. There is also the potential for misdiagnosis due to a meiosis II error or a chromosome abnormality of paternal origin. Moreover, CGH is unable to identify ploidy abnormalities and may not be significantly sensitive to detect embryonic aneuploidies caused by parental translocations, especially if the latter involve exchange of very small chromosomal segments (Malmgerm *et al.*, 2002; M. Simopoulou, personal communication). Hence, this method would be less likely to be applied for the PGD of structural chromosome rearrangements.

The most limiting factor, however, is the complexity of the actual technique. The whole protocol is very labour intensive and necessitates good knowledge of both molecular and cytogenetic methods, including expertise in recognising individual chromosomes. In addition, there is always the possibility of the failure to yield results, due to poor DNA quality of the sample. Hence a simplified approach would be required for the wider clinical application of CGH. The latter could come in the form of array CGH. This modified method involves the hybridisation of test and reference samples onto DNA microarrays, instead of metaphase slides, something which would significantly reduce hybridisation time and increase the accuracy and sensitivity of the method (Wells and Levy, 2003). Array CGH has been applied for the detection of amplifications in tumour DNA samples (Albertson *et al.*, 2000; Cai *et al.*, 2002; Pollack *et al.*, 2002). In a recent report, Hu and colleagues (2004) described the application of this type of CGH for the

examination of single lymphocytes that were either 46,XX or 46,XY, or were trisomic for 13, 15, or 18. The slides used were arrayed with chromosome-specific DNA libraries. The expected karyotype of all the cells analysed was confirmed, whilst hybridisation took place for just 30 hours. The authors of this report suggested that this modified method could be the most appropriate alternative for the wider application of CGH for the blastomere analysis in the context of PGS (Hu *et al.*, 2004).

1.8 Mosaicism in human preimplantation embryos

The advent of IVF and the continuously evolving molecular cytogenetic techniques that are employed in PGD have both enabled the extensive chromosome investigation of embryos that were not selected to be transferred back to the maternal womb. Such studies led to the detection of mosaicism in these embryos. The latter can be defined as the presence of two or more different cell lines in an individual derived from a single zygote. Initial studies into the extent of chromosome abnormality observed at this very early stage of human development, involved the karyotyping of small groups of embryos (Plachot *et al.*, 1987; Papadopoulos *et al.*, 1989; Wimmers and van der Merve, 1988; Angell, 1989; Jamieson *et al.*, 1994). Among them 16-40% were characterised as chromosomally abnormal with mosaicism being frequent. Mosaicism was first demonstrated by FISH analysis by Delhanty and colleagues (1993), during the investigation of eight embryos from one patient that was undergoing PGD for sex selection to avoid an X-linked disorder.

Harper and colleagues (1995) and Delhanty and colleagues (1997) were among the first to examine the full extent of chromosome abnormalities in human preimplantation embryos with the use of FISH. During the first investigation, 69 cleavage stage embryos underwent FISH for chromosomes X, Y, 1, and 17 in an attempt to determine the extent of sex chromosome and autosome 1 and 17 abnormality in normally developing, monospermic human preimplantation embryos (Harper *et al.*, 1995). The results for the chromosomes examined were very similar to the karyotyping studies. On the contrary, when abnormally developing embryos, or those generated from older IVF patients were tested for chromosomes X, Y, 18, and 16 in a similar investigation the rate of abnormalities increased to 70% (Munne *et al.*, 1993). Delhanty and colleagues (1997),

analysed a larger cohort of 93 morphologically normal embryos with FISH for chromosomes X, Y, and 1, and according to the chromosomal patterns that were observed, they classified them into the following categories:

(i) Uniformly normal with a diploid chromosome complement.

(ii) Uniformly abnormal having trisomies or monosomies of autosomal or sex chromosomes.

(iii) Mosaic having diploid cell lines and aneuploid, haploid or polyploid nuclei.

(iv) Chaotic having each cell with a different chromosome complement.

The authors also attempted to establish the actual origin of mosaicism, and attributed it to the loss or gain of a chromosome from some cells during post-zygotic development. Two mechanisms were thought to be causing this phenomenon, namely mitotic non-disjunction and anaphase lag the latter leading to chromosome loss alone. They determined the second cleavage division as the onset for aneuploid or ploidy abnormalities, while haploid nuclei were attributed to the production of binucleate cells (Delhanty *et al.,* 1997).

A slightly different classification was given in a review by Munne and Cohen (1998). They considered the chaotic embryos to be mosaic, and they grouped the mosaic abnormalities into different categories, according to ploidy (haploid, diploid, polyploid mosaics), and also according to the developmental stage in which they arose. Hence, they postulated that the mosaic chromosome abnormalities affecting a small proportion of the cells led to the generation of diploid/tetraploid embryos, which were also normally developing. If the chromosome abnormalities, scored were affecting the whole of the embryo then they would classify it as a chaotic mosaic, and such abnormalities would usually be noted in arrested or abnormally developing embryos (Munne and Cohen, 1998).

Numerous other studies were subsequently carried out, applying both conventional cytogenetic methods (Clouston *et al.,* 1997; 2000) but more frequently FISH with an increasing number of probes for the analysis of both normally and abnormally developing spare cleavage-stage embryos or even blastocysts (Munne *et al.,* 1998c; Evsikov and Verlinsky, 1998; Ruangvutilert *et al.,* 2000b; Sandalinas *et al.,* 2001; Magli *et al.,* 2001a,b; Bielanska *et al.,* 2003; Coonen *et al.,* 2004). The level of mosaicism observed in

these studies was in the range of 50% or over. Furthermore, it was concluded that certain patients had the tendency of generating chaotic embryos more frequently than others (Delhanty *et al.,* 1997). An association of mitotic non-disjunction with advanced maternal age was also demonstrated from the examination of embryos coming from patients undergoing PGS (Munne *et al.,* 2002a). These conclusions were drawn from the investigation of a limited number of chromosomes and led to the question of whether any embryo was uniformly chromosomally normal at this early stage of development (Delhanty, 2001).

The application of CGH for the analysis of two sets of cleavage-stage embryos carried out by two different groups (Wells and Delhanty, 2000; Voullaire *et al.,* 2000) gave the answer to this question. Wells and Delhanty (2000) analysed 64 blastomeres from 12 good quality embryos, with the use of the DOP PCR, followed by CGH. Among them, three embryos were classified as uniformly euploid for all 23 chromosomes. As would be expected, from the FISH results, many abnormalities were also detected, including a uniformly double aneuploid (trisomy 21 and monosomy X) embryo, another with monosomy for chromosome 1 in most of its cells, and one with a deletion of part of chromosome 1. In total, eight were mosaic, two of which were chaotic, and several abnormalities of meiotic origin were also detected (Wells and Delhanty, 2000). Comparable results were obtained from the other CGH study (Voullaire *et al.,* 2000). More recently, Malmgrem and colleagues (2002) employed CGH for the analysis of 28 cleavage-stage embryos, from 13 couples with structural chromosome abnormalities, including both reciprocal and Robertsonian translocations. Analysis revealed that all examined embryos were either highly mosaic, or even chaotic (Malmgrem *et al.,* 2002).

An effect of this high degree of chromosomal mosaicism, which is negatively associated with embryo survival is a phenomenon known as confined placental mosaicism (CPM). The latter can be defined as a dichotomy between the chromosomal constitution of the placental and embryonic/fetal tissues. This is observed in approximately 1-2% of all tested pregnancies, and in its most common type it involves a trisomic clone confined to the placenta (Kalousek *et al.,* 1989; Kalousek, 1990). In zygotes that are diploid, a

mitotic duplication of a specific chromosome taking place in a cell that could end up in the trophectoderm results in the generation of type I or II CPM (either trophoblast or chorionic stroma). Trisomic zygote rescue due to chromosome loss occurring in the embryonic progenitor cells has as an effect the formation of type III CPM (trisomy present in both the trophoblast and the chorionic stroma). (Kalousek *et al.,* 1993; Robinson *et al.,* 1997). The opposite is also feasible, i.e. the generation of a trisomic embryo with a diploid placenta. It has been shown that diploid placentas maintain fetuses that are trisomic for chromosomes 13 or 18 (Kalousek *et al.,* 1989).

As far as the general survival is concerned, embryos with extensive abnormalities, including more than one extra or missing chromosome in the majority or even a few cells have a very limited developmental potential. It is very likely that such embryos would either arrest prior to implantation, or if they do not, they will fail to implant (Wells and Delhanty, 2000). Data related to embryo survival, depending on which chromosomes show anomalies and their extent has been obtained by studies of embryos that were left to reach the blastocyst stage of development (Ruangvutilert *et al.,* 2000b; Clouston *et al.,* 2000; Sandalinas *et al.,* 2001). Analysis of 50 chromosomally abnormal embryos, diagnosed at the cleavage stage on a single cell that survived to the blastocyst stage, with probes for chromosomes X, Y, 1, 13, 15, 16, 18, 21, and 22 revealed that 17 of them were aneuploid, with 14 being trisomic and the other monosomic for 21 or X. None of these embryos were classified as highly mosaic or chaotic, whilst the majority of them were mosaic diploid/tetraploid. The latter is thought to be a normal part of TE development (Sandalinas *et al.* 2001). Similar results were obtained in the karyotyping study of 438 human blastocysts carried out by Clouston and colleagues (2000). The survival of the monosomic embryos, was confined to chromosomes 21 or X, as the one is relatively small and hence the deletion of genes is minimal, whilst the other one could be possibly inactivated in female embryos. Monosomies of larger chromosomes would not be compatible with survival, due to the loss of essential housekeeping genes, required for vital cellular functions (Wells and Delhanty, 2000).

The chromosomal mosaicism observed at this early stage of human development resembles the chromosome instability observed in cells coming from cancerous tumours.

The latter led to the suggestion that the normal cell cycle checkpoints may not be operating during the initial cleavage divisions (Delhanty and Handyside, 1995). A study carried out on mouse oocytes demonstrated the absence of the metaphase-anaphase checkpoint in these cells (Le Maire-Adkins *et al.,* 1997). This checkpoint is responsible for the correct alignment of chromosome onto the mitotic spindle, and the situation could be similar for the human oocytes as well. Since the first cleavage divisions are supported by the maternal genome with the embryonic genome being globally activated after the 4-cell stage (Braude *et al.,* 1988), it is possible that the absence of this checkpoint could be one cause of the complex chromosome abnormalities described above (Wells and Delhanty, 2000). Moreover, maternal genome support could result in the survival of embryos with multiple aneuploidy up until the blastocyst stage. Elimination of maternal mRNAs could lead to the arrest that is frequently observed prior to blastocyst formation for highly abnormal embryos (Wells and Delhanty, 2000).

IVF culture conditions could also be responsible for the high frequency of mosaicism. An example is a sudden decrease in temperature that could in turn affect cytokinesis, leading to the generation of diploid/polyploid embryos (Munne and Cohen, 1998). In addition, it has been shown that embryos produced by different stimulation protocols and cultured under different conditions have very diverse mosaicism rates (Munne *et al.,* 1997).

The factors regulating and causing post-zygotic chromosome abnormalities will be elucidated only by gene expression studies, which are very difficult to carry out in vitro. However, the incidence of such extensive anomalies in the chromosome complement is surely the main reason of the relatively low fertility observed in humans.

1.9 Oocyte studies

Cytogenetic analysis of preimplantation embryos has revealed that quite a few chromosome anomalies have their origin in meiosis. As was mentioned previously, female meiosis is a more complex process, compared to its male counterpart. The latter is confirmed by FISH studies carried out on sperm that demonstrated a chromosome-specific aneuploidy rate of 0.1-0.2% (Hassold, 1998). Extrapolation of this data to the full chromosome count, would result in a total of 2% of sperm with additional or missing chromosomes (Delhanty, 2001). Meiotic investigation of female gametes is more difficult

due to the inaccessibility of these cells. Such material is only obtained after IVF, and the oocytes that are available for examination are in their majority the ones that failed to fertilise after exposure to sperm. Moreover, they come from a selected group of patients that are being treated for infertility, which is not however necessarily of female origin (Delhanty, 2001).

These cells are arrested at metaphase II, and the latter has enabled their cytogenetic analysis, either via conventional karyotyping or FISH. Karyotyping of metaphase II oocytes demonstrated the gain or loss of whole chromosomes (Zenzes and Casper, 1992), or chromatids due to their premature division during meiosis I (Angell *et al.,* 1994; Angell, 1995; 1997). Both these mechanisms of aneuploidy have been described in section 1.4.1.

More recent studies on metaphase II oocytes have employed FISH or associated methods to establish the involvement of specific chromosomes in oocyte aneuploidy. The majority of these studies have used oocytes that failed to fertilise after their exposure to sperm. The polar body chromosomes were also investigated either separately, or in combination with the oocyte. Different fixation protocols have been employed including the ones suggested by Tarkowski (1966) or Kamiguchi and colleagues (1993) that allow the visualisation of whole chromosomes and chromatids.

One such study investigated the association of non-disjunction in meiosis II oocytes and 1st PBs with maternal age, and was carried out by Dailey and co-workers (1996). They examined 338 oocytes and corresponding 1st PBs from 107 patients using FISH, applying probes for chromosomes 13, 18, 21, and X in a single hybridisation round. Their results demonstrated both mechanisms of non-disjunction for the examined chromosomes and led to the conclusion that whole chromosome non-disjunction is age-related, with the aneuploidy rate increasing from 1.5% for women aged 25-34 to 24.2% for women over the age of 40. Chromatid predivision did not seem to increase with maternal age (Dailey *et al.,* 1996). In a subsequent study by the same group, that employed SKY for the analysis of 47 fresh metaphase II oocytes, an increase of balanced chromatid predivision was observed with advancing maternal age, from 6.5% for women of 34 years or less to 75% for women over the age of 40 (Sandalinas *et al.,* 2002). It has been shown that

balanced chromatid predivision could be caused by the oocyte ageing in culture (Dailey *et al.,* 1996). However, this was not the case with this study, as the oocytes were analysed just after retrieval (Sandalinas *et al.,* 2002).

Mahmood and colleagues (2000) and Cupisti and colleagues (2003) investigated spare meiosis II oocytes and 1st PBs using 3 sequential rounds of FISH employing probes for chromosomes 1, 9, 12, 13, 16, 18, 21 and X. Data was obtained for a total of 236 eggs (oocyte+PB), generated from 124 patients with an average age of 32.5 years (22-44). A total of 14 anomalies were scored, involving the presence of extra chromosomes and/or chromatids with chromosomes 13, 16, 18, and 21 being mostly affected. Both these studies were considering as abnormal oocytes and PBs with extra chromosomes only, as their absence could have been an artefact of the spreading process (Mahmood *et al.,* 2000; Cupisti *et al.,* 2003). The estimated hyperploidy rate was in the range of 4% for both oocytes and PBs, while both studies confirmed the presence of the above-mentioned non-disjunction mechanisms, and identified a third one, involving the presence of a trisomic cell line in the gonads of some patients (gonadal/germinal mosaicism). They also showed that the smaller chromosomes were more frequently participating in non-disjunction events, compared to larger ones (Mahmood *et al.,* 2000; Cupisti *et al.,* 2003).

Anahory and colleagues (2003) examined 104 unfertilised oocytes and 56 corresponding PBs by employing a double-label FISH procedure. In this study, centromeric or locus-specific probes were used in combination with whole painting probes for the examination of chromosomes 9, 13, 16, 18, 21, and X, taking place in 3 sequential rounds. These cells came from 45 women of average age 31.6 (21-42 years). The overall aneuploidy rate in this study was estimated to be 11.5%, and abnormalities involving extra chromosomes and/or chromatids were again observed (Anahory *et al.,* 2003).

A slightly increased aneuploidy rate of 22.1% was observed in the study of Pellestor and colleagues (2003) that employed an R-banding method for the examination of the whole chromosome complement of 3,042 oocytes coming from 792 women with an average age of about 34 years (19-46). This study was scoring as abnormal both the presence, but also the absence of chromosomes, as it used the gradual fixation method that is said not to be hampered by the artefactual loss of chromosomes, as is the Tarkowski one (Kamiguchi *et al.,* 1993). As with the previous studies, both whole chromosome and extra chromatids

were involved in the scored anomalies, and smaller chromosomes were preferentially participating (Pellestor *et al.,* 2003).

The average age of all the oocyte donors from the above studies was lower than that at which the aneuploidy risk becomes significant enough for prenatal diagnosis to be used. The latter suggests that age-independent factors could be operating to increase the risk of trisomic conceptions. Such factors could be causing the high rate of meiotic errors observed during oocyte maturation. As with post-zygotic cleavage divisions and the associated mosaicism, absent or malfunctioning checkpoint genes that regulate meiosis could lead to the frequent incidence of chromosome malsegregation. Examples of genes that have been identified as regulators of oogenesis include MAP kinases that increase in the GV stage and remain at high levels during the two meiotic processes and even after fertilisation (Sagata, 1997). The *c-mos* protooncogene and its corresponding protein regulate meiosis II arrest in oocytes (Sagata, 1997) and mask an epitope of the motorprotein CENP E at meiotic kinetochores (Duesbery *et al.,* 1997). CENP E itself regulates chromatid separation by delaying the anaphase onset until all centromeres are correctly attached to the spindle during mitosis (Abrieu *et al.,* 2000). Its role in oogenesis is not known yet, but it could be similar. Studies on mouse oocytes having the *c-mos* gene knocked out showed failure of arrest in metaphase II, and possession of aberrant spinldes (Araki *et al.,* 1996; Colledge *et al.,* 1994). Thus, the masking of the CENP E epitope by the C-MOS protein, is most likely involved in the regulation of these processes during oogenesis.

Another gene, *Rec8* is thought to be responsible for the maintenance of the cohesion of two sister chromatids at the centromere. Its removal is observed at anaphase I, enabling the termination of the chiasmata and the separation of the homologues. This is mediated by two other proteins, securin and separin whose mutations could lead to chromatid predivision (Cohen-Fix, 2000; Van Heemst and Heyting, 2000). *Mam1* has also been shown to enhance monopolar attachment of microtubules to sister chromatid kinetochores, ensuring in this way their correct segregation to different poles (Toth *et al.,* 2000). Finally, Steuerwald and colleagues (2001) observed that the transcripts of spindle attachment checkpoint genes, such as *MAD2* and *BUB1*, are detected in lower

concentrations in oocytes of older women, who are more likely to generate aneuploid gametes. This observation provided direct evidence that disturbances in the gene expression of meiosis specific genes may be directly associated with aneuploidy (Steuerwald *et al.*, 2001).

1.10 Aims and outline of the study

This study involved the investigation of chromosome abnormalities in preimplantation embryos, meiosis II oocytes and their corresponding first PBs with the application of two different molecular cytogenetic methods. It was divided into three parts, the last two being associated with each other. The aims and objectives of this study were the following:

- The first objective of this study was the development of reliable FISH-based protocols for their application in the PGD of chromosome anomalies and in the follow-up analysis of abnormal embryos. The aim was to extensively investigate the hypothesis that various types of chromosomal errors both meiotic and post-zygotic lead to the generation of highly mosaic and chaotic embryos that can be patient specific. Robust 3-colour FISH protocols were devised for seven patients and were applied clinically for five, with detailed follow-up analysis.
- The second objective of this study involved the in-depth investigation into the causal mechanisms of maternal aneuploidy, in an attempt to answer questions associated with chromosome size and participation in oocyte aneuploidy, frequency of whole chromosome versus single chromatid anomalies, the presence of gonadal/ germinal mosaicism, and an accurate estimation of both hyperhaploidy and hypohaploidy. Spare unfertilised oocytes and their corresponding first PBs were initially analysed with three sequential rounds of FISH for chromosomes of different sizes, and then by the application of CGH. This DNA-based technique was used to examine the whole of the maternal genome and would provide data about the participation of larger chromosomes in meiotic errors and the manner with which meiosis II oocytes were affected by hypohaploidy.

The main results of this investigation are described in three Chapters (3, 4, and 5). Chapter 3 describes the development and application of the FISH protocols for the diagnosis of five different chromosome abnormalities over nine PGD cycles, the chromosome complements seen in all generated embryos and an overall analysis of the levels of chromosomal mosaicism found in these embryos. Chapter 4 describes the

sequential FISH analysis of the unfertilised meiosis II oocytes and corresponding 1st PBs, including the probes used, and the conclusions drawn from this part of the study concerning the mechanisms underlying maternal aneuploidy. Chapter 5 describes the optimisation of the CGH protocol via some positive control experiments, with the aim of employing this in the extensive analysis of meiosis II oocytes and first PBs. The data obtained from its application for the examination of these cells is illustrated along with conclusions. A detailed discussion of this work with review of similar studies, both for the PGD of chromosome abnormalities and the cytogenetic analysis of female gametes, along with the general conclusions is presented in Chapter 6.

Chapter 2

Materials and Methods

2.1 Materials

The composition of reagents and solutions is described in detail in Appendix A

2.1.1 General reagents and equipment

2.1.1.1 Chemicals

The chemicals and reagents used in the laboratory were obtained from BDH Chemicals, Sigma Chemical Company or Gibco BRL, unless declared differently. They were all of Analar or biochemical grade. Preparation of solutions and buffers is described in Appendix 2A.1.

2.1.1.2 Enzymes

Details about the constitution of enzymes used for the nick translation for both FISH probes and CGH probes are shown in Appendix 2A.5.3 and came as part of the nick translation kit from Vysis/Abbott, UK. Pepsin (Sigma,UK) (10mg/ml) was applied for the pre-treatment of slides during the FISH procedure. Proteinase K (Roche,UK) (100μg/ml) was used for single cell lysis. Pronase (Sigma, UK) was used for the separation of oocytes and PBs. Finally, the Super Taq Plus DNA polymerase (5 units/μl), used during the *Alu* and DOP-PCR reactions was obtained from HT Biotechnologies.

2.1.1.3 Nucleic Acids

Oligonucleotide primers for the *Alu*, and the DOP-PCR reactions were supplied by Pharmacia and Oswel respectively (details seen in Appendix 2A.3.3 and 2A.4.3). DNA size standards (1kb Hypperladder IV) were from Bioline. Sonicated Herring Sperm DNA (10mg/ml) and human Cot^{-1} DNA (1μg/ml) were obtained from Sigma, UK, while the latter was also ordered from Gibco BRL. Deoxynucleotide triphosphates (dNTPs dATP, dCTP, dGTP, dTTP) for the DOP-PCR already mixed (10mM), were supplied by Promega. dNTPs were also used during the nick translation of FISH and CGH probes and came as part of the Vysis/Abbott nick translation kit (dATP, dCTP, dGTP, dTTP, each 0.3mM). Finally, labelled deoxynulceotide triphosphates (dUTPs, Spectrum Green, Spectrum Orange, Spectrum Red) also came from Vysis/Abbott.

2.1.1.4 Cell culture media and other equipment

Flasks for the culture of lymphocytes and fibroblasts, plastic pipettes, and microscope slides were all from BDH. Glass and plastic microcapillaries (internal diameter 75-100 mm) for embryo and oocyte manipulation and for single cell isolation and tubing were obtained from Laser. Iscoves modified Dulbeccos medium and Roswell Park Memorial Institute 1640 medium, both used for lymphocyte culture, were obtained from Sigma and Gibco BRL, respectively. Colcemid (100μg/ml) and fetal calf serum, used for lymphocyte culture, were both ordered from Gibco BRL. The former was stored at 4°C, whereas the latter at -20°C. Methotrexate (Sigma, UK) was used for lymphocyte culture. Hanks's medium and Versene medium, both used for skin fibroblast culture, were obtained from Gibco BRL. Other reagents for media preparation, antibiotics and growth supplements were from Sigma Chemical Company, Difco Bacto, Gibco BRL, and BDH. If any of the above were in desiccated form, they were made up to stock concentrations, filtered, and stored at -20°C.

2.1.1.5 FISH reagents

The chemical solutions and buffers used during the FISH procedure are shown in Appendix 2A.6. The detergent polyoxyethylene sorbitan monolaurate or Tween 20, the Poly-L-lysine adhesive reagent, along with the 4',6-diamidino-2-phenylindole (DAPI) solution, which counterstains DNA and enables the detection of nuclei under the fluorescence microscope, were all from Sigma Chemical Company. DAPI was mixed with the anti-fade mounting medium Vectarshield (Vector Laboratories). The first two solutions were stored at room temperature, whereas the other two at 4°C.

2.1.1.6 Microscopy and image analysis

Embryo and oocyte manipulation, as well as lymphocyte slide preparation took place with the use of dissecting and inverted microscopes, from Nikon and Olympus, respectively. Images were captured and analysed with the Olympus BX40 fluorescent microscope, equipped with a cooled charge-coupled device (CCD) camera, controlled by SmartCapture (Digital Scientific UK).

2.1.2 Ethical approval

Patient referrals for PGD came from different clinical genetics centres mostly in the UK, but overseas, as well. The IVF and PGD treatments were carried out exclusively at the Assisted Conception Unit (ACU), University College Hospital (UCH), London. Unfertilised oocytes were donated for research by patients undergoing routine IVF or ICSI treatments at both the ACU, UCH, London and the ACU at Tayside University Hospitals NHS Trust, Dundee, Scotland. The preliminary work on surplus embryos, the clinical application of PGD, and the research work carried out on human oocytes were all carried out under licence from the Human Fertilisation and Embryology Authority and were also approved by the Research Ethics Committees of University College London Hospital and Tayside Trusts. Donation of embryos and oocytes from patients occurred only after their informed consent.

2.1.3 Preimplantation Genetic Diagnosis

2.1.3.1 Patient Details

Seven couples were investigated, all of them being at risk of unbalanced pregnancy or offspring due to a chromosomal abnormality. In three of the seven, one of the two partners was a balanced carrier of a reciprocal translocation, in two cases one of the partners was carrying a Robertsonian translocation, whereas the remaining two were referred for suspected gonadal mosaicism for a trisomic cell line. The maternal age varied between 25-39 (average age: 32.6) The details of these patients including their reproductive histories are shown in Table 2.1.

2.1.3.2 FISH probe details

All the probes that were used in this part of the study were directly labelled and most of them were of commercial origin. Laboratory-prepared probes were also employed. The latter were provided in the form of agar stabs from various resource centres. DNA extraction for the plasmid and cosmid DNA probes took place with the application of the Wizard maxiprep kit from Promega, whereas yeast DNA was extracted with the Nucleon extraction and purification kit for yeast minipreps, obtained from Amersham. These probes were labelled via nick translation, using the Vysis nick translation kit. Details of the probes are shown in Table 2.2.

The various probe strategies employed for the PGD of these seven cases, along with the different probe combinations applied for spare embryo re-analysis are shown in Table 2.3.

Table 2.1: Details of the patients referred for PGD of chromosome abnormalities.

Case	Female partner details	Male partner details	Reproductive history
A	Karyotype: Normal 46,XX Age: 25	Karyotype: Balanced reciprocal translocation, 46XY,t(5;19)(p12;p12) Age:25	Primary infertility, due to severe oligospermia
B	Karyotype: Balanced reciprocal translocation 46,XX,t(11;22)(q23.3;q11.2) Age: 28	Karyotype: Normal 46XY Age: 39	Four spontaneous abortions
C	Karyotype: Normal 46XX Age: 28	Karyotype: Balanced reciprocal translocation, 46,XY,t(14;16)(q13;q11.1) Age: 32	Primary infertility, due to severe oligospermia
D	Karyotype: Normal, 46,XX Age: 34	Karyotype: Balanced Robertsonian translocation, 45,XY,der(13;21)(q10;q10) Age: 49	Primary infertility due to oligoasthenoteratozoospermia
E	Karyotype: Normal, 46,XX Age: 37	Karyotype: Balanced Robertsonian translocation, 45,XY,der(13;14)(q10;q1	Primary infertility due to oligospermia. Male partner fathered a pregnancy with previous partner after IVF, but ended in first

		0) Age: 45	trimester spontaneous abortion. Male partner's sister is also a balanced carrier of the translocation and has experienced early spontaneous abortions
F*	Karyotype: Normal, 46,XX. Age: 39	Karyotype: Normal, 46,XY Age: 36	Female partner experienced two ectopic pregnancies, which led to her having a bilateral salpingectomy. One pregnancy was achieved after IVF, which was affected by trisomy 21 and was terminated at 20 weeks.
G*	Karyotype: Normal, 46,XX. Age: 37	Karyotype: Normal, 46,XY Age: 35	Recurrent trisomy 21 pregnancies. Two Down's syndrome children and a termination of a Down's syndrome pregnancy

*Couples F and G were referred for possible gonadal mosaicism for trisomy 21 in one of the two partners. FISH analysis on 400 lymphocyte interphase nuclei coming from each of the two partners did not reveal any sign of mosaicism, in these somatic cells.

Table 2.2: Details of DNA probes employed for the PGD of chromosome abnormalities.

Probe name	**Probe details**	**Location of hybridisation**	**Origin**
Centromeric for chromosome 4	Alpha-satellite. Laboratory prepared probe. Labelled in SG, using the Vysis nick translation kit	4p11.1-q11.1	p4n1/4 D'Aiuto *et al.*, 1993
LSI EGR1 or "Cri du Chat" for chromosome 5	Locus-specific D57S21, D5S23. Dual coloured, SO and SG	5q31-SO 5p15.2-SG	Vysis/Abbott, UK
CEP 11 Centromeric	Alpha-satellite, DNA D11Z1. Labelled in SO	11p11.1-q11.1	Vysis/Abbott, UK
CEP 11 Centromeric	Alpha-satellite, DNA D11Z1. Labelled in SG	11p11.1-q11.1	Vysis/Abbott, UK
LSI 13	Locus-specific. Spans the retinoblastoma gene (RB1). Labelled in SG	13q14	Vysis/Abbott, UK
TelVysion 14q	Subtelomeric, D14S308. Labelled in SO	14qter	Vysis/Abbott, UK
CEP 15 Centromeric	Alpha-satellite. D15Z1. Labelled in SO	15p11.1-q11.1	Vysis/Abbott, UK
Centromeric for	Satellite III.	15q11.2	pMC15

chromosome 15	D15Z3 Laboratory-prepared. Labelled in SO, using the Vysis nick translation kit		Archidiacono *et al.*, 1995
Centromeric for chromosome 16	Beta-satellite. D16Z2. Laboratory-prepared. Labelled in SG, using the Vysis nick translation kit.	16q11.1	pZ16A Archidiacono *et al.*, 1995
CEP 16. Heterochromatic region	*Satellite II. DNA D16Z3 Labelled in SA*	*16q11.2*	Vysis/Abbott, UK
TelVysion 16p	Subtelomeric *D16S3400.* Labelled in SG	*16pter*	*Vysis/Abbott, UK*
CEP18. Centromeric	*Alpha-satellite, D18Z1. Labelled in SA*	*18p11.1-q11.1*	*Vysis/Abbott, UK*
YAC *probe for chromosome 19*	Locus-specific. Laboratory-prepared. Labelled in SO and SG, using the Vysis nick translation kit	19q13.2	S.cerevisiae strain. Frengen *et al.*, 1999
LSI 21	Locus-specific D21S529, D21S341,	21q13.2-q22.2	Vysis/Abbott, UK

	D21S342. Labelled in SO		
Chromosome 21 specific DNA probe	Band-specific. Labelled in red	21q22.2	Oncor, UK
TEL 21q	Subtelomeric. Labelled in red	21qter	Oncor, UK
TEL 21q	Subtelomeric. Labelled in red	21qter	Oncor, UK
LDI VCFS, or "Di George" for chromosome 22	Locus-specific D22S75. Dual coloured, labelled in SO and SG	22q11/TUPLE1 (HIRA)-SO 22q13/Arylsulphatase A (ARSA)-SG	Vysis/Abbott, UK
LSI 22	Locus-specific. Spans the bcr gene. Labelled in SG	22q11.2	Vysis/Abbott, UK
CEPX. Centromeric	Alpha-satellite DXZ1. Labelled in SO	Xp11.1-q11.1	Vysis/Abbott, UK
CEPY. Centromeric	Alpha-satellite DYZ3. Labelled in SO	Yp11.1-q11.1	Vysis/Abbott, UK

SA: Spectrum Aqua

SG: Spectrum Green

SO: Spectrum Orange- appeared red

Table 2.3: Probe combination developed for the PGD of chromosome abnormalities and for spare embryo re-analysis for the seven cases.

Case	Probe combination for diagnosis	Probe combination for re-analysis
A	LSI "Cri du chat" probe for chromosome 5 YAC probe for chromosome 19	
B	LSI "Di George" dual probe for 22 CEP 11 α-satellite for chromosome 11	Cycle 1: 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 CEP X centromeric α-satellite for chromosome X CEP Y α-satellite for chromosome Y
C	TelVysion 14q for chromosome 14 CEP 16 centromeric satellite II for chromosome 16 TelVysion 16p for chromosome 16	
D	LSI 13 for chromosome 13 LSI 21 for chromosome 21 TEL 21q for chromosome 21	Cycle 1: CEP 18 centromeric α-satellite for chromosome 18 CEP X centromeric α-satellite for chromosome X CEP Y α-satellite for chromosome Y
E	LSI 13 for chromosome 13 TelVysion 14q for chromosome 14	Cycle 1: 4 centromeric α-satellite for chromosome 4 15 centromeric satellite III for chromosome 15 CEP 18 centromeric α-satellite satellite for chromosome 18

		Cycle 2: CEP 18 centromeric α-satellite satellite for chromosome 18 CEP X centromeric α-satellite for chromosome X CEP Y α-satellite for chromosome Y
F	LSI 13 for chromosome 13 LSI 21 for chromosome 21	Cycle 2: CEP 15 centromeric α-satellite for chromosome 15 CEP 18 centromeric α-satellite for chromosome 18 LSI 22 for chromosome 22
G	Band-specific for chromosome 21 TEL 21q for chromosome 21	

2.1.3.3 Preimplantation embryos

The patients involved in this part of the study all underwent routine IVF procedures, as described in Ranieri *et al.*, 2001 and summarised in the Methods section 2.2.1. Diagnostic chromosome analysis with the application of FISH was carried out on eighty-nine embryos (147 blastomeres). Of the embryos biopsied, only three showed abnormal fertilisation, i.e. one or three pronuclei. The remaining were all normally fertilised, as indicated by the presence of two pronuclei 18 hours post-insemination. Two blastomeres were obtained from embryos having 6 cells or more, whereas one blastomere was obtained from embryos having 5 cells or less. Details of the oocytes collected and fertilised in each of the five PGD cases, along with the number of embryos biopsied and blastomeres obtained are shown in Table 2.4.

Table 2.4: Oocytes collected and embryos biopsied for each of the five PGD cases.

Case	No. of oocytes collected	No. of oocytes fertilised	No. of embryos biopsied	No. of blastomeres obtained
A				
Cycle 1	20	15	13	24
B				
Cycle 1	12	5	3	6
Cycle 2	14	9	8	11
D				
Cycle 1	9	7	6	11
Cycle 2	13	10	9	12
E				
Cycle 1	12	8	8	16
Cycle 2	13	13	8 + 1 with 3PN	13
F				
Cycle 1	19	17	17	24
Cycle 2	24	20	16	30

2.1.3.4 Preimplantation embryos grading criteria

Embryos produced via IVF procedures were divided into three categories, according to grading criteria set by Dawson *et al.*, 1995. These categories are the following:

Grade I Embryos at the correct stage of *in vitro* development with regular-shaped and even-sized blastomeres with no fragmentation.

Grade II Embryos show an intermediate morphology between grades I and III

Grade III Embryos show retarded development with unequally sized blastomeres with at least one degenerated blastomere and/or high level of fragmentation

2.1.3.5 Embryo classification according to chromosome abnormalities observed

Depending to the chromosome abnormalities observed during PGD analysis, embryos were classified into four groups: normal, abnormal, mosaic (diploid mosaic and aneuploid mosaic) and chaotic. Classification took place according to criteria set by Delhanty *et al.*, 1997 and designation to one of the four categories was based on the chromosome complement observed in majority of blastomeres constituting each embryo. Thus:

Normal Embryos which are uniformly normal for all tested chromosomes.

Abnormal Embryos which are uniformly abnormal for all tested chromosomes.

Mosaic

Diploid mosaic Embryos that are in the main euploid, but contain one or more cells that are aneuploid, polyploid or haploid.

Abnormal mosaic Embryos that are in the main aneuploid, polyploid or haploid, but also contain one or more cells that differ.

Chaotic Embryos showing a random chromosome complement, varying among different blastomeres. Chromosome status of initial zygote cannot be identified.

At least two cells with the same chromosome loss were required to demonstrate monosomy.

2.1.4 Human oocyte and 1st PB analysis with FISH

2.1.4.1 Patients

Meiosis II oocytes and corresponding first PBs were donated for research from women with no known chromosome abnormalities, whose age varied between 22 and 44 years (average age 32.5 years). These patients were undergoing routine IVF or ICSI procedures at the time, and the duration of infertility ranged between 1.5 to 10 years. The causes of infertility were the following:

- Male factor- 25%
- Tubal factor- 20%
- Endometriosis- 22%
- Polycystic ovaries- 11%
- Anovulation- 6%
- Low response to stimulation regimes- 6%

Two centres collaborated in this part of the study: the Assisted Conception Unit at Tayside University Hospitals NHS Trust, Dundee, Scotland, and the Assisted Conception Unit at the University College London Hospitals Trust. Fully informed consent was given.

The ovarian stimulation regime was the same in both centres and is described in the Methods section 2.2.1.

2.1.4.2 Oocytes

The majority of oocytes investigated in this part of the study had not demonstrated any signs of fertilisation, after either being incubated (IVF), or injected (ICSI) with sperm. Oocytes that were not fertilised either due to lack of sperm from the male partner, or because they were classified as immature were also studied. Culture time ranged between 24 to 48 hours. All oocytes were at the metaphase II stage of meiosis and had extruded the first PB, prior to them being spread on slides.

2.1.4.3 FISH probe details

All the probes that were used in this part of the study were directly labelled. Various commercial and lab-prepared probes were employed. The latter were all repetitive

sequence probes, initially cloned in plasmids or cosmids. Their treatment was exactly the same as described in 2.1.3.2. Details of the probes are shown in Table 2.5

Table 2.5: Details of DNA probes employed in the analysis of oocytes and 1st PBs.

Probe name	Probe details	Location if hybridisation	Origin
Repeat-sequence probe for chromosome 1 (heterochromatic region)	Satellite III. Lab-prepared probe. Labelled in SG using the Vysis nick translation kit.	1q12	pUC1.77 Cooke and Hindley, 1979
Centromeric for chromosome 4	Alpha-satellite. Lab-prepared probe. Labelled in SO using the Vysis nick translation kit	4p11.1-q11.1	p4n1/4 D'Aiuto *et al.*, 1993
Centromeric for chromosome 4	Alpha-satellite. Lab-prepared probe. Labelled in SG using the Vysis nick translation kit	4p11.1-q11.1	p4n1/4 D'Aiuto *et al.*, 1993
Centromeric for chromosome 9	Alpha-satellite Lab-prepared probe. Labelled in SO using the Vysis nick translation kit	9p11.1-q11.1	CCMP 9.27, 9qh; N.Carter Cambridge, UK
Centromeric for chromosome 12	Alpha satellite. Lab-prepared	12p11.1-q11.1	Baldini *et al.*, 1990

	probe. Labelled in SO using the Vysis nick translation kit.		
Centromeric for chromosome 12	Alpha satellite. Lab-prepared probe. Labelled in SG using the Vysis nick translation kit.	12p11.1-q11.1	Baldini *et al.*, 1990
LSI 13	Locus-specific probe. Spans the retinoblastoma gene (RB1). Labelled in SG	13q14	Vysis/Abbott, UK
Centromeric for chromosome 16	*Alpha-satellite. Lab-prepared probe. Labelled in SO, using the* Vysis nick translation kit.	*16p11.1-q11.1*	*pSE16, Greig et al., 1989*
CEP 16	Satellite II. DNA D16Z3. Labelled in SA	16q11.	Vysis/Abbott, UK
Centromeric for chromosome 17	Alpha-satellite. Lab-prepared probe. Labelled in SG using the Vysis nick	17p11.1-q11.1	pZ17-1.6A Archidiacono *et al.*, 1995

	translation kit		
Centromeric for chromosome 17	Alpha-satellite. Lab-prepared probe. Labelled in SO using the Vysis nick translation kit.	17p11.1-q11.1	pZ17-1.6A Archidiacono *et al.*, 1995
Centromeric for chromosome 18	Alpha-satellite. Lab-prepared probe. Labelled in SG using the Vysis nick translation kit	18p11.1-q11.1	L1.84 Devilee *et al.*, 1986
LSI 21	Locus-specific probe. D21S529, D21S341, D21S342. Labelled in SO	21q22.2	Vysis/Abbott, UK
LSI 22	Locus-specific probe. Spans the bcr gene. Labelled in SG.	22q11.1	Vysis/Abbott, UK
Centromeric for chromosome X	Alpha-satellite. Lab-prepared probe. Labelled in SO using the Vysis nick translation kit	Xp11.1-q11.1	pBAMX5 Willard *et al.*, 1983

SA: Spectrum Aqua

SG: Spectrum Green

SO: Spectrum Orange- appeared red

2.1.5 Human oocyte and 1st PB analysis with CGH

2.1.5.1 Patients

The genomic content of oocytes at the metaphase II stage of development and their corresponding first PBs were investigated. These came from routine IVF patients with no known karyotype abnormalities. Maternal age varied between 22 and 39 years (average age 31.9 years). The causes of infertility were broadly as described in 2.1.4.1. The Assisted Conception Unit at Tayside University Hospitals NHS Trust, Dundee, Scotland, provided material. Ovarian stimulation protocols were as described in 2.2.1.

2.1.5.2 Oocytes and 1st PBs

Similarly as before, the oocytes examined, had failed to fertilise after either IVF, or ICSI, or were unexposed to sperm. All of them however, had extruded the 1st PB. Culture time ranged between 24 to 48 hours. Oocyte and corresponding 1st PBs were separated and placed into tubes individually, as described in the Methods section 2.2.7.3.3.

2.1.5.3 Single cell lysis, and whole genome amplification

Single cells were lysed with the use of the enzyme Proteinase K (PK), provided by Roche and Sodium Dodecyl Sulphate (SDS), obtained from Sigma Chemical Company. Whole genome amplification was achieved with the application of the DOP- PCR reaction. The DOP primer was supplied by Oswel (details as seen in Appendix 2A.4.3).

2.1.5.4 Probe labelling, hybridisation and post-hybridisation washes

Amplified DNA was labelled with the application of the Vysis nick translation kit. Details of its components are given in section 2.1.1.3. Probes were hybridised onto CGH metaphase target slides, which were supplied by Vysis/Abbot UK. Sigma Chemical Company supplied Triton used during the post-hybridisation washes as a detergent.

2.1.5.5.Image analysis and interpretation

Image capturing took place with the use of a Zeiss Axioskop microscope, equipped with a Photometrics KAF 1400 cooled CCD (charged coupled device) camera, controlled by Path Vysion software (Vysis, UK). The captured images were then analysed in order for a comparison between the amplified test DNA (oocyte or PB), labelled in green and the amplified control DNA (normal male or female), labelled in red, along the length of each of the chromosomes to be achieved. Analysis and interpretation of the captured images was enabled by special computer software (Digital Scientific), that converted fluorescent intensities into a red-green ratio for each chromosome. Loss or gain of chromosome material was identified by the presence of deviation from a 1:1 ratio.

2.1 Methods

2.2.1 IVF and PGD procedures

2.2.1.1 IVF treatment cycles

All patients undergoing PGD followed a long IVF protocol, as described in Ranieri *et al.*, 2001. More specifically, initial pituitary suppression took place with administration of a luteinising hormone agonist in the form of buserelin nasal spay (Suprefact; Hoechst, Hounslow, Middlesex, UK). Ovarian stimulation began after 12 days, by providing patients with purified FSH (Metrodin HP; Serono, Welwyn Garden City, Hertfordshire, UK). The follicular development and growth was observed by transvaginal ultasonography, which started on day 6 of stimulation. Patients that had at least one follicle of 17 mm in diameter and two or more of 15 mm in diameter, between days 12 and 14 were administered with 10,000 IU of human chorionic gonadotrophin (hCG, Profasi; Serono, UK). Collection of oocytes took place 36 hours later by ultrasound guided transvaginal aspiration. These were inseminated with prepared spermatozoa, approximately four hours after retrieval, and fertilisation was evaluated 24 hours later, by the presence of two pronuclei. Intracytoplasmic sperm injection (ICSI) was carried out for patients with sperm problems, such as oligospermia. In such cases the protocol followed is as described in Van Steirteghem *et al.*, 1993.

Oocytes and embryos were cultured in IVF medium (Cook, Australia), while embryo suitability for biopsy was evaluated in the morning of day 3. Embryos that were assessed as normal following PGD were transferred to the uterus on day four post-insemination, and a pregnancy test (β-hCG assay) took place on day 15.

2.2.1.2 Embryo manipulation

Embryos that were considered to be normally fertilised were graded as far as morphology was concerned, as described in 2.1.3.4 on the morning of day 3, and subsequently biopsied. The protocol followed was as described by Piyiamongkol *et al.*, 2001. All embryo manipulation procedures were performed at 37°C in order to avoid embryo cooling. Prior to biopsy, embryos were immobilised by gentle suction with a holding pipette, controlled by a Leitz micromanipulator. Acid Tyrodes solution

(pH 2.4) was applied to the zona pellucida, using a fine pipette until a hole of around 30μm was made. The blastomere was aspirated from the embryo with a fine pipette, and was then transferred to the same medium droplet in the petri dish. The second blastomere was removed in the same way. If the blastomere lysed, during spreading or was anucleate, an extra blastomere was aspirated if the embryo cell number permitted. The remainder of the embryo was returned back to normal culture conditions immediately after the biopsy was completed. Two blastomeres were aspirated only from embryos whose cell number was six and above, so that the embryo mass was not drastically reduced.

2.2.1.3 Oocyte recovery

Oocytes that were investigated with application of FISH were obtained using the same IVF protocol as in 2. 2.1. Their culture conditions are described in 2.1.4.2

2.2.2 Cell culture

2.2.2.1 Peripheral lymphocyte culture

Two different protocols were used in order to obtain lymphocyte suspensions from normal individuals (controls), patients and their partners, that were then used for FISH investigations. Both of them involved a synchronised culture method, which would yield extended chromosomes from peripheral lymphocytes. Whole blood was collected in sterile lithium heparinised tubes.

Protocol 1

The blood culture was set up as soon as possible after whole blood collection. Firstly, under aseptic conditions, a 20ml culture was prepared using 1ml of peripheral venous blood. The culture was prepared in a 50ml culture flask by adding 17ml Iscoves modified Dulbeccos medium, (Appendix 1A.1) supplemented with 2ml (10%) GPS, 2ml FCS, and 200μl PHA. The culture was mixed by shaking and incubated at 37°C for 48 hours. On the third day 200μl of thymidine (30mg/ml stock) were added to the culture, which was then further incubated for 18 hours. On the following day 200μl of deoxycytosine (0.227mg/ml stock) were added to the culture and incubated for four

hours. After incubation, 200μl of colcemid (10μg/ml) were added and the culture was incubated for another 20 minutes. Colcemid arrests the cells at metaphase, by preventing the formation of the mitotic spindle. The cells were harvested employing standard methods, using 0.075M KCl hypotonic solution and methanol/ acetic acid fixative. The lymphocyte suspensions were stored at -20°C.

Protocol 2

The blood used to prepare the culture for this protocol was stored at 4°C for 3-4 days after being drawn. The medium used to set up the blood in this protocol was the RPMI 1640 medium (Appendix 1A.2). The latter was prepared in the following way. To 80ml of RPMI medium 15ml of FCS, 3ml of $NaHCO_3$, 1ml PS and 1ml L-Glutamine were added and the pH adjusted by adding up to 1ml of NaOH. The latter was indicated by the colour of the medium, which should be salmon pink. When this colour was achieved, 18.5ml of the medium were added to 50ml cell culture flasks, along with 0.4ml PHA and 1ml of whole blood. All the components were mixed gently, and the cultures were incubated at 37°C for 65-72 hours. When this period had passed, 200μl of methotrexate ($1x10^{-5}$ stock) were incorporated in each of the flasks, and the cultures were further incubated for 17 hours. The following day RPMI medium was prepared, as previously and warmed to 37°C. The cultures were again decanted into 10ml centrifuge tubes (two per flask) and they were then centrifuged at 1100rpm for 10 minutes. The supernatant was removed and 0.5ml were left above the cell pellets. Five ml of RPMI medium were added and the pellets were resuspended. Another centrifugation followed, the supernatant was removed and 5ml of RPMI medium were added, as before, along with 50μl of BrdU (1mg/ml stock). The cultures were incubated for 4 hours and 40 minutes. When this period had passed, 50μl of colcemid (10μg/ml stock) were added and another 20-minute incubation followed. Harvest of the cell culture took place subsequently, as before. The cell suspensions were left at 4°C overnight and were then stored in the –20°C freezer.

2.2.2.2 Skin fibroblast cell preparation for FISH

As part of the preliminary work for some PGD cases, the probes were tested on trisomic fibroblast cells, apart from lymphocytes and spare preimplantation embryos. The aim was to assess the efficiency of detection of trisomies, compared with disomies. Skin fibroblast cultures were obtained from frozen cell stocks from fetuses with trisomy 13 or trisomy 14. The cells were provided as growing monolayer cultures. The cell culture medium was poured off, and the cells were washed by first adding 3-5ml of Hank's medium (2.1.1.4), and then by adding 3-5ml of 0.25% trypsin/Versene solution (Appendix 1A.3). At that point they were left at room temperature for 1 minute, after which most of the trypsin/Versene solution was discarded, and only a thin layer was left. The cells were placed in the 37°C incubator for 3-5 minutes and then they were observed under the microscope to check if they were loose. They were washed with 5ml of PBS (Appendix 2A.1.1) and transferred to conical centrifuge tubes. Centrifugation at 1000rpm for 5 minutes followed, the supernatant was discarded and another wash with 5ml of PBS took place in exactly the same way as previously. The cells were fixed in exactly the same way as for the lymphocyte culture and were left at room temperature for 30 minutes. The fix was removed, and fresh fixative solution was added. The cell suspensions were then stored in -20°C.

2.2.3 Slide preparation

2.2.3.1 Slide preparation of cultured cells

Before preparing the slides, 20ml of fresh fix solution (Appendix 2A.1.6) and 10ml of 70% acetic acid (Appendix 2A.1.7) were prepared. Most of the old fix was removed and discarded from the lymphocyte or fibroblast suspension, and fresh fix was added to resuspend the cells. A small amount of cells was dropped onto a clean moist slide and the nuclei were spread by warming the slide on the back of the hand. With the use of a diamond marker a circle was drawn on the underside of the slide to mark where the nuclei were located. Once the slide was totally dry it was flooded with fix for 10 seconds. The latter was poured off, the slide was dried and flooded with 70% acetic acid for another 10 seconds. The slide was dried once again and observed under the

microscope to ensure that the nuclei were present. The slide was dehydrated through an ethanol series (70%, 90% and 100%), (5 minutes in each) and air-dried. Finally the initial cell suspension was topped up with fresh fix and stored in the -20°C.

2.2.3.2 Slide preparation of single blastomeres and embryos

Human embryos or blastomeres were spread on poly-l-lysine coated slides. These were prepared by firstly washing the standard microscope slides in methanol/HCl and then drying them with a cloth. The poly-l-lysine was diluted 1:10 with water, and poured into a coplin jar in which the slides were incubated for 5 minutes. They were then left overnight to dry at room temperature and then stored at 4°C.

The spreading solution (Appendix 2A.8.3) was made fresh each time. A small circle was drawn on the underside of the poly-l-lysine slide using a diamond marker, a small drop of spreading solution was placed on the side of the circle, and a small drop of PBS was placed on the corner of the slide. Under a dissecting microscope, a pulled capillary was primed with PBS and the blastomere or embryo was moved from the petri dish into the PBS on the slide. The capillary was then primed with spreading solution and the blastomere or embryo was transferred to the drop of spreading solution within the circle, ensuring at the same time minimum transfer of PBS, as this would prevent the cell(s) from lysing. The blastomere or embryo was located under an inverted microscope, and the spreading solution was gently removed and replaced until the cell membrane started to lyse, the cytoplasm was washed away and the nuclei were clear. The spreading solution was not allowed to dry before the cell lysed. The spreading of the nucleus took approximately 5 minutes, and once it had finished the slide was left to air dry. It was incubated in PBS for 5 minutes, and dehydrated through an ethanol series. Nuclei were located under the microscope and co-ordinates taken using an England Finder (Optech, UK). The embryo slides were stored for up to 2 weeks at room temperature.

2.2.3.3 Slide preparation of unfertilised oocytes

Generally, oocytes were spread on normal marked microscope slides and were fixed using the Tarkowski (1966) protocol with modifications (Mahmood *et al.*, 2000). More specifically, they were first treated with freshly made hypotonic solution (1% sodium citrate) for 6-7 minutes, and placed in fixative I (Appendix 2A.8.4) for 1

minute, during which the zona pellucida dissolved. The oocyte was transferred to the marked slide and 13μl fixative II (Appendix 2A.8.5) was gently dropped onto the oocyte. When the edge of the drying fixative reached the oocyte, another drop of fixative II was added and allowed to dry, and this process was repeated 2-3 times. The slide was dehydrated through an ethanol series, and air-dried. In some cases oocytes and 1st PBs were spread with an alternative protocol that used three fixative solutions instead of two. More specifically, initially oocytes were transferred to 0.9% sodium citrate in a culture dish and were left in this hypotonic solution for 10-15 minutes at room temperature. The oocytes were transferred very gently to the bottom of another culture dish, containing fixative solution I (Appendix 2A.8.6). This fixative gradually dissolved the zona pellucida. After about 2-3 minutes the oocytes showed signs of movement, indicating that this fixation step was complete. The oocytes were transferred to the centre of cleaned slides that were marked with a diamond pen. The oocytes were re-fixed by expelling a drop of fixative solution II (Appendix 2A.1.6) on top of them. The slides were then immediately immersed into a coplin jar containing fixative solution II and were incubated for 5 minutes. The slides were moved to another coplin jar containing fixative solution III (Appendix 2A.8.7), and were further incubated for 1 minute. This last step enabled the softening of the cytoplasm. Each slide was pulled out from the coplin jar very slowly and was wiped at the back. This protocol was suggested by Kamiguchi *et al.* (1993). Some oocyte groups were subsequently stained with either DAPI or Giemsa to check for the presence, the location and the morphology of the chromosomes. Other oocyte preparations were not stained at all and were identified under the phase microscope. Initially some oocytes were also spread on charged slides and in some cases the zona pellucida was removed with the use of Acid Tyrode's solution, prior to spreading. The slides were aged at room temperature for up to a month and then stored either at 4°C or at -20°C prior to FISH

2.2.4 Probe preparation

2.2.4.1 Bacterial culture

Repetitive sequence probes used mainly to examine the chromosome complement of unfertilised oocytes were isolated from *E. coli* strains carrying plasmid or cosmid vectors containing the DNA insert of interest. DNA was extracted with the application of maxiprep techniques. Bacterial culture started by inoculating *E.coli* cells into small cultures of 4ml of 2xTY medium (Appendix 1A.5), also containing 4µl of ampicillin (100mg/ml stock) or other antibiotic for which the bacterial strains were resistant. The cultures were left overnight in a shaking incubator at 37°C. The following day these small cultures were re-inoculated into larger cultures, containing 200ml of 2xTY medium, plus 200µl of ampicillin, and were again incubated overnight as above.

2.2.4.2 Maxiprep extraction of plasmid/cosmid DNA

The DNA was extracted using a commercial maxiprep kit (Wizard; Promega, USA). The procedure was the following: Firstly the cells were pelleted by centrifugation at 4,000 rpm for 10 minutes. The supernatant was discarded and the cells resuspended into 15ml of Cell Resuspension Solution (Appendix 2A.2.1). Subsequently, 15ml of Cell Lysis Solution (Appendix 2A.2.2) were added and mixed gently but thoroughly, by stirring or inverting. When the cellular mixture became clear, 15ml of Neutralisation Solution (Appendix 2A.2.3) were added, and immediately mixed by gently inverting the centrifuge bottles several times. The suspensions were centrifuged at 9,000rpm for 15 minutes at 22-25°C in a room temperature rotor. The supernatants were filtered through blotting paper and transferred into a 100ml graduated cylinder. After their volumes were measured, the supernatants were transferred to new centrifuge bottles. Half a volume of isopropanol was added to these supernatants, which was mixed by inversion. The suspensions were centrifuged at 9,000rpm for 15 minutes as above. The supernatants were discarded and the DNA pellets resuspended in 2ml TE buffer (Appendix 2A.1.4). The DNA purification was achieved with the use of the Wizard resin and vacuum pump. Finally, the DNA concentrations were measured by a fluorometer, as described in 2.2.4.5 below and were then stored at -20°C.

2.2.4.3 Yeast culture

The YAC probe for chromosome 19 (probe details in Table 2.2) was prepared by extracting total genomic DNA from yeast strains (*S.cerevisiae*). Initially six different yeast liquid cultures were set up from three frozen glycerol stocks of *S.cerevisiae* cells (Appendix 1A.7). A sterile needle was used to scrape some of the frozen stock and inoculate it into 10ml of S.D. medium (Appendix 1A.4), supplemented with 5μl ampicillin (100mg/ml, stock). The liquid cultures were incubated in a shaker at 30°C for three days.

2.2.4.4 Miniprep extraction of yeast DNA

The DNA extraction took place with the use of the "Nucleon extraction and purification kit" for yeast DNA extraction (Amersham). The protocol was as follows. Each of the 10ml cultures was divided into 1.5ml microcentrifuge tubes, which were then spun for 30 seconds at 13,000rpm. The supernatant was discarded and as much medium as possible was removed. The cells were completely resuspended by mixing in 540μl of Solution A. When each of the pellets was completely diluted, 60μl of Solution B were added and mixed thoroughly by rapid inversion. This mixture was incubated at 70°C for 10 minutes. 300μl of Solution C were added into each of the microcentrifuge tubes, which were mixed by shaking and were left to cool into ice for 3-5 minutes. The samples were centrifuged at 13,000rpm for 5 minutes. The supernatant was collected and 600μl of isopropanol were added to precipitate the DNA. Again the samples were centrifuged at 13,000 rpm for 5 minutes, the supernatant was discarded, and 200μl of cold 70% ethanol were added. The samples were again centrifuged at 13,000rpm for 5 minutes, and each tube was drained and allowed to dry by placing them at 70°C for 3 minutes. Finally, 50μl of Solution D were added and the tubes were placed at 70°C. The samples were mixed periodically, until the DNA was completely resuspended. These DNAs were stored at 4°C.

2.2.4.5 DNA reprecipitation

The miniprep procedure described above gave DNA yields of about 7μg/ml of original yeast culture. Thus, these DNAs were reprecipitated to increase the final concentration. The procedure was as follows. One tenth of the final volume, i.e. 5μl of 3M sodium acetate were added into each of the different samples, along with two volumes, i.e. 110μl of 100% ice-cold ethanol. The samples were either left overnight at -20°C or at -80°C for 1 hour. They were centrifuged at 13,000rpm for 20 minutes, the supernatant was removed and the pellets were left to dry at room temperature. They were resuspended in 1xTE (Appendix 2A.1.4) (50μl or less). The DNA concentrations were measured with the use of a flurometer, and varied between 350-565ng/μl. Each of the samples was stored at 4°C.

2.2.4.6 Fluorometry

Concentrations of plasmid, cosmid and yeast DNA were measured with the use of a Hoefer Scientific Instruments TKO fluorometer. Initially, the fluorometer was calibrated with a solution containing an intercalating DNA dye (0.1μg/ml Hoechst 33258 dye in 1xTNE buffer). The standard used was 100ng/ml calf thymus DNA. Readings obtained for the concentrations of the DNA samples were converted to ng/μl.

2.2.4.7 *Alu*-PCR

Alu-PCR was performed to amplify specifically the human sequences in the YAC DNA, in order to generate probes for the PGD case A. The *Alu*-PCR was performed as described by Romana *et al.*, (1993), with some modifications. The amplification was carried out with the use of two *Alu* primers: ALU1F and ALU1R (both Appendix 2A.3.3). Each were used in the PCR assay, along with 100ng DNA, 5μl of 10x PCR buffer (Appendix 2A.3.1), 2mM dNTPs (Appendix 2A.3.2), 0.125μl Super Taq enzyme and water to a volume of 50μl.

Two different sets of cycles were used for the amplification and these were the following: Initial denaturation at 95°C for 5 minutes, and then 30 cycles of PCR with denaturation at 95°C for 1 minute, annealing at 65°C for 1 minute, and extension at

72°C for 4 minutes. At the end of the last cycle a 10-minute extension took place at 72°C. The second set of PCR cycles involved an initial denaturation at 96°C for 1 minute, and then 30 cycles of PCR with denaturation at 96°C for 1 minute, annealing at 40°C for 30 seconds, and extension at 72°C for 6 minutes. At the end of the last cycle a 10-minute extension at 72°C took place. PCR products were analysed on 2% agarose gels, as described below.

2.2.4.8 Agarose gel electrophoresis

Products that were amplified via *Alu*-PCR were analysed on agarose gels, in order for the PCR efficiency to be assessed. These gels were made as follows: 2% agarose was melted in 1xTBE buffer (Appendix 2A.1.3) and 1μg/ml ethidium bromide was also added to enable visualisation of the gels. The melted agarose was poured into an electrophoresis tank, containing gel-slot formers and left to dry at room temperature. In the meantime, the DNA samples were prepared for the analysis, by mixing 10μl of sample with 1μl of loading buffer. When the agarose gel had set, it was immersed into 1xTBE buffer and the DNA samples were also loaded into the formed wells. Electrophoresis took place at 100V for 30 minutes. Visualisation of the gel occurred via ultra-violet trans-illumination.

2.2.4.9 Labelling of the FISH probes

FISH probes that were prepared in the lab acquired their fluorescent label via nick translation. The latter involves the introduction of random nicks in the DNA with the use of a DNAase I, specific for this process. These nicks act as priming sites for the synthesis of new DNA, that is enabled by DNA polymerase I. The labelled nucleotides are incorporated as the old strand is being degraded. A commercial kit was used for this purpose (Nick translation kit Vysis/Abbott, UK). This kit enables the substitution of half the amount of the dTTP with spectrum green or spectrum orange-labelled dUTP. The latter dilutes the label incorporation, and increases the DNA polymerase I efficiency. The procedure permits the incorporation of about 20% of the fluorescent-labelled nucleotide into the DNA, generating in this way a clear bright signal during hybridisation. Ethanol precipitation removes the unincorporated nucleotides.

2.2.4.9.1 Preparation of the nick translation kit

Initially spectrum green dUTP (Appendix 2A.5.1) 50nmol and spectrum orange dUTP (Appendix 2A.5.1) 50nmol concentrations were prepared at 0.2mM by adding 10μl of 0.1mM dUTP to 40μl nuclease-free water. dTTP was prepared in a concentration of 0.1mM with the addition of 10μl of 0.3mM dTTP (Appendix 2A.5.1) to 20μl nuclease-free water. A concentration of 0.1mM dNTP mix was achieved by mixing together 10μl of each of 0.3mM dATP, 0.3mM dCTP, 0.3mM dGTP (Appendix 2A.5.1).

In order to make sure that the added DNA was 1μg in a 50μl reaction mix, its volume had to be calculated in relation to the concentration of the DNA. The volume of nuclease-free water that was added was calculated using the formula 17.5-x, where x is the DNA volume.

2.2.4.9.2 Nick translation reaction

The components added in a microcentrifuge tube in order to begin the nick translation reaction were the following: 17.5-x nuclease-free water mixed with 1μg of DNA, 2.5μl of 0.2mM spectrum green or spectrum orange, 5μl of 0.1mM dTTP, 10μl of dNTP mix, 5μl of 10x nick translation buffer (Appendix 2A.5.2) and 10μl of nick translation enzyme (Appendix 2A.5.3).

The micocentrifuge tubes were briefly centrifuged and vortexed to mix the reaction components and incubated for 2 hours at 16°C. This temperature was crucial for probe efficiency and the success of the labelling method.

The nick translation reaction was stopped with the addition of 5μl of 0.5mM EDTA pH 8 (BDH, UK).

2.2.4.9.3 Probe preparation after labelling

Probe precipitation took place once the nick translation reaction was stopped. Different components were added in the microcentrifuge tubes, depending whether the labelled probes were locus-specific or hybridised onto the heterochromatic regions of chromosomes.

Thus, the following reagents were added for locus-specific probes: 100μl of human COT-1 DNA (Sigma, UK), 5μl herring sperm DNA (Sigma, UK), 16μl of 3M sodium

acetate and 1ml of 100% of ice-cold ethanol. For repetitive sequence probes the following components were mixed: 5µl of herring sperm DNA, 16µl of 3M sodium acetate, and 1ml of 100% ice-cold ethanol.

Precipitation took place by leaving the probes at -80°C for 1 hour. Once this period of time had passed, the probes were centrifuged for 10 minutes at 13,000rpm. The supernatant was discarded, and the resulting pellets were air-dried by leaving the tubes open in the dark. Finally, the repetitive sequence probes were resuspended in 100µl of hybridisation buffer (Appendix 2A.6.6), whereas the locus-specific probes were resuspended in 20µl of COSMIX buffer (Appendix 2.A.6.5).

2.2.5 FISH for PGD

2.2.5.1 Slide pretreatment

When all slides were prepared (control lymphocytes, blastomeres and embryos), they were incubated in 1N HCl and pepsin (10mg/ml, Sigma, UK) (Appendix 2A.6.1) at 37°C for 20 minutes. This incubation enabled the removal of the remaining protein from the cells, making in this way the DNA accessible to the probes. When the incubation period finished, the slides were washed briefly in double-distilled de-ionised water and PBS. Subsequently the slides were fixed by a 10-minute incubation in 1% paraformaldehyde (Sigma, UK) (Appendix 2A.6.2), in PBS at 4°C. Another wash in PBS and two washes in double-distilled de-ionised water followed, and the slides were dehydrated through an ethanol series and left to dry. All the above were carried out in 50ml volume coplin jars, unless otherwise stated. This method was described previously by Harper *et al.* (1994).

2.2.5.2 Probe preparation

The hybridisation mix for single-colour FISH consisted of the probes, different hybridisation buffers depending on the probes used and sometimes different volumes of water. The total volume of hybridisation mix used was in most cases 5µl. The hybridisation buffers used for commercial probes were the LSI, and CEP buffers (Vysis/Abbott, UK), and Hybridisation solution (Oncor, UK). The hybridisation buffer used for the YAC probes was the lab-prepared COSMIX buffer, whereas the

band-specific probe for chromosome 21 came already mixed with hybridisation solution.

The hybridisation mixes for the combination of different probes were made to a total volume of 5-6.5μl. Thus, the amounts of buffer and water were divided to accommodate the volume of each of the probes.

When blastomeres were tested during a PGD case, the hybridisation mix volume was always 3μl. Similarly with above, the amounts of probes and buffer were converted accordingly.

2.2.5.3 Denaturation and hybridisation

The probes and slides were denatured separately for all the PGD cases. More specifically, denaturation of nuclear DNA took place by covering the slides in denaturation mix (70% formamide in 2xSSC) and then placing them in an incubator set between 73-75°C for 5 minutes. Coverslips were removed and the slides immersed into ice-cold 70% ethanol for 5 minutes. Subsequently, slides were dehydrated through an ethanol series and left to dry, preserving the single-stranded DNA conformation. Locus-specific probes were mixed, denatured in an incubator set between 73-75°C for 5 minutes and then left to pre-anneal at 37°C with the COT1 DNA for a period of 30-60 minutes. In the case that the probe mix contained repetitive sequence probes as well, the latter were denatured in the same way in a separate microcentrifuge tube, but without the pre-annealing step. Once the denaturation was complete they were placed on ice. Probes were mixed prior to them being placed on the slides, under a 13mm coverlsip. Slides and probes were left to hybridise in a moist chamber at 37°C. Hybridisation varied according to the probes used. Thus, repetitive sequence probes required 45 minutes to 1 hour, whereas locus-specific probes required 16 hours on average. Combinations of locus-specific and repetitive sequence probes were left to hybridise overnight. In such cases, the coverslips were sealed with rubber cement to prevent probe evaporation.

2.2.5.4 Post-hybridisation procedures

The washes following the hybridisation of the probes controlled the stringency with which the latter bound onto the DNA. This was achieved by the adjustment of formamide and salt concentrations and also the temperature at which the post-

hybridisation washes took place. The stringency conditions were dependent on the probe type, i.e. whether these were locus-specific or centromeric. The post-hybridisation washes were all carried out in the dark to avoid possible bleaching of the fluorochromes. The coplin jars used were 50ml in volume and the washes that used formamide took place in a laminar flow cabinet. Once the hybridisation period was complete, any rubber cement was removed and the coverslips gently floated off by immersing the slides in the first wash solution for a brief period. When the probe mix included locus-specific probes, slides were washed at 45°C for 3x3 minutes in 50% formamide in 2xSSC and then 3x3 minutes in 2xSSC, with two subsequent 5 minute washes in 4xSSC/0.05% Tween at room temperature. In the case of repetitive sequence probes, there was an increase of formamide concentration to 60%, whereas the rest of the washes remained the same. The slides were finally dehydrated through an ethanol series and were left to air-dry.

A different set of washes was carried out for the PGD case E. Locus-specific probes were used for the diagnosis. The slides were initially washed at 46°C for 3x10 minutes in 50% formamide in 2xSSC, once for 10 minutes in 2xSSC, pH5.3 and then once for 5 minutes in 2xSSC/0.1% NP 40. They were left to dry in the dark.

The slides were mounted in Vectorshield antifade medium containing 1.25ng/ml 4',6-diaminidino 2 phenylindole (DAPI) counterstain and stored covered at 4°C.

2.2.5.5 Re-probing of slides

Embryos that were considered abnormal after PGD were not transferred and were analysed with the same probe mix to confirm the diagnosis. Re-analysis with other probes was often performed to further investigate chromosome status for these embryos. Re-probing of these embryos took place in the following way: The slides were immersed in a coplin jar containing 4xSSC/0.05% Tween and the coverslips were removed. Two 5-minute washes in 4xSSC/0.05% Tween and one 10 minute wash in PBS, all taking place at room temperature, followed. During these washes the slides were exposed to light, so the old probe mix faded. The slides were then dehydrated through an ethanol series and air-dried. The new probe mix was placed and the slides were denatured as before. The rest of the FISH steps remained as previously.

2.2.5.6 Scoring criteria for embryos

Embryo analysis took place with the use of a fluorescent microscope. The guidelines suggested by Hopman *et al.* (1988) were followed for signal scoring in embryonic interphase nuclei. Specifically, two signals closer than a signal diameter apart, were considered a single split signal. Two signals that were further apart than a signal diameter were considered as two separate signals.

2.2.6 FISH for metaphase II oocytes and 1st PBs

2.2.6.1 Slide pre-treatment

Material loss was experienced during the sequential rounds of FISH. To reduce the latter, the slides were handled in different ways prior to FISH. These included, an overnight incubation at 37°C or 65°C, or a combination of both and in all cases their ageing at room temperature for up to a month. Their pre-treatment, in order to make the chromosomes accessible to the probes was similar to that described in 2.2.5.1 with the following modifications. Incubation in 1N HCL and pepsin at 37°C took place for only 5 minutes, instead of the standard 20 minutes. It was thought that the longer the slides were left in pepsin the more likely it was for oocyte chromosomes to be lost afterwards. The remaining of the pre-treatment was exactly the same as for the lymphocytes and the blastomeres.

2.2.6.2 Probe preparation

Depending on whether the oocyte and PB chromosomes remained on the slides, a two- or three- stage FISH was carried out. Chromosomes were initially hybridised with repetitive sequence probes (Table 2.5), whereas the second and third rounds were carried out with the use of locus-specific probes The volume of the hybridisation mix applied on the slides was 4μl. The LSI hybridisation buffer (Vysis/Abbott, UK) was used in combination with locus-specific probes for chromosomes 13, 21 and 22. The latter were all commercial (Vysis/Abbott, UK, Table 2.5). Probe combinations applied during the three subsequent rounds of FISH are shown in Table 2.6.

Table 2.6: Probe combinations during the three sequential FISH rounds applied for oocyte analysis.

FISH Round	Probe combination
1st	• Satellite III for chromosome 1- SO Alpha-satellite for chromosome 12- SO+SG Alpha-satellite for chromosome X- SG • Alpha satellite for chromosome 12- SO+SG CEP 16 (Satellite II) for chromosome 16- SA Alpha-satellite for chromosome 18- SG • Alpha-satellite for chromosome 4- SG Alpha-satellite for chromosome 17- SO • Alpha-satellite for chromosome 4- SG or SO Alpha-satellite for chromosome 12- SO+SG Alpha-satellite for chromosome 17- SG or SO
2nd	• Satellite III for chromosome 1- SG CEP 16 (Satellite II) for chromosome 16- SA • LSI 13 locus-specific probe for chromosome 13- SG LSI 21 locus specific probe for chromosome 21- SO
3rd	• LSI 22 locus specific probe for chromosome 22- SG

SA- Spectrum aqua

SG- Spectrum green

SO- Spectrum orange- appeared as red

2.2.6.3 Denaturation and hybridisation

Oocytes and controls were denatured simultaneously. Thus, the probes were mixed in a microcentrifuge tube and placed on 13mm coverslips, onto which the oocytes were inverted. Denaturation took place in an incubator at 75°C, whereas the time the slides and probes were left varied between 3 and 5 minutes, depending on the probe set used for analysis. Hybridisation occurred at 37°C. Locus-specific probes were left to hybridise overnight. Repetitive sequence probes were left to hybridise either for 1-2 hours or overnight, according to resulting signal intensities, and as detailed in Mahmood *et al.*, 2000. In the case of overnight hybridisation, the coverslips were sealed with rubber cement to prevent probe evaporation.

2.2.6.4 Post-hybridisation procedures

The washes following the hybridisation of the probes controlled the stringency with which the latter bound onto the DNA, as mentioned in 2.2.5.4. Similarly with the FISH for the PGD cases, the post-hybridisation washes for the oocyte FISH were all carried out in the dark to avoid possible bleaching of the fluorochromes. The coplin jars used were of 50ml in volume and the washes that used formamide took place in a laminar flow cabinet. Once the required hybridisation period had passed, the coverslips were removed as described in 2.2.5.4. For locus-specific probes, slides were washed at 45°C once for 5 minutes in 50% formamide in 2xSSC and then once for 5 minutes in 2xSSC, with two subsequent 5 minute washes in 4xSSC/0.05% Tween at room temperature. For repetitive sequence probes, the formamide concentration increased to 60%, with the remaining washes staying as described above. The slides were finally dehydrated through an ethanol series, were left to air-dry in the dark and were mounted in Vectorshield antifade medium containing 1.25ng/ml 4',6-diaminidino 2 phenylindole (DAPI) counterstain. They were stored covered at 4°C.

2.2.6.5 Re-probing of slides

Analysis of the different oocyte and PB chromosomes was achieved in three sequential rounds of FISH. Re-probing of oocyte slides occurred as described in

2.2.5.5 (re-probing of embryo slides). Similar to the embryo slides, no further treatment or fixation took place on the oocytes that were spread on these slides. It was not possible to successfully re-FISH all of the oocytes and PBs due to some chromosome loss that was experienced during their processing.

2.2.6.6 Scoring criteria for oocytes and PBs

Analysis of oocytes was achieved with the use of a fluorescent microscope (Olympus BX 40). The guidelines suggested by Cupisti *et al.* (2003) were followed for signal scoring. More specifically, the morphology of oocyte and PB chromosomes varied, with the oocyte chromosomes being more distinct, and the PB chromosomes being more compact, less well spread and beginning to degenerate. It was not possible to confuse sperm and PB chromosomes, as the former tend to be very extended, whereas the sperm head itself had a typical compact shape.

Each oocyte and PB chromosome consists of two chromatids joined together on the centromere. Thus, repetitive sequence probes, which hybridised onto centromeric regions, were visualised either as one large signal or a doublet if chromatids were close to each other. Premature separation of chromatids would result to two distinct signals for the repetitive sequence probes. The locus-specific probes used in this study hybridised onto long arm regions of chromosomes 13, 21, and 22. Thus, they always demonstrated some degree of separation.

During this study, only the presence of extra signals indicating the gain of either a whole chromosome or a chromatid was scored. Absence of signals was not considered as a true abnormality, as it could be influenced by the artifactual loss of chromosomes that could have taken place either during the spreading of the cells or the FISH procedure.

2.2.7 CGH of human meiosis II oocytes and corresponding 1st PBs

2.2.7.1 DNA extraction from blood

DNA extraction from whole blood was carried out according to a protocol suggested by Lahiri *et al.* (1991). The blood was initially collected in tubes containing 15% EDTA. The procedure was as follows: 5ml of whole blood were placed in centrifuge

tubes and 5ml of low salt solution (TKM1, Appendix 2A.9.1), and 125μl of Nonident-40 (NP40, Sigma) were also added, in order for the cells to be lysed. The contents of the tubes were mixed well by inversion and were spun at 1000 rpm for 10 minutes in a Centaur™ centrifuge. The resulting supernatant was discarded and each of the pellets washed in 5ml of TKM1. NP40 was added again and another centrifugation followed, similarly with above. The TKM1 and NP40 washes were repeated for three more times, or until the pellet became white, something which indicated that all the red blood cells were removed.

The pellets were resuspended in 50μl of TKM1. Lysis of the white blood cells was achieved by addition of 800μl of high salt solution (TKM2, Appendix 2A.9.2) and 50μl of 10% (w/v) SDS. After mixing thoroughly, the suspension was incubated at 55°C, with occasional agitation, for a minimum of 30 minutes, or until it became clear. Once the latter took place, 300μl of 6M NaCl were added into each of the tubes, and the suspensions were mixed thoroughly so as to ensure that all cells had lysed.

A centrifugation followed at 10,000 rpm for 5 minutes and the resulting supernatants were transferred to new tubes, into which 2 volumes of ice-cold 100% ethanol were added. Inversion of each of the tubes was carried out for several times until the DNA had precipitated. The DNA strands were transferred to microcentrifuge tubes that contained 1ml of ice-cold 70% ethanol with the use of sterile inoculation loops. Another centrifugation at 10,000 rpm for 5 minutes took place, the supernatants were removed and the DNA pellets were air-dried. Each of the pellets was dissolved in an appropriate volume of TE solution (Appendix 2A.1.4) and stored at 4°C.

2.2.7.2 DNA extraction from skin fibroblasts

DNA from skin fibroblast cultures carrying trisomies for different chromosomes (e.g. trisomy 18, trisomy 22) was used during initial positive control experiments to demonstrate that the CGH technique was able to detect these trisomies, during analysis and interpretation of the captured images. The extraction procedure started by removing the cell culture medium and washing the cells with Hank's balanced salt solution, pre-warmed at 37°C. This solution was discarded after a few seconds and 5ml of Versene, containing 2.5% trypsin (Appendix 1A.3) were added into the tissue culture flasks, which were then incubated at 37°C for 5 minutes. The Versene/trypsin

solution was also pre-warmed at 37°C. Incubation of the cells at 37°C resulted in their detachment from the flasks, something, which was confirmed by observing the cells under an inverted microscope. The trypsin was inactivated with the addition of a few drops of fetal calf serum. The cell suspensions were placed into centrifuge tubes and were spun at 1000 rpm for 5 minutes. Most of the supernatant was removed and a small amount was left in order for the pellets to be resuspended in it. Addition of 2ml of sterile PBS followed and the cells were again centrifuged at 1000 rpm for 5 minutes. The cells were lysed with the addition of 2.5ml of lysis buffer (Appendix 2A.10.1) and a 30 minute incubation at 37°C. After this period of time had passed, equal volumes of isopropanol were added into the tubes, in order for the DNA to be precipitated. Similar to the extraction of DNA from whole blood, the DNA was transferred into microcentrifuge tubes with the use of sterile inoculation loops, which were spun at 10,000 rpm for 5 minutes. Removal of the supernatants followed and the DNA pellets were left to dry at room temperature. They were finally resuspended into appropriate volumes of TE and stored at 4°C.

2.2.7.3 Single cell collection

2.2.7.3.1 Buccal cells

Buccal cells were collected from both male and female individuals with a normal karyotype (46,XY or 46,XX) by gently scraping the inside of the cheek with a sterile mouth swab. These cells served as the reference DNA against which the test DNA was hybridised. The buccal cells were transferred into 1.5ml of sterile PBS containing 1% polyvinyl alcohol (PVA).

2.2.7.3.2 Skin fibroblasts

Single fibroblasts from cell cultures that were carrying trisomies for different chromosomes were used in initial positive control experiments for the same purpose as the DNA. The collection of these cells took place in the following way. Initially, the culture medium and any dead cells were removed from the tissue culture flasks. Live cells that were attached to the flasks were washed for a few seconds with Hank's balanced salt solution that was pre-warmed at 37°C. The latter was then removed, and the cells covered with Versene, containing 2.5% trypsin and incubated at 37°C for 5 minutes. The Versene/trypsin solution was also pre-warmed at 37°C. Incubation of the

cells at 37°C resulted in their detachment from the flasks. Once the latter was achieved the cells were removed and spun at 3,000 rpm for 5 minutes.
The supernatants were discarded and the pellets were resuspended in 2-5ml PBS containing 1% PVA.

2.2.7.3.3 Human oocytes and PBs

The meiosis II oocytes and corresponding first PBs were separated either with the use of acid Tyrodes solution or with pronase (Appendix 2A.7.1).
The acid Tyrode's solution was used for the first two groups of oocytes and PBs. The procedure was as follows. The oocyte was placed in a drop of acid Tyrode's solution in a small petri dish. The oocyte was observed continuously under a dissecting microscope. Gentle aspiration with a mouth pipette also took place in order to enable the lysis of the zona pellucida. Once the latter was achieved, both oocyte and corresponding PB were placed in different drops of PBS/PVA.
The pronase separation was applied for the third group of oocytes and PBs. Similar to above, the oocyte was placed in a drop of pronase in a small petri dish. The petri dish was covered, and put in an incubator set at 37°C for 5-10 minutes. Once this period of time had passed, the petri dish was removed from the incubator and the oocyte observed under a dissecting microscope. If the zona pellucida had disappeared completely the oocyte and PB were recovered and placed in different drops of PBS/PVA. If the zona was still there, the oocyte was gently aspirated and released using a mouth pipette until the zona lysed. Both cells were again transferred in different drops of PBS/PVA.

2.2.7.4 Isolation and lysis of clumps or single cells

Once the cells were collected in PBS/PVA, an aliquot was placed on a petri dish and observed under a dissecting microscope. Another petri dish containing 10μl droplets of PBS/PVA was also prepared and the cells from the initial aliquot were passed through at least three different droplets of PBS/PVA. In this way, either clumps (3-5 cells) or single cells were isolated and washed. In the case of buccal cells that during the CGH were used as the reference DNA with which the test DNA was compared only clumps were isolated, in order to ensure that there would be at least one cell that would yield hybridisation results. Fibroblasts were isolated either as single cells or in

clumps, whereas all oocytes and corresponding PBs were washed individually in four droplets of PBS/PVA.

Once the above procedure of isolation and washing was complete, the buccal cells and fibroblasts were transferred individually to microcentrifuge tubes containing 2μl of proteinase K (PK, 125μg/ml) and 1μl of SDS (17μM) and overlaid with oil. Lysis of the cells followed, to allow their DNA to be released. This was achieved by incubation at 37°C for 1 hour. After this, the PK was inactivated by another incubation at 95°C for 15 minutes. All cells were stored at -80°C until they were used.

Three differently prepared groups of oocytes and corresponding PBs were investigated with CGH during this study. All of them were lysed on the day that the whole genome amplification reaction was set up. More specifically, the cells of the first group were placed in microcentrifuge tubes containing 100μl of sterile PBS and were stored at -80°C. In this case, when they were to be tested, they were first centrifuged at 6,000 rpm for 10 minutes, 98μl of the PBS removed, and 2μl of PK and 1μl of SDS (lysis mixture) were added to the tubes. They were covered with oil and lysed as described above. For the second group of oocytes and PBs, all cells were placed in microcentrifuge tubes, suspended in 2μl of sterile PBS, and they were stored at -80°C. In this way, the initial centrifugation at 6,000 rpm was avoided. Lysis mixture was added to each of the tubes, which were then covered with oil and lysed. Similarly, in the third group, all cells were suspended in 2μl of sterile PBS but were also overlaid with oil, prior to their storage at -80°C. They were lysed as above.

2.2.7.5 Degenerate Oligonucleotide Primed PCR

The Degenerate Oligonucleotide Primed (DOP) PCR was applied for the whole genome amplification of the buccal cells, fibroblasts, oocytes, and PBs and for the genomic and fibroblast DNAs for all the CGH experiments. The protocol was carried out as suggested by Wells *et al.* (1999) with some modifications. Thus, the reactions were performed in a total volume of 50μl and consisted of the following reagents: 10x SuperTaq Plus buffer (Appendix 2A.4.1); 10mM dNTPs (Appendix 2A.4.2); 83μM degenerate oligonucleotide primer (CCGACTCGANNNNNNATGTGG) (Appendix 2A.4.3); 2.5 U SuperTaq Plus polymerase and nuclease-free H_2O.

Thermal cycling was carried out as follows: 94°C for 4.5 minutes; 10 cycles of 95°C for 30 seconds, 30°C for 1 minute, a 1°C/second ramp to 68°C, and 68°C for 3 minutes; 40 cycles of 95°C for 30 seconds, 56°C for 1minute, and 68°C for 3 minutes; and finally, 68°C for 8 minutes. Once the amplification was complete 5-10μl of each of the samples were taken and stored at 4°C for further analysis. The DOP-PCR was carried out in an Omnigene thermal cycler.

Strict precautions were taken against contamination, as suggested by Wells and Sherlock (1998) and Wells *et al.* (2002). More specifically, all equipment and reagents used to set up the DOP-PCRs were reserved only for single cell use. In addition, all reactions were set up in a room that was designated for single cell work only, was separate from the main laboratory and was kept under constant positive pressure, to avoid the entry of dust and amplified DNA products. All reagents, equipment, gowns, gloves and overshoes remained in this room.

The possible incidence of contamination was investigated by the presence of negative control tubes, which contained all DOP reagents apart from DNA. These were subjected to the entire DOP-PCR and the remaining of the CGH protocol. Absence of DNA in these tubes was demonstrated as absence of fluorescence during the analysis of the samples.

2.2.7.6 Agarose gel electrophoresis

DOP-PCR amplification and efficiency were assessed by analysing the DOP products on 1% agarose gels. These gels were prepared by mixing 1% agarose in 1xTBE. Ethidium bromide (1μg/ml) was also added, so as for the gels to be visualised under UV illumination. As far as sample preparation was concerned, 5μl of each of the DOP products was mixed with 1μl loading buffer. The remaining of the procedure was as described in 2.2.4.8.

2.2.7.7 Amplified product precipitation

All DOP-PCR products were precipitated prior to their fluorescent labelling. This was achieved by mixing the 40-45μl of the samples with 4.5μl of 3M Sodium Acetate (Sigma) and 125 of ice-cold 100% ethanol. They were then placed at -80°C for 24 hours. Once this period of time had passed, the samples were centrifuged at 13,000

rpm for 30 minutes, their supernatants were discarded and their pellets were left to dry at room temperature for 5 minutes.

2.2.7.8 Labelling of the amplified products.

A nick translation reaction was applied in order for the DOP PCR products to be fluorescently labelled. The reaction took place with the use of a commercial kit (Nick translation kit Vysis/ Abbott, UK). The process was similar to the labelling of FISH probes (described in 2.2.4.9) with some modifications to accommodate for the nature of the CGH probes. More specifically, and for all experiments, the test DNA (i.e. fibroblasts, oocytes and PBs) was labelled in spectrum green (SG), whereas the reference DNA (buccal cell clumps, genomic) was labelled in spectrum red (SR). The preparation of the nick translation kit is described in 2.2.4.9.1, whereas the nick translation reaction is described in 2.2.4.9.2. The modifications were the following: all DOP-PCR product pellets were resuspended in 17.5μl of nuclease-free H_2O, and the nick translation reaction took place at 15°C for up to 2 hours. The incubation period at 15°C was directly associated with the size of the labelled DNA fragments. The desired sizes ranged between 500bp-1Kb, and were assessed by agarose gel electrophoresis of a 10μl sample from the labelled products. The reaction was stopped by incubating all samples in a water-bath set at 70°C for 10 minutes. Test and control DNAs were mixed and then 30μg of COT 1 DNA, 13μl of 3M Sodium Acetate, and 360μl of ice-cold 100% ethanol were also added. The samples were left to precipitate at -80°C. The duration of the precipitation varied between 2-24 hours. Similar with above and after this period was complete, all samples were centrifuged at 13,000 rpm for 40 minutes, their supernatants were removed, and the pellets were covered and left to dry at room temperature for 5 minutes.

2.2.7.9 Comparative Genomic Hybridisation

2.2.7.9.1 Slide and probe preparation

The fluorescent DNA pellets were resuspended in 6μl of COSMIX buffer and were left to dissolve for 20 minutes in a water-bath set at 37°C. In the meantime, the slides that were used as templates onto which the CGH probes were to be hybridised were prepared. These were normal 46,XY metaphase slides and were provided by Vysis/Abbott, UK. The slides were observed under a phase microscope and

hybridisation areas were marked, using a diamond pen. They were dehydrated by passing them through an ethanol series (70%, 90%, and 100%, 3 minutes in each), and were left to air-dry. They were then placed onto a hot-plate set at 42-45°C.

2.2.7.9.2 Denaturation and hybridisation

Denaturation of the slides was carried out by placing them in a 50ml coplin jar containing 70% formamide, 2xSSC in a water-bath set at 75°C, for 5 minutes. The denaturation process was stopped by immersing the slides in an ice-cold ethanol series (70%, 85% and 100%) and incubating them 3 minutes in each. The slides were left to air-dry and were again placed on the hot plate as above.

Denaturation of the probes was performed in an incubator set at 75°C and it lasted for 10 minutes. After this period of time had passed, the probes were left covered at room temperature for 2 minutes. They were transferred on 22mm round glass coverslips, placed over the marked areas on the slides and sealed with rubber cement. Probes and slides were placed in a humidified chamber, and were left to hybridise in an incubator set at 37°C for 72 hours.

2.2.7.9.3 Post-hybridisation procedures

Similar to the post-hybridisation washes during FISH, stringency conditions were critical for CGH. In this case formamide was avoided, while the slides were sequentially washed in decreasing temperatures. The protocol followed was described in Wells *et al.* (2002). All washes were carried out in 50ml coplin jars and in the dark to avoid possible bleaching of the fluorochromes. Thus, after the hybridisation period had passed, the rubber cement was removed from the slides, which were then immersed briefly in a coplin jar containing 2x SSC at 73°C. Coverlsips were gently floated off and the slides incubated in this solution for 5 minutes. Three 5 minute washes at 37°C followed, the first and third in 4x SSC, while the second was in 4x SSC+ 0.1% Triton-X. The slides were finally washed in 2x SSC at room temperature, for another 5 minutes, they were briefly immersed in double-distilled deionised H_2O, and were dehydrated through an ethanol series as before. Once they were dry, they were mounted in DAPI/Vectorshield and stored at 4°C until they were observed. Image analysis and interpretation are described in 2.1.5.5.

3.1 Development of a FISH protocol for the PGD of chromosomal abnormalities

FISH protocols were devised for seven couples, and three different types of chromosomal abnormalities. Patient details are shown in 2.1.3.1 and Table 2.1. The abnormalities investigated included three different reciprocal translocations, two different Robertsonian translocations, and two cases of possible gonadal mosaicism for a trisomic cell line. Maternal age varied between 25-39 years (average age 32.6 years). The reproductive histories of these patients are shown individually in Table 2.1.

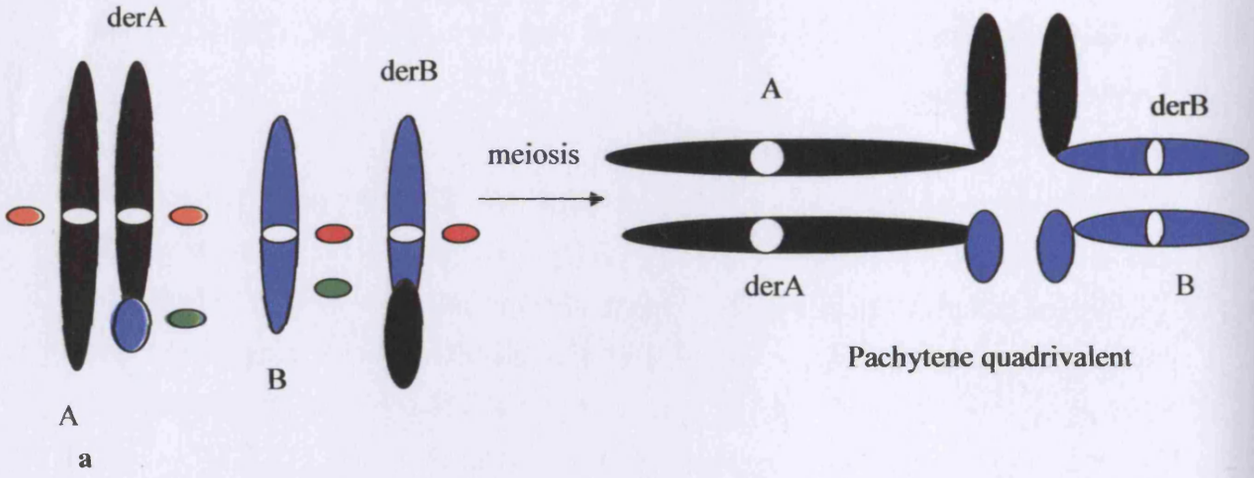
Characterisation of the patient karyotype had already taken place by the clinical cytogenetic centre from which these couples were referred. The development of each individual PGD protocol initially involved the prediction of the possible behaviour of the normal and derivative chromosomes during meiosis. Note was taken of all modes of segregation, depending on the type of chromosomal abnormality. Probe combinations were selected that would distinguish all abnormal chromosome compliments. Each of the chosen probes was tested individually and in combination on control (46, XY) and patient lymphocytes. In this way, confirmation of the binding position of the selected probe combination on the patient metaphases and evaluation of the efficiency of the FISH protocol were both achieved.

The aims of this part of the study were the following:

- To develop robust and reliable FISH protocols for their clinical implementation in the PGD of chromosomal abnormalities.
- To extensively analyse all untransferred embryos so as to investigate the hypothesis that different types of errors both meiotic and mitotic result in the generation of highly mosaic embryos that could also be patient specific.

Description of each of these PGD cases and their outcome will follow.

Fig.3.1: FISH strategy for the PGD of reciprocal translocations



a

b Mode of segregation	Segregating chromosomes	Signals visible	Embryo outcome
2:2 Alternate	A,B Or derA, derB	2, 2, 2	Normal or balanced
2:2 Adjacent-1	A, derB	2, 1, 2	Partial monosomy B
	B, derA	2, 3, 2	Partial trisomy B
2:2 Adjacent-2	A, derA	3, 2, 1	Partial trisomy A
	B, derB	1, 2, 3	Partial monosomy A
3:1 Tertiary aneuploidy	A, B, derA	3, 3, 2	Tertiary trisomy A
	derB	1, 1, 2	Tertiary monosomy A
3:1 Tertiary aneuploidy	A, B, derB	2, 2, 3	Tertiary trisomy B
	derA	2, 2, 1	Tertiary monosomy B
3:1 Interchange aneuploidy	A, derA, derB	3, 2, 2	Interchange trisomy A
	B	1, 2, 2	Interchange monosomy A
3:1 Interchange aneuploidy	B, derA, derB	2, 3, 3	Interchange trisomy B
	A	2, 1, 1	Interchange monosomy B
4:0 Double aneuploidy	A, B, derA, derB	3, 3, 3	Trisomy A, B
	0	1, 1, 1	Monosomy A, B

Fig.3.1: a. Triple-colour FISH strategy developed for the PGD of reciprocal translocations. Out of the three probes, two (red, green) flank the breakpoint on one of the two chromosomes involved in the translocation, and one probe (orange 50:50 red:green) is located on the other chromosome. The homologous segments of the two normal and the two derivative chromosomes pair-up during meiosis and form the pachytene quadrivalent. **b.** The pachytene quadrivalent theoretically segregates in 8 different ways: three 2:2, four 3:1 and one 4:0. These generate 16 different gamete types. The signal patterns are different for each of the different modes of segregation. Identification of all unbalanced segregations is feasible with this probe strategy. The signal pattern is identical for the normal and balanced segregants.

3.2 PGD for three couples with reciprocal translocations

During the first meiotic division in the gametes of balanced reciprocal translocation carriers, the two normal and two derivative chromosomes align with homologous material and form a structure called the pachytene quadrivalent. The FISH strategy employed in our centre for the detection of all possible modes of segregation of this structure was initially developed by Conn and colleagues (1999) and involves the use of two probes that flank the breakpoint on one chromosome and a third probe specific for the other chromosome. The "flanking" probes can hybridise at any distance apart, as long as one is proximal and the other distal to the breakpoint on that chromosome. With the application of this strategy a different combination of signals is achieved for all unbalanced chromosome constitutions and all imbalances due to both 2:2 and 3:1 segregations are detected. However, this strategy cannot distinguish between a normal and a balanced chromosome complement (Fig. 3.1).

FISH protocols were devised for three couples that were referred to our centre because one of the partners was a carrier of a reciprocal translocation. Out of these, two cases reached the biopsy stage, whereas the third couple is was treated after completion of this thesis. The patient karyotypes were the following: 46,XY, t(5;19)(p12;p12), 46,XX, t(11;22)(q23.3;q11.2), and 46,XY, t(14;16)(q13;q11.1). A combination of locus-specific, subtelomeric and centromeric probes was employed for all cases. All probes used were commercial, apart from one locus-specific probe for chromosome 19 that was developed from a YAC clone.

3.2.1 Case A: Reciprocal translocation 46,XY, t(5;19)(p12;p12)

This couple was referred for PGD due to a paternal balanced reciprocal translocation 46,XY, t(5;19)(p12;p12). The couple had experienced several years of infertility due to severe oligospermia. Maternal age was 25 at the time of treatment.

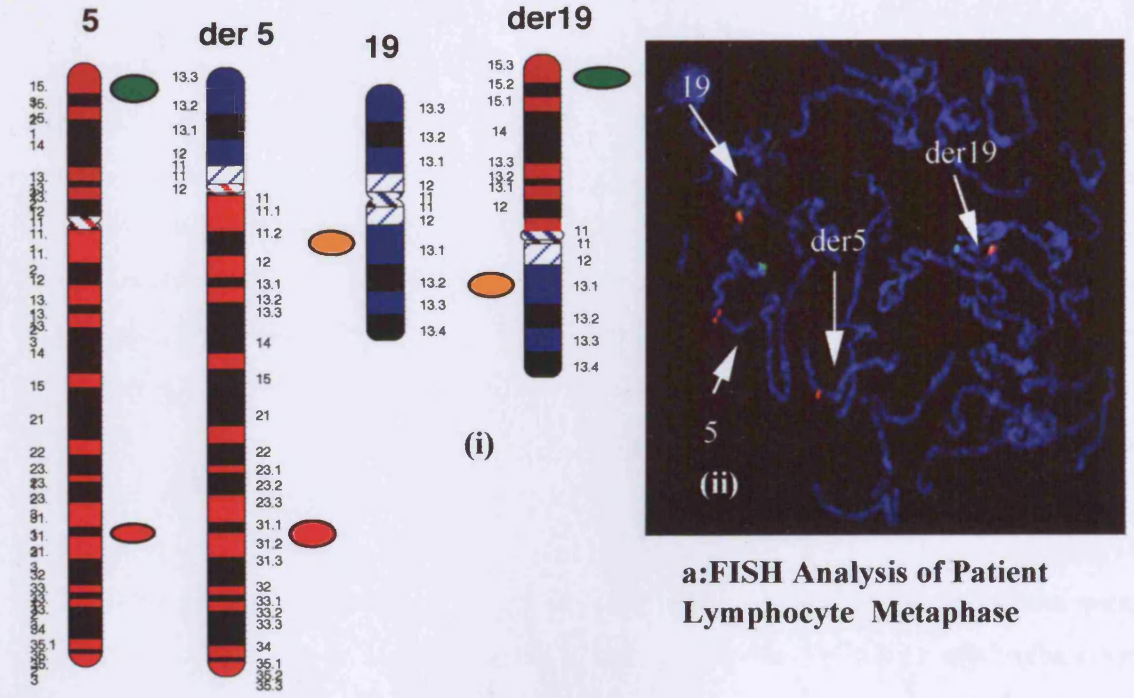
The FISH protocol devised for this case employed only locus-specific probes, as the centromeric probes for chromosomes 5 and 19 cross-hybridised. Thus, the probe combination used consisted of the commercial LSI EGR1 or "Cri du chat" dual probe that flanked the breakpoint on chromosome 5 (binding positions: 5q31-SO, seen as red and

5p15.2-SG, seen as green) and a locus-specific probe for chromosome 19 that was developed from a YAC clone, due to lack of a commercially available probe at the time of treatment. The latter mapped on 19q13.2 and was labelled in SO, red +SG, green to give orange fluorescence. This combination was tested on both control (46,XY) and patient lymphocytes giving a FISH efficiency of 96.75%. Signal appearance was evaluated on lymphocytes and a spare embryonic nucleus. In both cases the commercial probe for chromosome 5 gave intense and clearly visible signals. The YAC probe for 19 was also clearly seen, but its signals were diffuse.

One PGD cycle was carried out for this couple, and ICSI was used, due to the severe oligospermia of the male partner. Twenty oocytes were recovered, and fifteen fertilised (fertilisation rate 75%). On day 2 all fifteen embryos consisted of 4-5 cells, whereas on day 3 all of them were at the 6-8 cell stage. Out these 15 embryos, 13 were considered suitable for biopsy, and two cells were taken from each of them.

Of the 26 blastomeres investigated, results were obtained from 19, as some of them lysed during spreading, or were lost during the FISH procedure. Unfortunately, only one of these blastomeres showed the expected signals for the "Cri du chat" and YAC probes to be considered normal or balanced, i.e. two SO and two SG signals for chromosome 5 and two orange signals (red+green) for chromosome 19. The second blastomere from this embryo had the same signals for the probe hybridising to the two positions on chromosome 5, and two additional red signals, but no green signals. The latter was explained as possible failure of hybridisation for the labelled in green YAC probe, as during preliminary work, its signals were fainter compared to the red YAC probe and the "Cri du chat" probe. The other possible explanation was that the chromosome complement of this blastomere included two chromosomes 5, two derivatives of 5 and no chromosome 19. The latter however, would not result in a viable pregnancy. This embryo was transferred, but pregnancy did not follow. The blastomeres from the remaining embryos were all considered as abnormal. All remaining non-transferred embryos were analysed after the embryo transfer took place, in order to confirm the initial diagnosis. As the yeast strain carrying the YAC insert failed to grow after further culturing, and all the DNA had been labelled for the blastomere diagnosis, the non-transferred embryos were analysed only with the "Cri du chat" probe for chromosome 5.

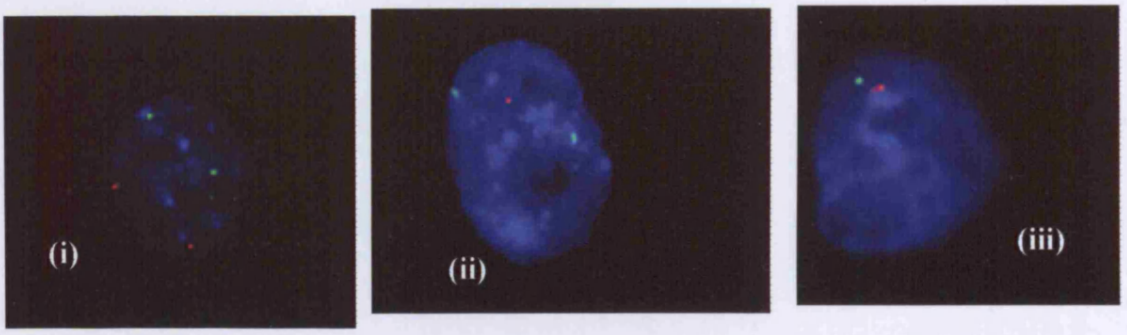
Fig. 3.2: PGD for Case A, 46,XY,t(5;19)(p12;p12)



a:FISH Analysis of Patient Lymphocyte Metaphase

Dual locus specific probe-5p 15.2 (SG), 5q31 (SO)

YAC probe- 19 q 13.1 (SG & SO)



b: FISH Analysis of Biopised Blastomeres for PGD

Fig.3.2: a (i) PGD triple probe strategy selected for Case A, 46,XY,t(5;19) (p12;p12).

(ii) Triple-colour FISH using the probes from (i) on patient chromosomes.

b The same probe combination applied to blastomeres coming from preimplantation embryos during the PGD cycle for this couple.

(i) Blastomere with normal or balanced chromosome complement (2 green, 2 red, 2 orange).

(ii) Blastomere with monosomy 5q31->qter (1red, 2 green).

(iii) Blastomere with monosomy 5 (1 red , 1 green).

The latter meant that information was obtained for chromosome 5 and the two derivatives, but not for chromosome 19 alone. Of the 12 spare embryos, eight were categorised as chaotic (66.66%) and 4 as aneuploid or aneuploid mosaic (33.3%). Due to the high incidence of chaotic embryos, the meiotic segregation was determined only for two and was in one case alternate and the other adjacent-2. Table 3.1 shows the results obtained from the biopsied cells, the non-transferred embryos, their classification and the possible segregation in the sperm. Detailed analysis of the non-transferred embryos using the ISCN nomenclature is shown in Appendix B. FISH analysis of a patient metaphase along with a normal/balanced and two unbalanced blastomeres can be seen in Fig. 3.2.

Sperm analysis (carried out by B. Smith) demonstrated that alternate segregation was observed in 54% of the gametes, while the rest were as follows: 18% adjacent-1, 11% adjacent-2, and 13% 3:1 disjunction.

This couple did not come through for a second cycle, as the female partner was able to conceive naturally one year later.

Table 3.1: Case A, 46,XY,t(5;19)(p12;p12); FISH analysis of biopsied cells and non-transferred embryos in one PGD cycle.

Embryo	Biopsied cell(s)	Cells from remainder of embryo	Embryo classification	Possible segregation (paternal gamete type)
1	A: Monosomy 5q11->qter, monosomy 19 B: Lost during FISH procedure	1: Trisomy 5p12->pter nullisomy 19p12->pter. 2: normal/balanced for 5 3: Monosomy 5q11->qter and monosomy 19q11->qter 4: Trisomy 5p12->pter, nullisomy 19p12->pter	Chaotic	Unknown
2	A: Failure of hybridisation B: Monosomy 19	1: Balanced for chromosome 5 2: Trisomy 5p12->pter, monosomy 19q11->qter, nullisomy 19p12->pter 3: Monosomy 5 4: Nullisomy 19 5: Trisomy 5q11->qter, monosomy 19p12->pter 6: Monosomy 5q11->qter monosomy19q11->qter, nullisomy 19p12->pter	Chaotic	Unknown

Embryo	**Biopsied cell(s)**	**Cells from remainder of embryo**	**Embryo classification**	**Possible segregation (paternal gamete type)**
5	No results as cells lysed during spreading	1: Tetrasomy 5p12->pter, nullisomy 19p12->pter 2: Tetrasomy 5 3: Tetrasomy 5p12->pter, nullisomy 19p12->pter 4: Tetrasomy 5p12->pter, nullisomy 19p12->pter 5: Trisomy 5, monosomy 19q11->qter 6: Trisomy 5p12->pter, monosomy 19q11->qter, nullisomy 19p12->pter	Chaotic	Unknown
7	A: Failure of hybridisation of SG YAC probe, possibly normal/balanced B: Normal/balanced	Transferred	Normal or balanced	Alternate
8	A (binucleate) A1: Trisomy 5p12->pter, nullisomy 19p12->pter A2: Trisomy 5p12->pter, monosomy 19q11->qter, nullisomy 19p12->pter	1: Trisomy 5p12->pter, monosomy 19q11->qter, nullisomy 19p12->pter 2: Trisomy 5 3: Trisomy 5 4: Trisomy 5p12->pter, monosomy 19q11->qter, nullisomy 19p12->pter	Chaotic	Unknown

Embryo	Biopsied cell(s)	Cells from remainder of embryo	Embryo classification	Possible segregation (paternal gamete type)
9	A: Lost during FISH procedure B: Monosomy 5, monosomy 19	Embryo lost during spreading	Abnormal	Inconclusive
10	A: Monosomy 5q11->qter, monosomy 19p12->pter B: Monosomy 19	1: Trisomy 5q11->qter 2: Trisomy 5q11->qter 3: Trisomy 5, monosomy 19 4: Trisomy 5, monosomy 19	Mosaic aneuploid chaotic	Unknown
11	Both cells lost during the FISH procedure	1, 2, 3, 4: Monosomy 19 5, 6: Monosomy 5	Mosaic aneuploid	Unknown
13	A (binucleate) A1, A2: Monosomy 5p12->pter, trisomy 19p12->pter B: Lost during the FISH procedure	1: Monosomy 5 2: Monosomy 19 3: Monosomy 5p12->pter, monosomy 19p12->pter, nullisomy 19q11->qter	Chaotic	Unknown
14	A: Monosomy 5q11->qter, monosomy 19q11->qter, nullisomy 19p12->pter B: Monosomy 5	1, 2, 3, 4: Monosomy 5q11->qter, monosomy 19q11->qter, nullisomy 19p12->pter 4: Monosomy 5p12->pter, monosomy 19p12->pter 5: Monosomy 5	Chaotic	Unknown

Embryo	Biopsied cell(s)	Cells from remainder of embryo	Embryo classification	Possible segregation (paternal gamete type)
15	No results, as cells lysed during spreading	1, 2, 3, 4: Tetrasomy 5p12->pter, monosomy 5q11->qter, trisomy 19q11->qter	Uniformly abnormal	Does not fit any standard segregation pattern
17	Both cells lost during FISH procedure	1: Trisomy 5q11->qter, monosomy 5p12->pter 2-6: Monosomy 5p12->pter, and monosomy 19p12->pter	Mosaic aneuploid	2:2 Adjacent-2
18	A: Trisomy 5, nullisomy 19 B: Lost during FISH procedure	1: Trisomy 5p12->pter, monosomy 19q11->qter 2: Trisomy 5q11->qter, monosomy 19p12->pter 3, 4: Trisomy 5	Chaotic	Unknown

3.2.2 Case B: Reciprocal translocation 46,XX, t(11;22)(q23.3;q11.2)

The balanced reciprocal translocation carrier in this case was the female partner, whose karyotype was 46,XX, t(11;22)(q23.3;q11.2). The couple had experienced four early spontaneous abortions in the 12 months preceding treatment. Maternal age was 28 at the time of the two PGD cycles.

The FISH protocol devised for this case employed one dual locus-specific probe for chromosome 22 and one centromeric probe for the heterochromatic region of chromosome 11. More specifically, the probe combination applied during both PGD cycles involved the commercial LSI VCFS or "Di George" dual probe (Vysis/Abbot) flanking the breakpoint on chromosome 22 (binding positions: 22q11/TUPLE1-SO, seen as red and 22q13/ARSA-SG, seen as green) and another commercial centromeric probe, the CEP11 (Vysis/Abbott) for chromosome 11 hybridising on the alpha-satellite region of this chromosome. An orange colour was achieved for this probe by mixing SO and SG probes together. This combination was tested on both control (46,XY) and patient lymphocytes giving a FISH efficiency of 98%. Signal appearance was evaluated on lymphocytes only. Both probes gave signals that were bright and clear on metaphase and interphase nuclei. The signals for the locus-specific probe would sometimes appear as split. The latter was an expected observation on the lymphocyte cells as the probe hybridised to unique sequences on the two chromatids of chromosome 22. Embryonic nuclei are much more compact compared to lymphocyte interphases, and thus locus-specific probe signals are much more discreet.

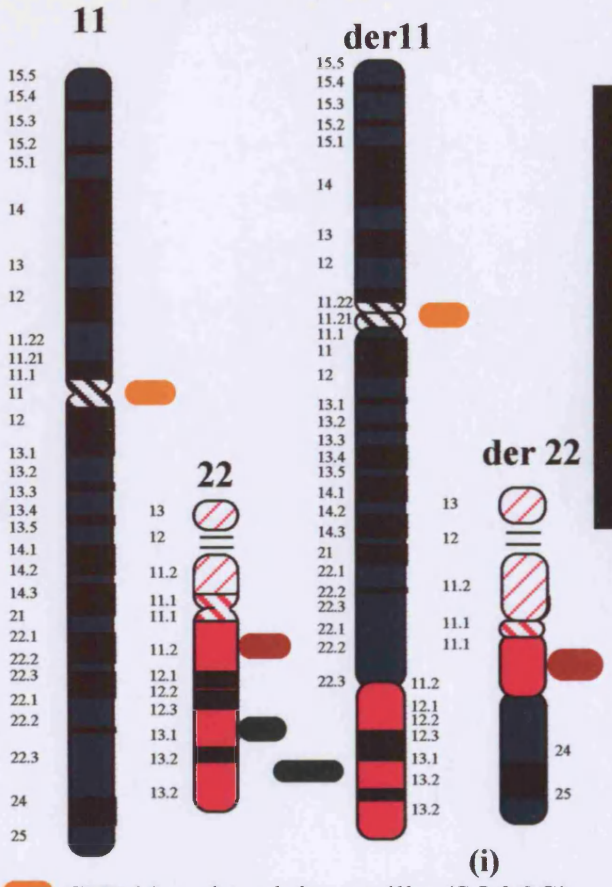
Two IVF cycles were carried out for this couple. In the first standard IVF cycle, twelve oocytes were collected, but only five of these fertilised, leading to a fertilisation rate of 41.6%. Three embryos consisted of 6-8 cells on day 3, all of which were biopsied and had two blastomeres taken from each. FISH analysis of the biopsied blastomeres was carried out by A. Mantzouratou. During diagnosis, FISH results were obtained for all cells, which showed a chaotic chromosome complement with nuclei coming from the same embryo not agreeing as far as the observed signal patterns, were concerned. Thus, no embryo was considered suitable for transfer in this first PGD cycle.

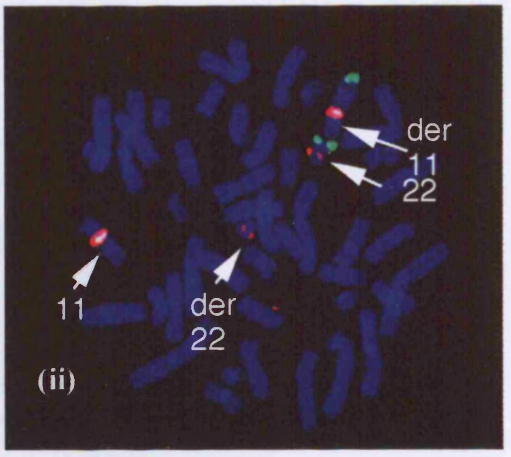
Case B Results

The three biopsied embryos, along with the remaining two that were not considered fit for biopsy on day 3 of development, were analysed with the probe combination used for the PGD diagnosis, in order to confirm the latter. These embryos were re-analysed with another probe set including a lab-prepared centromeric probe for chromosome 15 labelled in SO, another lab-prepared centromeric probe for chromosome 16 labelled in SG and a commercial centromeric probe for chromosome 18 (CEP18, Vysis/Abbott) labelled in SA, in order to further investigate their chromosome complement. Details of these probes are shown in Table 2.2. Out of these five embryos, the three that were biopsied were classified as fully chaotic (60%), showing a different signal pattern and thus, chromosome complement, in almost each of their nuclei. The remaining two gave inconclusive results, due to their nuclei either appearing as fragments or being very faint without any signals. Details of these results are seen in Table 3.2. Because of the chaotic nature of these embryos, segregation patterns could not be determined, and the unfertilised oocytes were not provided for analysis.

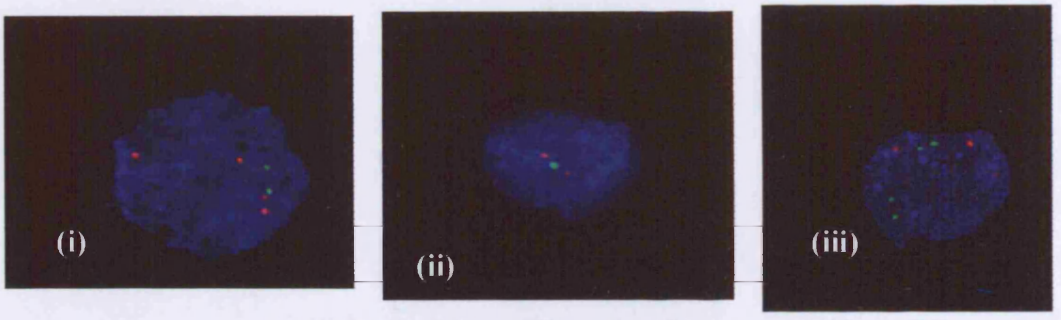
ICSI was used for the second treatment cycle for this couple, due to the low fertilisation rate during the first cycle. Fourteen oocytes were recovered, nine were fertilised (fertilisation rate 64.3%), and eight embryos were considered suitable for biopsy on day 3 of preimplantation development. Out of these, two blastomeres were obtained from only two embryos, which also had the best morphology. One blastomere was obtained from the rest, as they consisted of less than 6 cells and were of poorer morphology on the day of the biopsy. FISH results were obtained from 8 of the biopsied cells, as some of them were either covered with cytoplasm or were lost during the FISH procedure. The same probe combination used for the 1st PGD cycle was employed this time as well. However, this batch of probes was not initially tested on lymphocytes, as they had arrived the day previous to the actual biopsy. During diagnosis, it was observed that the SG CEP11 probe had almost completely failed to hybridise on the embryonic nuclei, and had hybridised poorly on the control lymphocyte slide. Even so, with considered judgement, two embryos were identified as normal or balanced and were transferred on day 4. Diagnosis was based on two blastomeres from the first and on one blastomere from the second. Both were at the morula stage of preimplantation development. All the remaining embryonic nuclei were considered as abnormal.

Fig.3.3: PGD for Case B, 46,XX,t(11;22)(q23.3;q11.2)





a: FISH Analysis of Patient Lymphocyte metaphase



b: FISH Analysis of Biopsied Blastomeres for PGD

Fig.3.3: a (i) PGD triple probe strategy selected for Case B 46,XX,t(11;22) (q23.3;q11.2).

(ii) Triple-colour FISH combination using the probes from (i) on a patient metaphase.

b The same probe combination applied to blastomeres from the embryos generated by this couple during 2 PGD cycles.

(i) Blastomere with normal or balanced chromosome complement (2 red, 2 green, 2 orange).

(ii) Blastomere with monosomy 11 (1 orange) and monosomy 22 (1 red, 1 green).

(iii) Blastomere with one der11 (1 orange, 1 green) and trisomy 22 (3 red, 3 green).

This double embryo transfer resulted in a clinical pregnancy and a normal live birth.

The non-transferred embryos were analysed with the probe combination applied during the 2nd PGD cycle, in order to confirm the initial diagnosis. However, an older batch of SG CEP11 probe was used, to obtain more accurate results. They were also re-analysed with another commercial (Vysis/Abbott) probe set, including the centromeric probe for chromosome 18 labelled in SA, the centromeric probe for chromosome X labelled in SG and the centromeric probe for chromosome Y labelled in SO. Details of these probes are shown in Table 2.2. This re-analysis took place in order to further investigate the chromosome complement of the non-transferred embryos. Out of the six non-transferred embryos, one was lost after the 1st round of FISH, whereas three more were lost after the second round of FISH. According to the signal patterns after both rounds of FISH, the embryos were classified in the following categories: one aneuploid mosaic and chaotic for the sex chromosomes, one balanced aneuploid mosaic, one balanced chaotic mosaic, one uniformly abnormal, and two fully chaotic. The segregation could be determined as follows: Alternate for the two transferred embryos, 3:1 interchange combined with mitotic non-disjunction for the mosaic aneuploid and the mosaic aneuploid balanced embryos, and 2:2 adjacent-1 for the uniformly abnormal embryo (results based on biopsied cell). Similarly to the 1st PGD cycle, unfertilised oocytes were not provided for FISH analysis.

Details of the chromosome constitutions of these embryos are shown in Table 3.2. A patient metaphase demonstrating the binding positions of the probes used for diagnosis and pictures of a normal and two abnormal blastomeres are shown in Fig.3.3. Detailed cytogenetic analysis of all embryonic nuclei is described in Appendix B.

In summary, during both PGD cycles, twenty-six oocytes were collected, fourteen fertilised, and eleven embryos were considered suitable for biopsy on day 3. Out of these, 2 were considered as normal or balanced (18.2%), 5 as fully chaotic (45.4%), while the remaining of the abnormal chromosome complements observed varied. Even though this couple produced a relatively low number of embryos during both cycles and the majority were considered as abnormal a healthy pregnancy was achieved, which resulted in the birth of a normal male.

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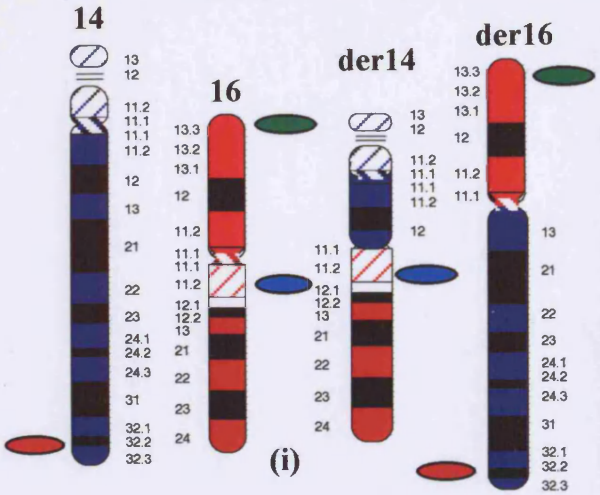
Table 3.2: Case B, 46,XX,t(11;22)(q23.3;q11.2); FISH analysis of biopsied cells and non-transferred embryos in two PGD cycles.

Embryo 1st cycle	**Biopsied cell(s) FISH probes for 11 and 22**	**Cells from remainder of embryo FISH probes for 11, 22, 15, 16, 18**	**Classification**	**Possible segregation (maternal gamete type)**
1	Not biopsied	Fragmented nuclei 1: Nullisomy 11, nullisomy 18 2: Nullisomy 11, nullisomy 16	Inconclusive	Unknown
2	Not biopsied	1: Monosomy 22q11.2->qter 2: Monosomy 11, nullisomy 22 No signals visible upon re-FISH	Inconclusive	Unknown
10	A: Apoptotic, no signals B: Monosomy 11	1: Monosomy 11, monosomy 16, nullisomy 15 and 18, inconclusive for 22 2: Monosomy 11q22.3->pter, trisomy 22q11.2->qter, monosomy 16, nullisomy 15 and 18	Chaotic	Unknown
11	A: nullisomy 11, tetrasomy 22q11.1->p13, monosomy 22q11.2->qter B (binucleate) B1: nullisomy 11q22.3->pter, nullisomy 22q11.2->qter	1: Nullisomy 11q22.3->pter, monosomy 22q11.2->qter, nullisomy 15, monosomy 18 2: Nullisomy 11q22.3->pter, monosomy 22q11.2->qter 3: Nullisomy 11, monosomy 22q11.2->qter, monosomy 15 and 16, nullisomy 18 4: Nullisomy 22q11->p13, nullisomy 11q23.3->qter, monosomy 15 and 16 5: Tetrasomy 11q23.3->qter, monosomy 15, nullisomy 16 and 18	Chaotic	Unknown

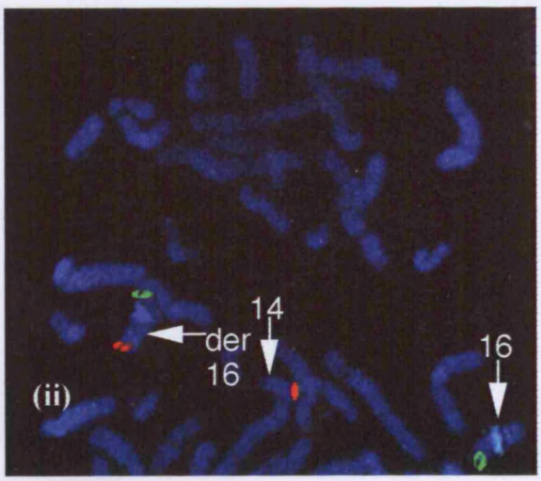
Embryo 1st cycle	**Biopsied cell(s) FISH probes for 11 and 22**	**Cells from remainder of embryo FISH probes for 11, 22, 15, 16, 18**	**Classification**	**Possible segregation (maternal gamete type)**
12	Both nuclei gave inconclusive results	1: Nullisomy 11, trisomy 16 2: Nullisomy 11, monosomy 16	Chaotic	Unknown
Embryo 2nd cycle	**Biopsied cell(s) FISH probes for 11 and 22**	**Cells from remainder of embryo FISH probes for 11, 22, 18, X, Y**	**Classification**	**Possible segregation (maternal gamete type)**
1	A: No signals, as nucleus covered with cytoplasm	1: Monosomy 11, trisomy X 2: Monosomy 11, trisomy X, disomy Y 3: Monosomy 11, trisomy 22, disomy Y	Mosaic aneuploid, chaotic for sex chromosomes	3:1 Interchange, oocyte with chromosome 22 only
2	A: Monosomy 22 B: Lost during FISH process	1: Trisomy 22 2: Balanced for both 11 and 22 Both nuclei lost after 2nd round of FISH	Mosaic balanced aneuploid	Alternate, combined with mitotic non-disjunction
3	A: Normal or balanced	Transferred	Normal or balanced	Alternate
5	A: Lost during FISH process B: Inconclusive	1: Trisomy 22q11.1->p13 2: Monosomy 11 and 22 Both nuclei lost after 2nd round of FISH	Chaotic	Unknown

Embryo 2nd cycle	**Biopsied cell(s) FISH probes for 11 and 22**	**Cells from remainder of embryo FISH probes for 11, 22, 18, X, Y**	**Classification**	**Possible segregation (maternal gamete type)**
6	A: Normal or balanced B: Normal or balanced	Transferred	Normal or balanced	Alternate
7	A: Tetrasomy 11q23.3->qter, nullisomy 22q11.2->qter	1: Monosomy 11 2: Balanced for both 11 and 22 3: Monosomy 11, trisomy 22 Nuclei lost after 2nd round of FISH	Mosaic balanced chaotic	Unknown
8	A (binucleate) A1, A2: Monosomy 11q23.3->qter, trisomy 22q11.2->qter	Lost during FISH process	Uniformly abnormal	2:2 Adjacent-1
9	A: Fragments, no signals	1: Nullisomy 11 and 18, monosomy 22q11.1->p13 2: Nullisomy 11, and 18, monosomy 22q11.2->qter 3: Nullisomy 11q22.3->pter, monosomy 18, trisomy 22q11.1->p13 4: Trisomy 11q22.3->pter, trisomy 18, monosomy 22q11.1->p13, disomy X, trisomy Y	Chaotic	Unknown

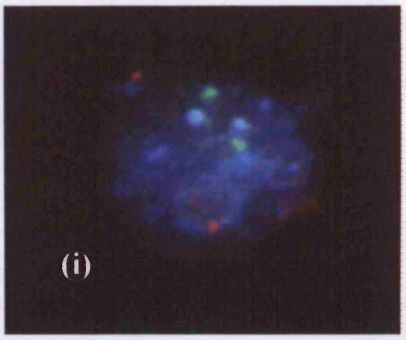
Fig 3.4: PGD for Case C, 46,XY,t(14;16)(q13;q11.1)

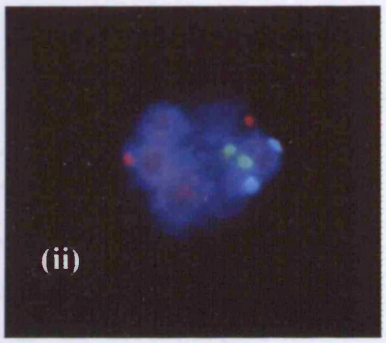


TelVysion 14q probe- 14qter (SO)
TelVysion 16p probe-16pter (SG)
CEP 16 probe- beta satellite (SA)



a: FISH Analysis of Patient Lymphocyte metaphase





b: FISH Analysis of Spare embryonic blastomeres

Fig. 3.4: a (i) Triple-colour FISH strategy selected for Case C, 46,XY,t(14;16)(q13;q11.1).

(ii) Three-colour FISH using the probes from (i) on patient chromosomes. The der14 was missing from this metaphase.

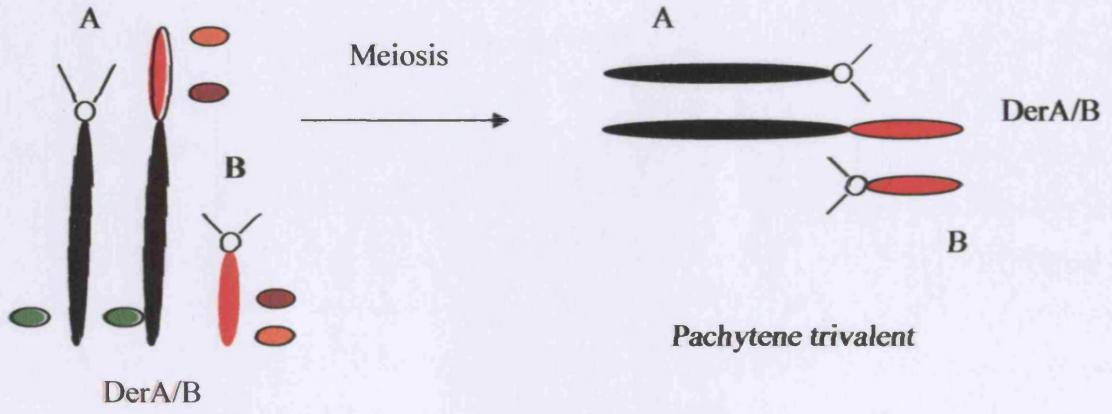
b (i), (ii) Application of the same three probes on spare embryonic nuclei. Both were identified to have a normal complement for the examined chromosomes (2 green, 2 aqua, 2 red).

3.2.3 Case C: Reciprocal translocation 46,XY, t(14;16)(q13;q11.1)

This couple was referred for PGD because of a balanced reciprocal translocation identified in the male partner, whose karyotype was 46,XY, t(14;16)(q13;q11). At the time the couple had experienced two years of infertility due to severe oligospermia. This was followed by further three years of infertility before treatment commenced. The maternal age was 28 at the time of referral.

The FISH protocol devised for this case employed one commercial subtelomeric probe for the long arm of chromosome 14, and two commercial probes flanking the breakpoint on chromosome 16, all from Vysis/Abbott. Thus, the probe combination involved the centromeric CEP 16 probe, labelled in SA and mapping on the beta-satellite region of this chromosome, the subtelomere probe for the short arm of chromosome 16 labelled in SG, and the subtelomere probe for chromosome 14 labelled in SO. Evaluation of the FISH efficiency of this combination took place on control (46,XY) and patient lymphocytes giving a FISH efficiency of 95.7%. Signal appearance was evaluated initially on lymphocyte metaphases and interphases. All probes gave bright and clear signals. However, there was some interference of the SA fluorochrome with the DAPI used to stain the nuclei, and both subtelomere probes gave split signals. The latter made the testing of this probe combination on a spare embryo essential for reasons mentioned in the previous section. One normally fertilised spare embryo was tested. The signals for all three probes were much more intense and sharp, and the embryo was considered normal for the chromosomes tested. Fig.3.4 demonstrates a patient metaphase showing the binding positions of the probe, and two spare embryonic nuclei showing signal appearance. The couple were finally treated in May 2004, after preparation of this thesis.

Fig.3.5: FISH strategy for the PGD of Robertsonian translocations



a

b Mode of segregation	Segregating chromosomes	Signals visible	Embryo outcome
Alternate	A, B	2, 2, 2	Normal
	DerA/B	2, 2, 2	Balanced
Adjacent	A, derA/B	2, 2, 3	Trisomy A
	B	2, 2, 1	Monosomy A
Adjacent	B, derA/B	3, 3, 2	Trisomy B
	A	1, 1, 2	Monosomy B
3:0 Double aneuploidy	A, B, derA/B	3, 3, 3	Trisomy A, B
	0	1, 1, 1	Monosomy A, B

Fig.3.5: a. Triple-colour FISH strategy developed for the PGD of Robertsonian translocations. Two probes are used for the detection of the chromosome that is most likely to result in a viable trisomy, e.g. 21. In this case one probe (green) hybridises on A and two probes (red, orange obtained by combining 50:50 red:green) on B. During meiosis chromosomes A and B pair-up with homologous regions from the derA/B. This results in the formation of the pachytene trivalent. **b.** This arrangement theoretically segregates in four different ways generating eight different gamete types. Hence, the alternate mode of segregation leads to the formation of either normal or balanced gametes, the adjacent produces interchange aneuploidy and the 3:0 leads to aneuploidy for both chromosomes involved in the rearrangement. The embryo outcome for PGD is represented for each type, assuming that the other parent has a normal karyotype. The application of this FISH strategy enables the detection of all unbalanced chromosome constitutions, due to the resulting signal patterns being different, depending on the segregation. As with the FISH approach for the PGD of reciprocal translocations, this FISH strategy cannot distinguish between normal and balanced chromosome constitutions.

3.3 PGD for two couples with Robertsonian translocations

The first meiotic division in the gametes of balanced Robertsonian translocation carriers is somewhat different from reciprocal translocation carriers. More specifically, the derivative metacentric chromosome and the two normal homologues synapse together and behave as a trivalent.

In such cases, centromeric probes cannot be used, due to sequence homology between chromosomes 13 and 21 and 14 and 22. Thus, the FISH strategy used involved two or three locus-specific probes, labelled in different colours, which hybridised to a position on the long arm of each of the two acrocentric chromosomes that formed the derivative chromosome (Fig. 3.5).

FISH strategies were devised for two couples that were referred in our centre, as one of the two partners was a balanced carrier of a Robertsonian translocation. Two PGD cycles were carried out for both cases. The patient karyotypes were as follows: 45,XY, t(13;21)(q10;q10), 45,XY, t(13;14)(q10;q10). A three-colour FISH protocol was applied in the first case, with two probes hybridising on chromosome 21 and one on chromosome 13, whereas two locus-specific probes were used for the second case, one mapping on the long arm of chromosome 13 and the other on the long arm subtelomere of chromosome 14. All probes were commercially available.

3.3.1 Case D: Robertsonian translocation 45,XY, t(13;21)(q10;q10)

This couple was referred to our centre after having experienced four years of primary infertility, due to severe oligoasthenoteratozoospermia. Cytogenetic investigations of the male partner revealed that he was a balanced carrier of a Robertsonian translocation. His karyotype was identified to be: 45,XY, t(13;21)(q10;q10). The maternal age was 34 at the time of treatment.

The FISH protocol used for this case involved the application of two locus-specific probes and one subtelomeric probe. Thus, one probe was used for the detection of chromosome 13. The latter hybridised on position 13q14, was labelled in SG and was commercially available (Vysis/Abbott, UK). Two probes were used to identify chromosome 21, as this chromosome was associated with the more viable trisomy. Out of these, the first mapped on positions 21q22.13-q22.2, was labelled in SO and was

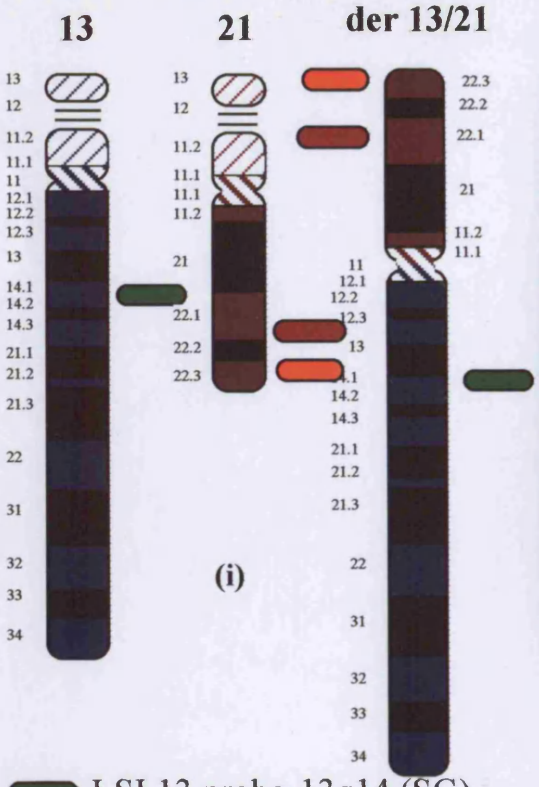
provided by Vysis/Abbott. The second mapped on the telomere of the long arm of chromosome 21, was provided by Oncor, UK and was labelled in orange by mixing red and green together. This triple probe combination was initially tested on control (46,XY) and patient lymphocytes, resulting in a FISH efficiency of 94%. Observation of metaphase chromosomes and interphase nuclei showed that all probes gave clear signals. However, the two locus-specific probes were relatively more intense compared to the subtelomeric probe. Split signals were also seen on some nuclei.

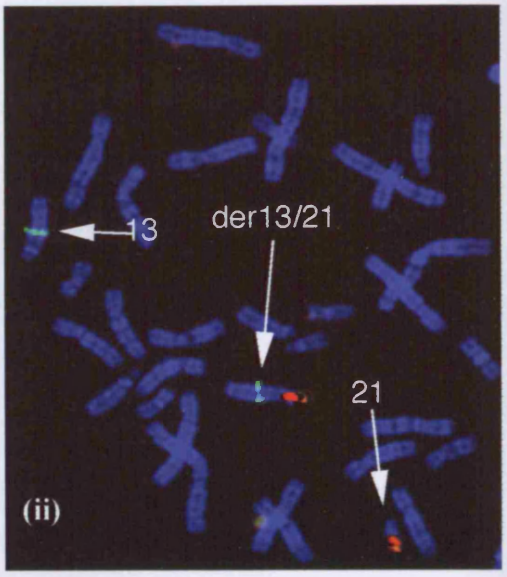
Two treatment cycles were carried out for this couple. ICSI was used both times, in order for fertilisation of the oocytes to be achieved. During the first PGD cycle nine oocytes were collected, and seven fertilised, resulting in a fertilisation rate of 77.7%. Out of the seven embryos created, six were considered fit for biopsy on day 3. Eleven blastomeres were obtained in total, but FISH results were acquired only from seven. The rest were either lost during spreading, or they were too faint to be detected under the phase microscope. Diagnosis led to the identification of three normal or balanced embryos. The latter were all transferred, but no clinical pregnancy was achieved.

The non-transferred embryos from this first cycle were biopsied for a second time on day 4, and the blastomeres that were taken were to be analysed with the application of CGH. These embryos were then placed on slides and analysed with the same probes used on the day of the diagnosis, to confirm the latter. Further investigations of their chromosome constitution took place with the use of another commercial centromeric probe set for chromosomes 18 labelled in SA, X labelled in SG, and Y labelled in SO (Vysis/Abbott, UK). Details of all probes and combinations are shown in Tables 2.2 and 2.3. Out of the three spare embryos, FISH results were obtained for two, as one was lost during spreading. Thus, one was identified as fully chaotic, while the other one gave inconclusive results after both rounds of FISH. CGH analysis (carried out by M. Simopoulou) revealed a mosaic balanced aneuploid chromosome complement.

During the second treatment cycle, thirteen oocytes were collected, twelve were injected, and ten were fertilised (fertilisation rate: 83.3%). Nine embryos were biopsied, leading to FISH analysis of twelve blastomeres (two were obtained from 4 embryos and 1 from the

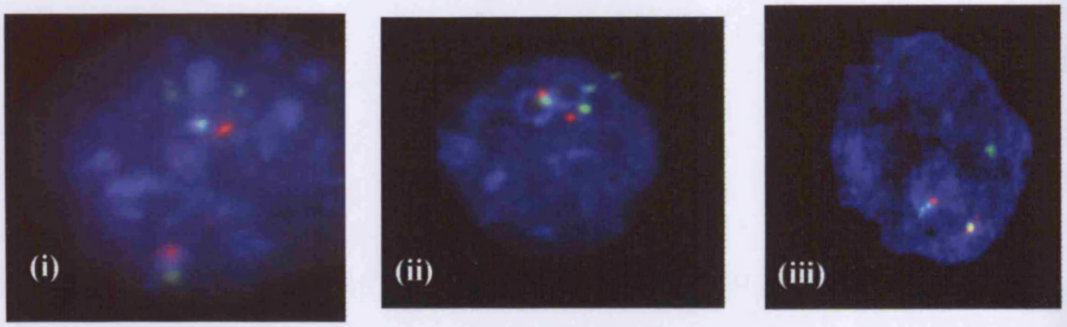
Fig. 3.6: PGD for Case D, 45,XY,der(13;21)(q10;q10)





a: FISH Analysis of Patient Lymphocyte metaphase

LSI 13 probe-13q14 (SG)
LSI 21 probe-21q22.13-q22.2 (SO)
TEL 21q probe- 21qter (green/red- orange)



b: FISH Analysis of Biopsied Blastomeres for PGD

Fig. 3.6: a (i) Triple probe strategy devised for the PGD for Case D, 45,XY,der(13;21)(q10;q10).

(ii) The same probe combination applied on a patient metaphase.

b Blastomeres from embryos generated by this couple during two PGD cycles:

(i) Blastomere with a normal or balanced chromosome complement (2 green, 2 red, 2 orange).

(ii) Blastomere trisomic for 13 (3 green), and monosomic for 21 (1 red, 1 orange).

(iii) Blastomere monosomic for 21 (1 red, 1 orange) and normal for 13 (2 green). The red and one of the two green signals appeared to be very close together.

remaining five. Results were obtained from all cells, apart from one that was lost during the FISH procedure. The same probe combination used in the first cycle was also employed for this second cycle. Similarly to the first cycle, three embryos were identified as normal or balanced and were transferred, but no clinical pregnancy ensued.

The six non-transferred embryos were analysed only with the probes used for diagnosis, to confirm the latter and investigate their chromosome status. No further analysis took place, as all nuclei were of very poor morphology, being very faint and fragmented in some cases. All were classified as chaotic, showing different chromosome complements in almost all their cells. Details of the chromosome constitutions observed in embryos from both cycles are shown in Table 3.3. Cytogenetic analysis of all nuclei according to the ISCN nomenclature is described in Appendix B. Fig. 3.6 demonstrates a patient metaphase with the probes used for the FISH diagnosis, along with pictures of a normal and two abnormal blastomeres.

Sperm was available for FISH analysis (carried out by B.Smith) in this case. From the results, it was evident that 89% of the male gamete chromosomes were segregating in an alternate mode. This agreed with the identification of six normal or balanced embryos out of the fifteen that were analysed in both cycles.

In summary, in the two treatment cycles that took place for this couple, twenty-two oocytes were collected, seventeen fertilised, and fifteen embryos were considered of appropriate morphology and cell number to be biopsied on day 3 of preimplantation development. Six embryos were classified as normal or balanced (40%), one was classified as mosaic balanced/aneuploid (6.6%), one as aneuploid (6.6%), based on the biopsied cell results, and seven were characterised as chaotic (46.6%). Conn and colleagues (1998) observed that the high incidence of chaotic embryos is patient-specific and tends to lead to a poor PGD outcome. The latter, along with the fact that all embryos from this couple were of relatively poor morphology, could explain their failure to achieve pregnancy in either of the two PGD cycles.

Table 3.3: Case D, 45,XY,der(13;21)(q10;q10); FISH analysis of biopsied cells and non-transferred embryos in two PGD cycles.

Embryo 1st cycle	**Biopsied cell(s) FISH probes for 13 and 21**	**Cells from remainder of embryo FISH probes for 13, 21, 18, X, and Y**	**Embryo classification**	**Possible segregation (paternal gamete type)**
2	A: Lost during spreading B: Trisomy 13	Lost during spreading	Aneuploid Results obtained from 1 cell only	Inconclusive, possibly adjacent-1
3	A: Normal	Transferred	Normal or balanced	Alternate
4	A: Trisomy 21, no information on 13 B: Lost during spreading	1: Trisomy 21, normal for 13, 18, X, and Y 2: Monosomy 13, 21, nullisomy 18, X and Y 3: Trisomy 21, lost after 2nd round of FISH 4: Monosomy 13, 21, nullisomy 18 and X	Chaotic	Unknown
5	A, B: Normal	Transferred	Normal or balanced	Alternate
7	A: Lost during FISH B: Normal	Transferred	Normal or balanced	Alternate
9	A, B: Lost during spreading or FISH	1: Nullisomy 13	Inconclusive from FISH Mosaic balanced aneuploid after CGH analysis	Unknown

Embryo 2nd cycle	Biopsied cell(s) FISH probes for 13 and 21	Cells from remainder of embryo FISH probes for 13 and 21	Embryo classification	Possible segregation (paternal gamete type)
1	A,B: Normal	Transferred	Normal or balanced	Alternate
2	A: Trisomy 13	Nuclei of poor morphology 1: No signals 2: Monosomy 21 3: Monosomy 13 and 21 4: No signals	Chaotic	Unknown
3	A: Monosomy 21	1: Trisomy 13 2: Normal/balanced 3: Trisomy 13 4: Trisomy 13, monosomy 21	Chaotic	Unknown
4	A: Trisomy 13, monosomy 21	1: Monosomy 13, nullisomy 21 2: Monosomy 21 3: Monosomy 13, nullisomy 21 4: Trisomy 13, monosomy 21	Chaotic	Unknown
5	A: Lost during FISH B: Normal	Transferred	Normal or balanced	Alternate

Embryo 2nd cycle	Biopsied cell(s) FISH probes for 13 and 21	Cells from remainder of embryo FISH probes for 13 and 21	Embryo classification	Possible segregation (paternal gamete type)
6	A: Normal	Transferred	Normal or balanced	Alternate
7	A: Trisomy 13	1: Trisomy 13, 2: Trisomy 13 3: Six copies of 13 4: Trisomy 13 5: Monosomy 13 6: Monosomy 21	Chaotic	Unknown
8	A: Trisomy 21	1: Monosomy 21 2: Trisomy 21, 5 copies of 13 3: Trisomy 21, 5 copes of 13 4: Trisomy 13 5: Trisomy 13, 21 6: Trisomy 13, 21	Chaotic	Unknown
9	A: Normal B: Tetrasomy 13, trisomy 21	1: Trisomy 21 2: Monosomy 21, nullisomy 13 3: Normal/balanced	Chaotic	Unknown

3.3.2 Case E: Robertsonian translocation 45,XY, t(13;14)(q10;q10)

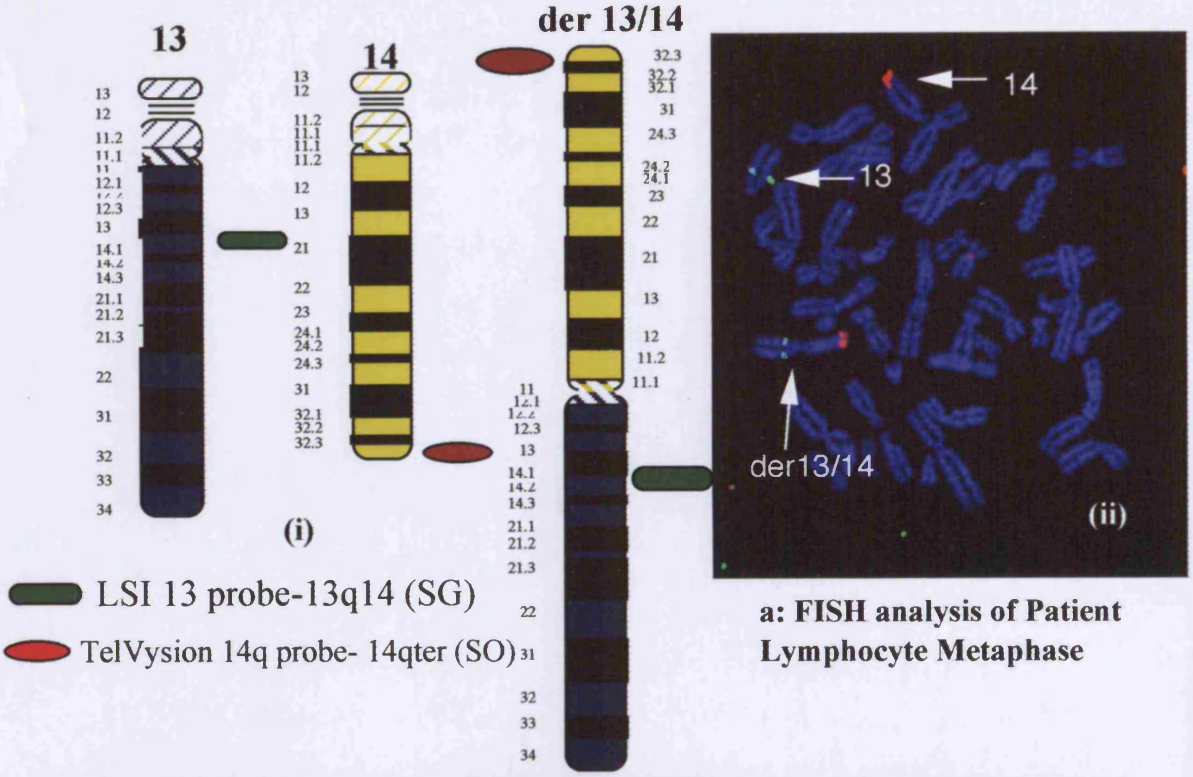
This couple had experienced many years of infertility, as the male partner was oligospermic. He had had IVF treatment in the past with a previous partner. This treatment resulted in a clinical pregnancy, which aborted spontaneously during the first trimester. Cytogenetic analysis revealed that he was a balanced carrier of a Robertsonian translocation, his karyotype being: 45,XY, t(13;14)(q10;q10). His sister was identified to be carrying the balanced form of this translocation and had also experienced early spontaneous abortions. The maternal age was 37 at the time of both PGD cycles.

A dual colour FISH protocol was developed for this case. The probes used included the locus-specific probe for chromosome 13, hybridising on region 13q14 and labelled in SG and the subtelomere probe for the long arm of chromosome 14, mapping on 14qter and labelled in SO. Both probes were commercially available (Vysis/Abbott, UK). As with all the previous cases, this probe combination was tested on lymphocytes from both control individuals (46,XY) and from the patient as well. FISH efficiency was calculated to be 98%, and the probes gave intense and clearly visible signals on both metaphase and interphase nuclei. The subtelomere probe would sometimes appear as a split signal for reasons already mentioned above (3.2.1).

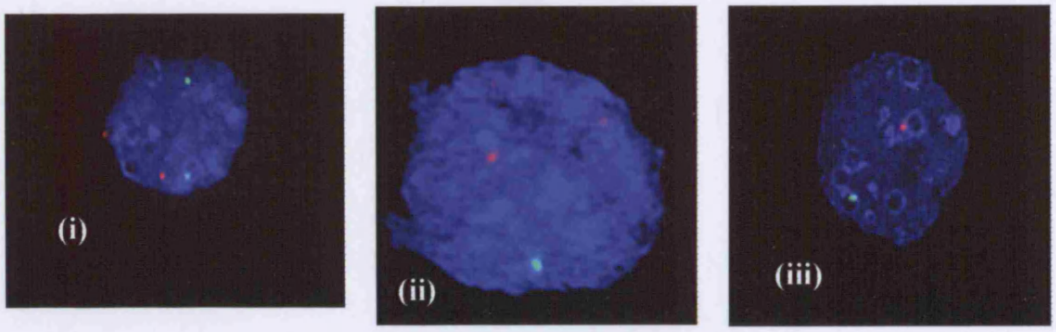
Two PGD cycles were carried out for this couple and ICSI was used in both in order for fertilisation to be achieved. During the first treatment twelve oocytes were collected from the female partner and eight of them fertilised, resulting in a fertilisation rate of 66.66%. All eight resulting embryos were biopsied giving a total of sixteen blastomeres (two from six embryos with 6-8 cells, 3 from an embryo with 10 cells and 1 from an embryo with 5 cells). FISH analysis was carried out by A. Mantzouratou. Results were obtained from 10 blastomeres, as the remainder were either fragmented, or had no signals due to being too cytoplasmic, or apoptotic. According to the observed signal patterns, three embryos were identified as normal and were transferred. Unfortunately no clinical pregnancy followed; the female partner suffered a kidney infection and this could have prevented implantation.

The remaining five non-transferred embryos were initially examined with the probe combination used on the day of the diagnosis, in order to confirm the latter. They were

Fig. 3.7: PGD for Case E, 45,XY,der(13;14)(q10;q10)



a: FISH analysis of Patient Lymphocyte Metaphase



b: FISH Analysis of Biopsied Blastomeres for PGD

Fig. 3.7: a (i) Probe strategy devised for the PGD of Case E, 45,XY,der(13;14)(q10;q10)
(ii) The same probe combination applied on a patient metaphase.
b Blastomeres coming from preimplantation embryos, generated by this couple during two PGD cycles:
(i) Blastomere showing a normal or balanced complement for the examined chromosomes (2 green, 2 red).
(ii) Blastomere monosomic for 13 (1 green) and normal for 14 (2 red).
(iii) Blastomere monosomic for both 13 and 14 (1 red, 1 green).

further investigated with the use of another probe combination, consisting of the lab-prepared centromeric probe for chromosome 4 labelled in SG, the lab-prepared centromeric probe for chromosome 15 labelled in SO and the commercial (Vysis/Abbott, UK) centromeric probe for chromosome 18 labelled in SA. These five embryos were classified as follows: one normal or balanced (20%), one aneuploid mosaic (20%), and three fully chaotic embryos (60%). Details of the results of the biopsied cells and the spare embryos are shown in Table 3.4, and detailed cytogenetic analysis of all embryos with the use of ISCN nomenclature is shown in Appendix B.

During the second treatment cycle thirteen oocytes were collected and all of them fertilised after ICSI (fertilisation rate 100%). Eight normally fertilised embryos along with one that showed three pronuclei (3 PN) were biopsied in day 3, resulting in a total of thirteen blastomeres for FISH analysis (two from six embryos consisting of 6-9 cells and one from the remaining two that had 4-5 cells). Results were obtained from nine cells, as the rest were either fragmented and did not have any signals, or they were lost during spreading or FISH. Two embryos were characterised as normal and were transferred to the female partner, but no clinical pregnancy was achieved.

The chromosome constitution of the six non-transferred embryos was examined with the application of two sets of probes. The first included the two probes used on the day of the diagnosis, while the second involved three commercial (Vysis/Abbott, UK) centromeric probes for chromosomes 18 labelled in SA, X labelled in SG and Y labelled in SO. Embryo classification for this second PGD cycle was as follows: two embryos were classified as mosaic balanced/aneuploid (33.3%), one was mosaic aneuploid (16.6%), and three were classified as fully chaotic (50%). Details of these results, along with those from the biopsied cells are shown in Table 3.4, and cytogenetic analysis of all embryos with the use of ISCN nomenclature is shown in Appendix B. Fig. 3.7 demonstrates a patient metaphase showing the exact position of the probes on the chromosomes involved in the translocation. One normal and two abnormal blastomeres are also illustrated.

Segregation modes in the sperm of the male partner were established as alternate for the embryos that were classified as normal or balanced and were transferred. The chromosome complements identified for the remaining embryos were attributed to

post-zygotic errors such as mitotic non-disjunction. Sperm was not available for analysis in this case.

Thus, during both PGD cycles for this Robertsonian translocation twenty-five oocytes were collected, twenty-one fertilised and sixteen embryos reached the biopsy stage. Both cycles led to the detection of six normal or balanced embryos in total (37.5%), five of which were transferred. Three embryos were classified as balanced aneuploid mosaic (18.75%), one as aneuploid mosaic (6.25%), and six as fully chaotic (37.5%). No clinical pregnancy was achieved in any of the two cycles. The relatively high incidence of abnormal embryos, combined with the advanced maternal age and the kidney infection of the female partner could be attributing factors.

Table 3.4: Case E, 45,XY,der(13;14)(q10;q10); FISH analysis of biopsied cells and non-transferred embryos in two PGD cycles.

Embryo 1st cycle	**Biopsied cell(s) FISH probes for 13 and 14**	**Cells from remainder of embryo FISH probes for 13, 14, 4, 15, and 18**	**Classification**	**Possible segregation (paternal gamete type)**
1	A: Fragmented, no signals B: Monosomy 14, normal for 13	1,2,3: Normal for 13 and 14, nullisomy 4,15,18 4,5,6,7: Normal for 13, nullisomy 14, 4,15,18 8: Normal for 13,4, nullisomy 14,15,18 9: Normal for 14,18, trisomy 13, monosomy 15, nullisomy 4	Chaotic	Unknown
2	A: Covered with cytoplasm, no signals B: Normal	Transferred	Normal or balanced	Alternate
3	A,B: Normal	Transferred	Normal or balanced	Alternate
4	A,B: Normal	1,2,3: Normal for 13,14, 4,15,18 4 (fragment): Monosomy 13,14,4,18, nullisomy 15 5 (fragment): Monosomy 13,14,4, normal for 15,18	Normal or balanced Fragments could be part of the same nucleus, but cannot conclude from signal patterns	Alternate

Embryo 1st cycle	**Biopsied cell(s) FISH probes for 13 and 14**	**Cells from remainder of embryo FISH probes for 13, 14, 4, 15, and 18**	**Classification**	**Possible segregation (paternal gamete type)**
5	A,B: Fragmented, no signals C: Normal	Transferred	Normal or balanced	Alternate
6	A: Faint and apoptotic, no signals B: Normal	1: Normal for 13,14,4,15,18 2: Normal for 13,4, monosomy 14,15, trisomy 18 3: Nullisomy for 13,14,4,15,18	Mosaic balanced/aneuploid	Alternate combined with post-zygotic errors
7	A,B: Monosomy 13, nullicomy 14	1,2: Monosomy 13,14, normal for 4,15,18 3: Monosomy 13,14, nullisomy 4,15,18 4: Trisomy 13, monosomy 14, nullisomy 4,15,18 5: Trisomy 13, monosomy 14,4,15, nullisomy 18 6: Normal for 13,14,4,15,18 7: Monosomy 14, normal for 13, trisomy 4,15,18 8: Nullisomy 13,14, lost after 2nd FISH round	Chaotic	Unknown
8	A,B: Trisomy 13,14	1,2: Normal for 13,14, nullisomy 4,15,18 3: Normal for 13,14, monosomy 15, nullisomy 4,18 4: Monosomy 13,14,15, nullisomy 4,18	Chaotic	Unknown

Embryo 1st cycle 8 cont.		**Cells from remainder of embryo FISH probes for13, 14, 4, 15, and 18** 5: Monosomy 13,14,4,15, nullisomy 18 6,7: Nullisomy 13,14, normal for 4, monosomy 15,18 8: Trisomy 13,4, monosomy 14, normal for 15,18 9: Normal for 13, monosomy 14,4,15,18 10: Nullisomy 14, monosomy 15, normal 13,4,18 11: Trisomy 13, normal 14,4,15,18		
Embryo 2nd cycle	**Biopsied cell(s) FISH probes for 13 and 14**	**Cells from remainder of embryo FISH probes for 13, 14, 18, X, and Y**	**Classification**	**Possible segregation** (paternal gamete type)
1	A: Monosomy 13, and 14 B: Lost during FISH	1: Normal for 13,18, tetrasomy 14, disomy X,Y 2: Normal for 13,14, trisomy 18, disomy X,Y 3: Normal for 13, tetrasomy 14, monosomy 18, trisomy X, disomy Y	Chaotic	Unknown
2	A: Monosomy 13	1: Monosomy 13 2: Monosomy 14 3: Monosomy 14 4: Normal for 13,14 5: Trisomy 13, monosomy 14 Nuclei lost after 2nd FISH round	Chaotic	Unknown

Embryo 2nd cycle	**Biopsied cell(s) FISH probes for 13 and 14**	**Cells from remainder of embryo FISH probes for 13, 14, 18, X, and Y**	**Classification**	**Possible segregation (paternal gamete type)**
3	A: Lost during FISH B: Normal	Transferred	Normal or balanced	Alternate
5	A(fragmented): Monosomy 14 B: Fragments, no signals	1: Trisomy 14 2,5,6: Normal for 13 and 14 3: Monosomy 14 4: Monosomy 13 Nuclei lost after 2nd FISH round	Mosaic balanced/aneuploid	Alternate combined with post-zygotic errors
6	A,B: Normal	Transferred	Normal or balanced	Alternate
7	A: Lost during FISH	1: Monosomy 13,14 2: Normal for 13,14 Nuclei lost after 2nd FISH round	Mosaic balanced/aneuploid	Alternate combined with post-zygotic errors
8	A: Monosomy 14, normal for 13 B: Monosomy 13,14	Lost during spreading	Chaotic	Unknown
9	A: Monosomy 13, trisomy 14	1: Normal for 13,18,Y, trisomy 14, disomy X 2: Monosomy 13, trisomy 14, normal for 18,Y, disomy X 3: Normal for 13,18, trisomy 14, tetrasomy X, disomy Y 4: Normal for 13,18,Y, trisomy 14, disomy X	Mosaic aneuploid	Adjacent-1 combined with post-zygotic errors

3.4 PGD for two couples with possible gonadal mosaicism for trisomy 21

Two couples that were phenotypically normal were referred to our centre in order for PGD for trisomy 21 to be carried out. Both couples had trisomy 21 conceptions in the past, and both had experienced terminations of affected pregnancies. Cytogenetic investigation of lymphocytes took place for both partners in each of the two cases, and all karyotypes appeared to be normal 46,XX and 46,XY. Maternal ages were 39 and 37, respectively.

Two different strategies were applied for these two couples. In both protocols dual FISH probe combinations were employed. More specifically, in the first case two locus-specific probes were used for the detection of chromosomes 13 and 21. In the second case one locus-specific and one telomeric probe, both mapping on chromosome 21 were used.

3.4.1 Case F: PGD for a couple with ectopic pregnancies and one trisomy 21 conception

The female partner had experienced two ectopic pregnancies, which led to a bilateral salpingectomy. These pregnancies were not karyotyped. Another pregnancy was achieved after an IVF cycle, but was terminated at 20 weeks gestation, as the fetus was prenatally diagnosed to have Down's syndrome. The karyotypes of both partners were normal 46,XY and 46,XX. Two PGD cycles and a frozen embryo transfer were carried out for this couple. Maternal age was 39 at the time of treatment.

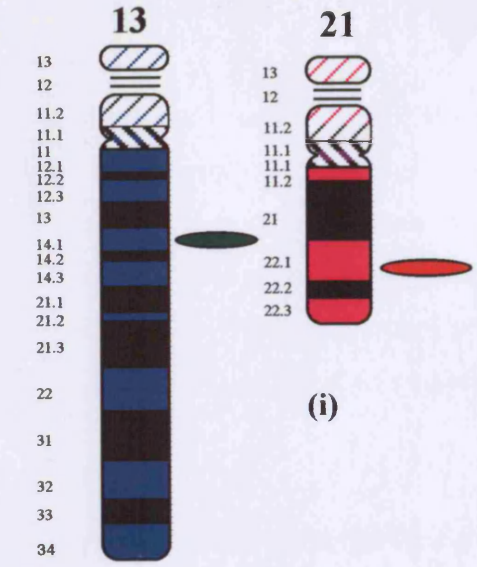
The FISH protocol devised for this couple employed two probes, one hybridising on chromosome 13 and the other on chromosome 21. Both probes were locus-specific and commercially available (Vysis/Abbott, UK). Chromosome 13 was investigated for two reasons. The first was the two ectopic pregnancies that the female partner had experienced in the past. These were not karyotyped, but it has been suggested that chromosomal abnormalities are more frequent in ectopic pregnancies (Karikoski *et al.*, 1993). Moreover, the second probe would act as an additional indicator of chromosome status. Thus, the probe for chromosome 13 mapped on 13q14 and was labelled in SG, whereas the probe for chromosome 21 hybridised on 21q13.2-q22.2 and was labelled in SO. This probe combination was tested on control (46,XY) lymphocytes, resulting in a FISH efficiency of 98%. Signals for both probes were intense and clearly visible on both

metaphase chromosomes and interphase nuclei. This probe combination was subsequently tested on lymphocytes of both the male and the female partner. Four hundred interphase nuclei were scored, in order to identify if either of the two partners was mosaic for trisomy 21. The results obtained were comparable to those seen on lymphocyte controls, i.e. 95% of nuclei had the expected number of signals for both probes. Thus, neither of the two partners appeared to be a trisomy 21 mosaic in these somatic cells.

Standard IVF was used for fertilisation during the first PGD cycle. Nineteen oocytes were collected from the female partner and 17 fertilised normally (fertilisation rate 89.5%). All seventeen embryos were considered suitable for biopsy on day 3 of preimplantation development. Two blastomeres were taken from twelve embryos consisting of 6-8 cells, whereas one blastomere was biopsied from the remaining 5, as they contained 4-5 cells. In total 24 blastomeres were spread and FISH results were obtained from ten. The remaining fourteen were either lost during spreading or FISH, or no signals were visible for various reasons. According to these results, six embryos were characterised as chromosomally balanced. Out of these, two were transferred to the female partner, while the rest were frozen. No clinical pregnancy was established after this first cycle.

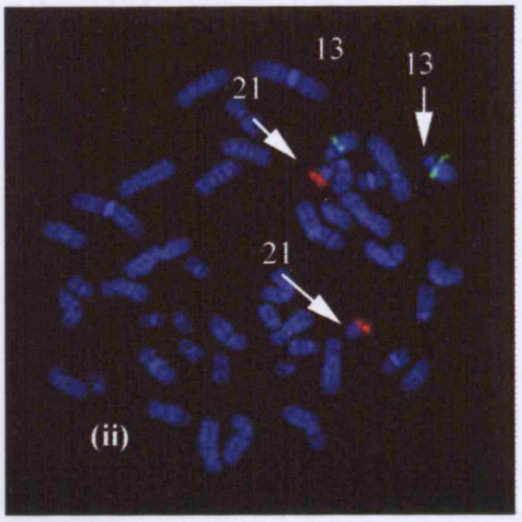
The ten non-transferred embryos along with one that had showed three pronuclei and another one that was of poor quality, both of which were not biopsied, were spread onto slides. FISH analysis took place with the application of the same probe combination that was used on the day of the diagnosis, so as for the latter to be confirmed. These embryos were not investigated by further FISH analysis, as their nuclei were of very poor morphology. FISH results were obtained for eight of these embryos, which were classified as follows: one mosaic diploid/aneuploid (12.5%), one mosaic balanced/chaotic (12.5%), one mosaic aneuploid/chaotic (12.5%), and five fully chaotic (62.5%). Another embryo transfer followed this first PGD cycle, using two of the embryos that were identified as normal and were frozen at the time, but again no pregnancy ensued.

Fig. 3.8: PGD for Case F, Possible Gonadal Mosaicism for trisomy 21

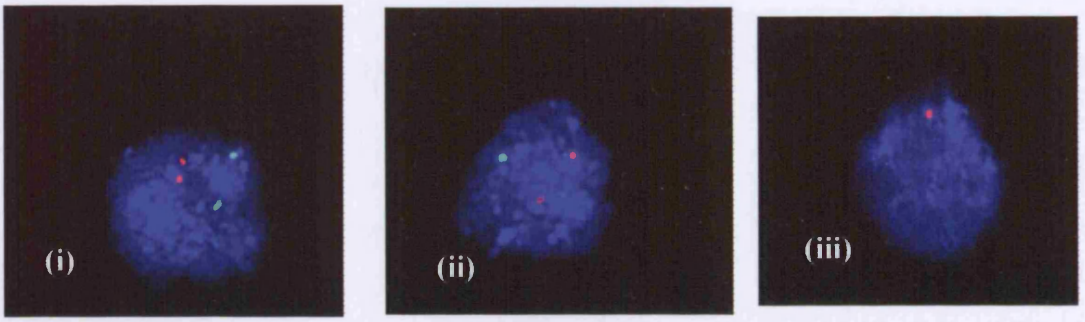


LSI 13 probe-13q14 (SG)

LSI 21 probe-21q22.13-q22.2 (SO)



a: FISH Analysis of Patient Lymphocyte Metaphase



b: FISH Analysis of Biopsied Blastomeres for PGD

Fig. 3.8: a (i) Dual-colour probe combination devised for the PGD for Case F.
(ii) The same probes applied on a metaphase from the female partner of this couple.
b Blastomeres from preimplantation embryos generated by this couple during two PGD cycles:
(i) Blastomere with a normal complement for the chromosomes examined (2 green, 2 red).
(ii) Blastomere monosomic for 13 (1 green), normal for 21 (2 red).
(iii) Blastomere monosomic for 21 (1 red), nullisomic for 13 (0 green).

Case F Results

In the second PGD cycle, twenty-four oocytes were collected, and twenty fertilised (fertilisation rate 83.3%). Fourteen normally fertilised embryos, one of which initially showed 0 pronuclei (0 PN), and one that showed three pronuclei (3 PN) were considered fit for biopsy. Two blastomeres were obtained from the fourteen normally fertilised embryos, all of which consisted of 6-9 cells, whereas one blastomere was obtained for the 0 PN and the 3 PN embryos. FISH results were obtained from twenty-one blastomeres, and three embryos were identified as chromosomally normal for the chromosomes tested. These were transferred to the female partner. Similarly with the other two transfers, no clinical pregnancy was achieved.

The non-transferred embryos were analysed with the same probe combination used on the day of the diagnosis. These embryos and the biopsied blastomeres from the embryos that were transferred were also examined further with a probe set, including the centromeric probe for chromosome 15 labelled in SO, the centromeric probe for chromosome 18 labelled in SA and the locus-specific probe for chromosome 22 labelled in SG. All probes were commercial (Vysis/Abbott, UK) and are shown in Tables 2.2 and 2.3. From the results obtained after the two rounds of FISH the embryos were classified as follows: five were characterised as mosaic aneuploid/diploid (41.66%), five as mosaic aneuploid (41.66%), one as chaotic (8.3%%) and one as normal for all the chromosomes examined (8.3%). The biopsied blastomere from this embryo was lost during the FISH procedure, and hence no results were obtained during diagnosis. Details of chromosome constitutions for biopsied cells and non-transferred embryos for both cycles are shown in Table 3.5. Cytogenetic analysis of all nuclei according to the ISCN nomenclature is described in Appendix B. Fig. 3.8 demonstrates a patient metaphase with the probes employed during diagnosis, along with pictures of a normal and two abnormal blastomeres.

In summary, during two PGD cycles that took place for this couple, fourty-three oocytes were collected and thirty-seven fertilised, resulting in a fertilisation rate of 86%. Out of the thirty-three embryos that were biopsied, conclusive FISH results were obtained from twenty-nine. Ten (34.5%) of those were classified as normal, six (20.7%) as mosaic diploid/aneuploid, five (17.24%) as mosaic aneuploid, one (3.45%) as mosaic aneuploid/chaotic, one as mosaic balanced/chaotic (3.45%) and six (20.7%) as fully chaotic. Oocytes and sperm from the couple were not available for FISH analysis, and thus the possibility of one of them being a gonadal mosaic for trisomy

21 was not investigated directly. However, examination of the spare embryos especially from the second PGD cycle, revealed abnormal numbers for chromosomes other than those that were investigated during the diagnosis, combined with chromosome 21. The inability of this couple to establish a clinical pregnancy could be attributed to various factors, including the advanced maternal age (39 years at the time of treatment), and the high number of abnormal or mosaic embryos that were produced in both cycles. The origin of the abnormalities scored in the non-transferred embryos was different between the two cycles. Hence, in the first cycle all 8 spare embryos were highly mosaic, chaotic in their majority (5). In the second cycle, however, most of the non-transferred embryos (12) were either mosaic aneuploid (5) or mosaic diploid/aneuploid (5), and only one was characterised as fully chaotic. Of all the embryos showing aneuploidy, only two were deduced to be of meiotic origin. Both (14, 20) were monosomic for 21, and embryo 14 was also trisomic for 13. Taking into account the three embryos that were transferred to the mother, the latter results in an aneuploidy rate of 2 in 16 for chromosome 21 in the second cycle. The mosaicism observed during this second cycle was mostly due to mitotic non-disjunction, contrary to the chaotics observed during the first PGD cycle. It is possible, that the advanced maternal age was the factor leading to both the meiotic abnormalities, but to the post-zygotic as well. Evidence for the latter has also been provided by Munne and colleagues (2002b) in their study of mosaicism at the cleavage-stage of embryo development and the effect of maternal age.

Table 3.5: Case F, possible gonadal mosaicism for trisomy 21; FISH analysis of biopsied cells and non-transferred embryos in two PGD cycles.

Embryo 1st cycle	**Biopsied cell(s) FISH probes for 13 and 21**	**Cells from remainder of embryo FISH probes for 13 and 21**	**Embryo classification**
1	Nucleus lost during spreading	1,2,3,4,5: Normal for 13 and 21 6,7,8,9,10,11,12: Tetrasomy 13 and 21 13,14: Trisomy 13, normal 21	Mosaic diploid/aneuploid
2	A: Normal for 13 and 21 B: Lost during spreading	Frozen	Normal for chromosomes 13 and 21
3	A: Lost during FISH B: Normal for 13 and 21	Frozen	Normal for chromosomes 13 and 21
4	A: No signals visible	Fragmented nuclei with no signals	Inconclusive
5	A,B: Normal for 13 and 21	Transferred	Normal for chromosomes 13 and 21
6	A: Normal for 13 and 21	Frozen	Normal for chromosomes 13 and 21
7	A,B: Normal for 13 and 21	Frozen	Normal for chromosomes 13 and 21

Embryo 1st cycle	**Biopsied cell(s) FISH probes for 13 and 21**	**Cells from remainder of embryo FISH probes for 13 and 21**	**Embryo classification**
8	Both nuclei lost during spreading	1,2: Tetrasomy 13, normal 21 3,4: Nullisomy 13, normal 21 5,6: Nullisomy 13, monosomy 21 7,8,9: Tetrasomy 13,21 10: Normal 13,21 11: Tetrasomy 13, five copies for 21 12: Nullisomy 13, tetrasomy 21	Chaotic
9	A: Signals not visible clearly during initial diagnosis. Nucleus was re-investigated, whilst embryo was frozen. Signals were not clear during re-investigation B: Lost during FISH	Frozen due to required re-investigation	Inconclusive
10	A: Inconclusive, due to background fluorescence	Very faint nuclei, inconclusive results	Inconclusive
11	Not biopsied due to poor quality	1: Normal for 13, tetrasomy 21 2: Nullisomy 13,21	Chaotic
12	A: Normal for 13, trisomy 21	1,2: Tetrasomy 13,21 3,4: Nullisomy 13,21	Chaotic

Embryo **1st cycle**	**Biopsied cell(s)** **FISH probes for 13 and 21**	**Cells from remainder of embryo** **FISH probes for 13 and 21**	**Embryo classification**
13	A: Normal for 13, monosomy 21 B: Lost during FISH	Fragmented nuclei with no signals	Inconclusive
14	A,B: Lost during FISH C: Trisomy 13,21	1,2,3: Nullisomy 13, tetrasomy 21	Chaotic
15	A: Normal for 13 and 21 B: Large hole in middle of nucleus, one signal for 13	Frozen	Inconclusive
16	A,B: Normal for 13 and 21	Transferred	Normal for chromosomes 13 and 21
17	A: Lost during FISH B: Normal for 13, trisomy 21	1: Normal for 13, tetrasomy 21 2,3: Tetrasomy 13,21 4: Normal for 13 and 21	Chaotic
18	A: Normal for 13,21 and one micronucleus with monosomy 21 B: Lost during spreading	1,2,3,4: Tetrasomy 13, trisomy 21 5,6,7,8,9,10,11,12,13,14,15,16,17: Normal for 13,21 18,19,20,21,22,23: Tetrasomy 13,21 24,25,26,27: Nullisomy 13,21 28: Eight copies of 13,21 Two binucleate cells: Normal 13,21 in each of the nuclei	Mosaic balanced/chaotic
3PN	Not biopsied	1,2,3,4,5,6,7,8,9,10,11: Trisomy 13,21 12: Normal for 13, trisomy 21 13: Trisomy 13, normal for 21 14: Monosomy 13, trisomy 21 15,16: Normal for 13,21	Mosaic aneuploid/chaotic

Embryo 2nd cycle	**Biopsied cell(s) FISH probes for 13, 21, 15, 18, and 22**	**Cells from remainder of embryo FISH probes for 13, 21, 15, 18, and 22**	**Classification**
1	Not biopsied due to poor quality	1,4: Normal for 13,21,15.18.22 2: Normal for 13,21, monosomy 15,18,22 3: Monosomy for 13,21, lost after 2nd round of FISH 5: Nullisomy 13,21,18,22, monosomy 15 6: Normal for 13,21,18,22, monosomy 15	Mosaic diploid/aneuploid
2	A: Lost during FISH	1,2,3,4,5,6: Normal for 13,15,18,21,22	Normal for all chromosomes tested
3	A: Nucleus covered with cytoplasm, two signals for 21 visible, no signals visible for 13 B: Normal for 13,21 Nuclei lost after 2nd round of FISH	Transferred	Normal for 13 and 21
4	A: Normal for 13,21,15,18,22 B: Lost during FISH	Transferred	Normal for chromosomes tested
5	A: Monosomy 13, normal for 21 B: Normal for 13 and 21	1: Monosomy 13,15,18,22, normal for 21 2: normal for 13,15,18,21,22, 3: Normal for 13,21,15,22, trisomy 18 4: Monosomy 13,21, trisomy 18, normal for 15,22	Mosaic aneuploid

Embryo 2nd cycle	**Biopsied cell(s) FISH probes for 13, 21, 15, 18, and 22**	**Cells from remainder of embryo FISH probes for 13, 21, 15, 18, and 22**	**Classification**
6	A: Normal for 13 and 21 B: Normal for 13, trisomy 21	1: normal for 13,21, trisomy 22, nullisomy 15,18 2: monosomy 13, normal for 15,18,21,22 3: Trisomy 13,21, lost after 2nd round of FISH 4,6: Normal for 13,21,15,18,22 5: Trisomy 13, normal for 21, lost after 2nd round of FISH 7: Normal for 13,21,15,22, monosomy 18 8: Normal for 13, monosomy 21, lost after 2nd round of FISH 9: Normal for 13,21,15,22, trisomy 18	Mosaic diploid/aneuploid
7	A: Normal for 13 and 21 B: Normal for 13, monosomy 21	1: trisomy 13, normal for 21,22, monosomy 15,18 2: normal for 13,21,15,18, trisomy 22 3,4,5: Normal for 13,15,18,22, monosomy 21 6: Trisomy 13, normal for 21,15,18,22 7: Normal for 13,21, lost after 2nd round of FISH	Mosaic aneuploid

Embryo 2nd cycle	**Biopsied cell(s) FISH probes for 13, 21, 15, 18, and 22**	**Cells from remainder of embryo FISH probes for 13, 21, 15, 18, and 22**	**Classification**
9	A: Trisomy 13, normal for 21 B: Lost during FISH	1: Monosomy 13,21, normal for 15,18,22 2: Normal for 13,21,15,18,22 3: Monosomy 13,21,15,18,21 4: Normal for 13,21,18,22, trisomy 15 5: Monosomy 13, normal for 21,18, trisomy 15,22 6: Monosomy 13, normal for 21,15,22, five copies of 18	Mosaic diploid/aneuploid
10	A: Very faint nucleus, no signals visible B: Lost during FISH	All nuclei were fragmented and no signals were visible	Inconclusive
11	A: Nullisomy 13, monosomy 21 B: Anucleate	1,6,9: Normal for 13,21,18,22, monosomy 15 2: Normal for 13,15,18,22, monosomy 21 3,4: Normal for 13,21,15,18, trisomy 22 5: Monosomy 13, normal for 21,15,18,22 7: Normal for 13,21,15, trisomy 18, monosomy 22 8: Normal for 13,21, lost after 2nd round of FISH 10: Normal for 13,21,15,18,22	Mosaic diploid/aneuploid

Embryo 2nd cycle	**Biopsied cell(s) FISH probes for 13, 21, 15, 18, and 22**	**Cells from remainder of embryo FISH probes for 13, 21, 15, 18, and 22**	**Classification**
12	A: Trisomy 13,21 B: Trisomy 13, normal for 21	1: Nullisomy 13,21, trisomy 22, normal for 15,18 2: Monosomy 13,21,15, trisomy 22, normal for 18 3: Nullisomy 13,21,22, trisomy 15, monosomy 18 4: Normal for 13,18, monosomy 21,15, trisomy 22 5: Trisomy 13,21, normal for 15,18,22 6: Trisomy 13, normal for 21, lost after 2nd round of FISH	Mosaic aneuploid
13	A: Normal for 13,21 B: Nullisomy 13,21	Nuclei lost during 1st round of FISH	Inconclusive
14	A: Trisomy 13, monosomy 21 B: Nucleus covered with cytoplasm, no signals visible	1,2,3: Trisomy 13, monosomy 21, lost after 2nd round 4: Trisomy 13,22, monosomy 21, normal for 15,18 5: Trisomy 13, monosomy 21, normal for 15,18,22	Mosaic aneuploid
15	A,B: Normal for 13,15,18,21,22	Transferred	Normal for all chromosomes tested

Embryo 2nd cycle	**Biopsied cell(s) FISH probes for 13, 21, 15, 18, and 22**	**Cells from remainder of embryo FISH probes for 13, 21, 15, 18, and 22**	**Classification**
16	A: Normal for 13,21 B: Normal for 13, monosomy 21	1: Monosomy 13, trisomy 22, normal for 21,15,18 2: Normal for 13,15,18,22 monosomy 21 3: Normal for 13,18, trisomy 21,15, monosomy 22 4: Normal for 13,21,18,22, trisomy 15 5: Normal for 13,21, trisomy 15, monosomy 18,22 6: Trisomy 13, normal for 21, monosomy 15,22, tetrasomy 18	Mosaic aneuploid
19	Not biopsied	1: Five copies of 13, trisomy 21, four copies of 15, monosomy 18,22 2: Lost during 1st round of FISH	Inconclusive, due to lack of information, possibly chaotic (results of 1 nucleus)
20 (0 PN)	A: Normal for 13, monosomy 21	1: Normal for 13, monosomy 21,18,22, nullisomy 15	Mosaic diploid/aneuploid (results of 2 nuclei)
21 (3 PN)	A: Lost during FISH	1: Monosomy 13, normal for 21,15,18,22 2: Tetrasomy 13, trisomy 21,18,22, normal for 15	Chaotic (results of 3 nuclei)

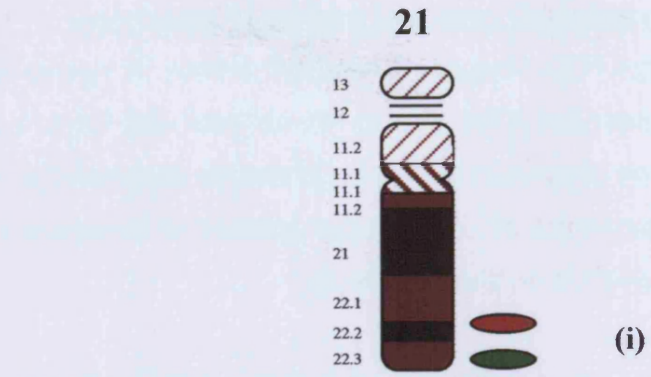
3.4.2 Case G: PGD for a couple with recurrent trisomy 21 conceptions

This couple was referred for PGD because of recurrent trisomy 21 conceptions. More specifically, the female partner had given birth to two children with Down's syndrome, and one more pregnancy was terminated after being prenatally diagnosed to be trisomic for chromosome 21. The karyotypes of both partners appeared to be normal 46,XY and 46,XX, and maternal age was 37 at the time of referral.

A dual colour FISH strategy was developed for this case, involving the use of a band-specific probe for chromosome 21 labelled in red, and the subtelomere probe for the same chromosome, labelled in green. Both probes were commercially available (Oncor, UK). Testing of this probe combination on control lymphocytes revealed that both hybridised to the expected regions of chromosome 21 and gave clear signals, which were sometimes split. When observing a metaphase spread, both probes hybridised very close together. This was not the case for interphases, though, and as embryonic blastomeres are at the interphase stage, the latter would not pose a problem during diagnosis. Scoring of control interphases (n=200) resulted to an efficiency of 94.5% for this probe combination.

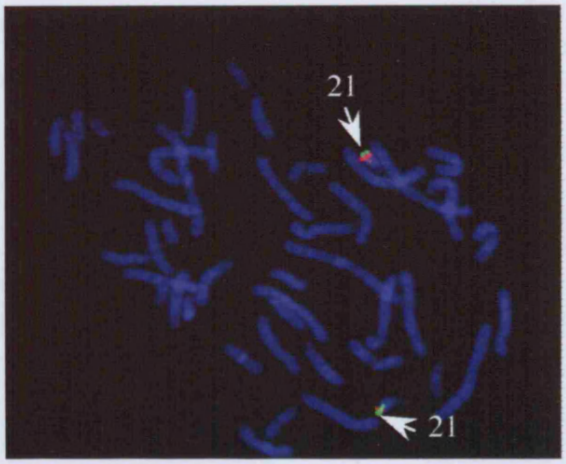
This probe combination was then evaluated on lymphocytes of both the male and the female partner. The signals observed for both probes were equally good as those observed on control lymphocytes. In order to identify if either of the two partners was mosaic for trisomy 21 or had a structural rearrangement involving this chromosome, 400 interphase nuclei were scored for both the male and the female partner. Thus, for the female partner the two probes showed the expected number of signals in 93.4% of nuclei. The latter was comparable to the probe combination efficiency observed in the control lymphocytes. Different results were obtained from the scoring of the lymphocytes of the male partner. In his case, approximately 10 (25%) cells of the 400 scored consisted of three signals for both probes, resulting in an efficiency of detection of disomy of 91.25%. The detection of 25% of cells as trisomic for chromosome 21 is a significant finding in view of the family history. From the above it was suspected that the male partner could be a gonadal mosaic for trisomy 21. However, to confirm this, FISH analysis of his sperm would have to take

Fig. 3.9: PGD for Case G, Possible Gonadal Mosaicism for trisomy 21

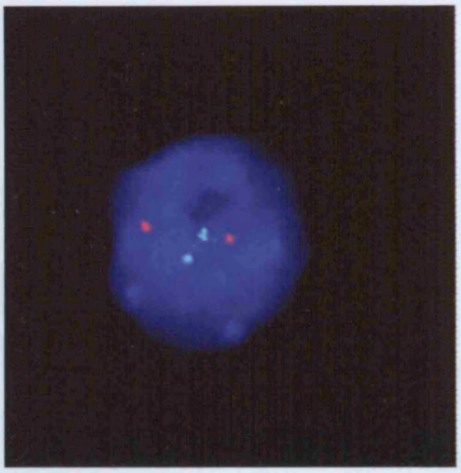


Band-specific probe for chromosome 21- 21q22.1 (Red)

Subtelomere probe for chromosome 21- 21qter (Green)



a: FISH Analysis of a Patient Lymphocyte metaphase



b: FISH Analysis of a Patient interphase

Fig, 3.9: (i) The two different probes targeting chromosome 21. This strategy was devised for the PGD of Case G.

a This probe combination applied on a metaphase from the female partner of this couple.

b The same probe combination applied on a lymphocyte interphase from the male partner.

place. This couple decided not to proceed with treatment, because of family problems. Thus, no further investigations were carried out. Fig. 3.9 demonstrates a metaphase spread showing the positions of hybridisation for both probes, and also an interphase nucleus after FISH with these probes.

3.4 Summary of the outcome of nine PGD cycles

FISH protocols were devised for seven couples, all of who were referred to our centre, as one of the two partners was a carrier of a chromosomal abnormality. Out of these, five were treated, whereas from the remaining two, one couple came through in 2004, and the other decided against PGD treatment due to family problems.

The optimisation of different 3-probe combinations and the extensive chromosomal examination of all embryos characterised as abnormal during diagnosis in order to establish parental segregation patterns and elucidate the underlying mechanisms leading to the generation of highly mosaic and chaotic embryos in this patient group were addressed in this part of the project.

Nine cycles (IVF or ICSI) took place and PGD was carried for two balanced reciprocal translocations (3 cycles), two balanced Robertsonian translocations (4 cycles), and one couple with possible gonadal mosaicism for trisomy 21 (2 cycles plus one frozen embryo transfer). Maternal ages ranged from 25 to 39 years with a mean age of 32.6 years.

During these nine PGD cycles 136 oocytes were retrieved and 104 were fertilised successfully (76.5%). Eighty-eight (84.6%) of the embryos created were considered suitable for biopsy on day 3 of preimplantation development. Results of FISH analysis of the blastomeres obtained revealed that only 21 (23.8%) of these embryos were characterised as normal or balanced and were transferred to the female partners. Ideally the identification of the balanced embryos was based on FISH results being acquired from two biopsied cells. The latter was not feasible in every case, as nuclei were either lysed or lost during spreading or the FISH procedure, or were covered with cytoplasm, or were of poor morphology or quality without any signals. Eight cycles led to the transfer of embryos, one of which resulted in a clinical pregnancy and subsequently a normal live birth. Table 3.6 demonstrates the summary outcome of these nine PGD cycles.

The remaining non-transferred embryos and some others that were not biopsied due to abnormal fertilisation (0 PN or 3 PN) were analysed with the same probe combination

that was applied on the day of the diagnosis. In two of the five couples being treated, two non-transferred embryos were identified as balanced during this re-analysis. In the first case, the biopsied cell, had also given a normal result, but this embryo was not selected for transfer, as three more were discovered to have a balanced chromosome complement and for these, diagnosis was based on two embryonic nuclei. In the second case, the biopsied nucleus was covered with cytoplasm, making the visualisation of signals impossible. Re-analysis of this embryo showed a normal complement for the chromosomes in question, but also for another set of probes that were applied to investigate its chromosome constitution further. In six out of the nine cycles, the spare embryos were further examined for chromosomes not involved in the initial diagnosis. Poor morphology and the DNA quality of embryonic nuclei did not permit this for the three remaining cycles. The purpose of this second FISH analysis was to gain more information on embryonic chromosomes, leading in this way to an accurate classification of abnormalities, but also identification of specific segregation modes in the parental gametes, or different types of mitotic errors at the post-zygotic stages of embryonic development. Tables 3.7-3.9 show the different groups of embryos for two patients with reciprocal translocations, two with Robertsonian translocations, and one with possible gonadal mosaicism for trisomy 21.

In total 94 embryos were investigated, some of which being characterised as 0 PN or 3 PN initially. Out of these, twenty-five (26.6%) were grouped as normal or balanced, while 69 (73.4%) were characterised as abnormal. The latter category consisted of the following:

1. Embryos that were uniformly abnormal or with inconclusive result (13.8%)
2. Embryos classified as diploid mosaic (10.6%)
3. Embryos classified as aneuploid mosaic (10.6%)
4. Embryos classified as chaotic (38.3%).

A summary of these results is seen in Table 3.10.

It is obvious from the above percentages that chaotic embryos predominate among the abnormal ones, and were produced by all couples in this study in different frequencies. It has been postulated that the generation of highly mosaic or even chaotic embryos is largely patient specific and can be used as a predictor of PGD

outcome (Delhanty *et al.*, 1997). The latter hypothesis was confirmed in this study. Hence, the couple in case B produced the second lowest number of chaotic embryos in both cycles, and they were able to achieve a clinical pregnancy and a normal live birth after the second PGD cycle. The female partner was the second youngest in this group of patients. On the contrary, the couples in cases A and F both produced a high number of chaotic embryos and failed to establish a clinical pregnancy. The female partner in this case A was the youngest in the group, whilst the female partner in case F was the oldest. Maternal ages and number of mosaic and chaotic embryos were similar for the remaining cases. Hence, even though all the rest of the couples had embryos transferred in all cycles, no pregnancies ensued. Attributing factors, apart from the frequent generation of highly abnormal embryos, could include the advanced maternal age, embryo morphology and their general developmental potential. These will be discussed in a subsequent section.

To conclude, the frequent observation of highly abnormal embryos being produced from couples investigated for chromosomal abnormalities, illustrates the necessity of the biopsy of two blastomeres, where possible. In this way, the risk of misdiagnosing an abnormal embryo as normal is significantly reduced.

Table 3.6: Summary of nine PGD cycles for five couples investigated for a chromosomal abnormality.

Case	Parental karyotype	PGD cycles	Oocytes collected	Oocytes fertilised	Embryos Biopsied/Not biopsied	Normal or Balanced	Abnormal	Embryos transferred
A	46,XY,t(5;19)(p12;p12)	1	20	15	13/2	1	12	1
B	46,XX,t(11;22)(q23.3;q11.2)	2	26	14	11/2	2	11	2
D	45,XY,der(13;21)(q10;q10)	2	22	17	15/0	6	9	6
E	45,XY,der(13;14)(q10;q10)	2	25	21	16/0	6	10	5
F	46,XX , 46,XY, suspected gonadal mosaicism for trisomy 21	2 + 1 frozen embryo transfer	43	37	33/4	10	27	7

Table 3.7: Summary of embryo classification and segregation patterns observed for three PGD cycles for two couples carrying reciprocal translocations.

Case	Parental karyotype	PGD cycles	Normal	Uniformly abnormal or inconclusive FISH result	Diploid mosaic	Aneuploid mosaic	Chaotic*	Segregation patterns in parental gametes: no. of embryos
A	46,XY,t(5;19)(p12;p12)	1	1	2	0	2	8	Alternate: 1 2:2 Adjacent 2: 1 Unknown: 8 Inconclusive: 2
B	46,XX,t(11;22)(q23.3;q11.2)	2	2	3	0	1	7	Alternate: 3 3:1 Interchange: 2 2:2 Adjacent 1: 1 Unknown: 3

*Also includes mosaic aneuploid/chaotic and mosaic balanced/chaotic

Table 3.8: Summary of embryo classification and segregation patterns observed for four PGD cycles for two couples with Robertsonian translocations.

Case	Parental karyotype	PGD cycles	Normal	Uniformly abnormal or inconclusive FISH result	Diploid mosaic	Aneuploid mosaic	Chaotic*	Segregation patterns in parental gametes: no. of embryos
D	45,XY,der(13;21)(q10;q10)	2	6	1	0	1	7	Alternate: 6 Adjacent 1: 1 Unknown: 8
E	45,XY,der(13;14)(q10;q10)	2	6	0	3	1	6	Alternate: 6 Alternate with post-zygotic errors: 5 Adjacent 1 with post-zygotic errors: 1 Unknown: 4

*Also includes mosaic aneuploid/chaotic and mosaic balanced/chaotic

Table 3.9: Summary of embryo classification and segregations observed for two PGD cycles for one couple investigated for possible gonadal mosaicism for trisomy 21.

Case	Parental karyotype	PGD cycles	Normal	Uniformly abnormal or inconclusive FISH result	Diploid mosaic	Aneuploid mosaic	Chaotic*	Segregation in embryos
F	46,XX , 46,XY	2 + 1 frozen embryo transfer	10	7	7	5	8	Diploid zygote status with post-zygotic errors: 5 Mitotic non-disjunction:6 Meiotic errors: 2 Unknown: 7

*Includes mosaic aneuploid/chaotic

Table 3.10: Summary of chromosome constitutions observed in embryos from five couples treated for chromosome abnormalities with PGD.

Case	Parental karyotype	PGD cycles	Normal	Uniformly abnormal or inconclusive	Diploid mosaic	Aneuploid mosaic	Chaotic*
A	46,XY,t(5;19)(p12;p12)	1	1	2	0	2	8
B	46,XX,t(11;22)(q23.3;q11.2)	2	2	3	0	1	7
D	45,XY,der(13;21)(q10;q10)	2	6	1	0	1	7
E	45,XY,der(13;14)(q10;q10)	2	6	0	3	1	6
F	46,XX, 46,XY, possible gonadal mosaicism for trisomy 21	2 + 1 frozen embryo transfer	10	7	7	5	8
Combined totals		Total embryos: 94 (100%)	25 (26.6%)	13 (13.8%)	10 (10.6%)	10 (10.6%)	36 (38.3%)

*Includes mosaic aneuploid/chaotic, and mosaic balanced/chaotic

Chapter 4-Results

The investigation of chromosomal abnormalities in human oocytes and corresponding polar bodies using FISH

4.1 Human metaphase II oocytes and corresponding 1st PBs

This part of the study involved the analysis of human oocytes, arrested at the metaphase stage of the second meiotic division and their corresponding 1st PBs, when these were available, with the application of FISH. 453 oocytes and 51 PBs were spread on slides, obtained from a total of 168 patients, undergoing routine IVF or ICSI treatments. All had either failed to fertilise after exposure to sperm or were unexposed. Time in culture varied between 24-48 hours.

Of the 453 oocytes, 265 were considered suitable for FISH analysis. The remaining were discarded because they did not contain any chromosomes, were of too degenerate morphology, had too few chromosomes due to over spreading, or were immature, being arrested at meiosis I. The fact that the majority of the analysed oocytes did not have PBs, could be attributed either to the latter degenerating during culture, or being lost during the spreading process.

The patients participating in the study were not known to have abnormal karyotypes with maternal ages ranging between 22-44 years (mean 32.5). Most of the couples were being treated for infertility due to a male factor. Some of these patients came from the Assisted Conception Unit at the University College London Hospitals Trust, but the great majority of them were treated at the Assisted Conception Unit at Tayside University Hospitals NHS Trust, Dundee, Scotland. In the latter case, all eggs (oocyte+PB) were spread on slides in Scotland and were received by post.

The aim of this part of the study was to investigate the variety of anomalies arising during the first maternal meiosis. Two hypotheses were being tested:

1. Several mechanisms are involved in maternal aneuploidy.
2. Chromosomes of all sizes could be participating. The latter was evident from molecular cytogenetic investigations of cleavage stage embryos.

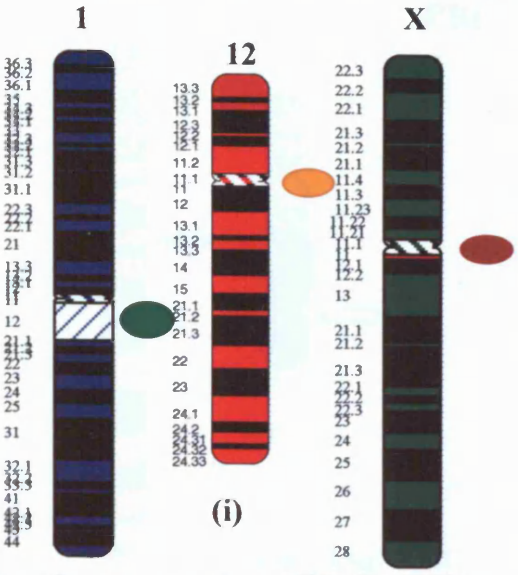
4.2 Fixation and FISH efficiency

Oocytes and PBs were fixed together and the protocol followed for most of them was as suggested by Tarkowski (Tarkowski, 1966), with some modifications described by Mahmood *et al.* (2000). An alternative spreading protocol was also applied for some cells, involving their gradual fixation on the slides (Kamiguchi *et al.*, 1993). Details of both protocols are described in 2.2.3.4.

It was not possible to successfully subject all 265 cells to three sequential rounds of FISH, since some chromosome loss occurred during processing. Thus, after the first round, results were obtained for 51 preparations, after the second round for 29, while after the third round only seven oocytes could be detected and scored. In total 180 cells were lost during this study either prior or after the different rounds of FISH analysis. Different pre-treatments of the slides were used before FISH, in an attempt to reduce this high chromosome loss. The protocol identified as the most effective involved not staining the cells with either DAPI or Giemsa, locating them under the phase microscope, and reducing both time of incubation in HCL/pepsin during the FISH procedure (5 minutes instead of 20), but also the actual pepsin concentration from 10mg/ml to 5mg/ml, as suggested by Clyde *et al.* (2001).

The details of the probes used for the investigation of all oocytes and PBs during the three consecutive FISH rounds are shown in Table 2.5 whereas the actual probe combinations applied are described in Table 2.6. Lymphocyte control slides were employed for every FISH procedure, in order to evaluate signal morphology and efficiency of the different probe combinations. All probes, either centromeric or locus-specific, gave clear and intense signals on both metaphase and interphase nuclei as FISH efficiency was calculated by scoring 200 interphase nuclei and ranged between 85-95%, depending on which probes were used for the investigation. Fig. 4.1a and b shows pictures of lymphocyte metaphases demonstrating the various probe combinations employed during the three consecutive rounds of FISH.

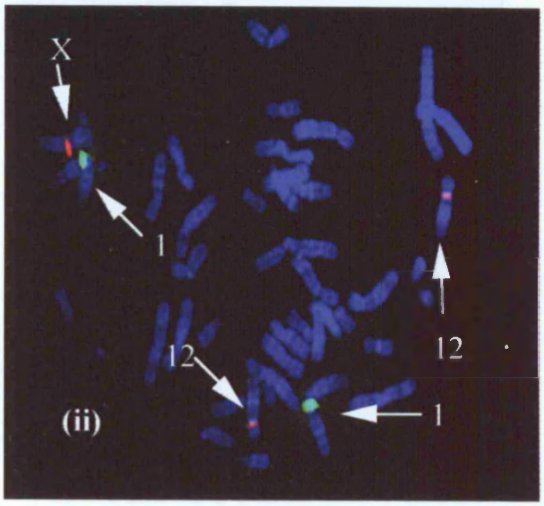
Fig. 4.1a: Two sets of centromeric probes employed for the first round FISH analysis of metaphase II oocytes and 1st PBs



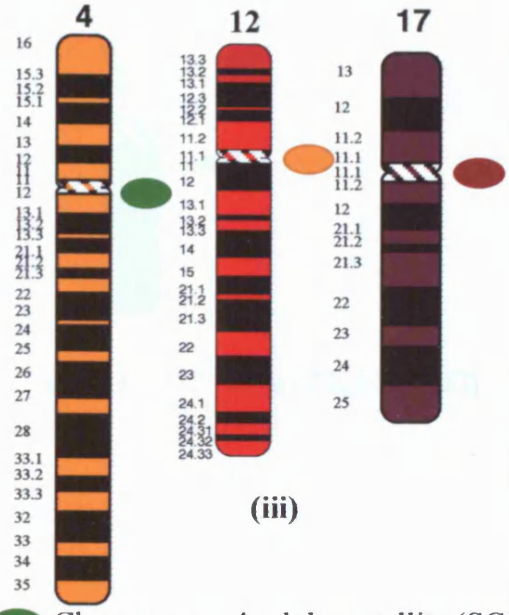
Chromosome 1- satellite III (SG)

Chromosome 12- alpha-satellite (orange)

Chromosome X- alpha-satellite (SO)



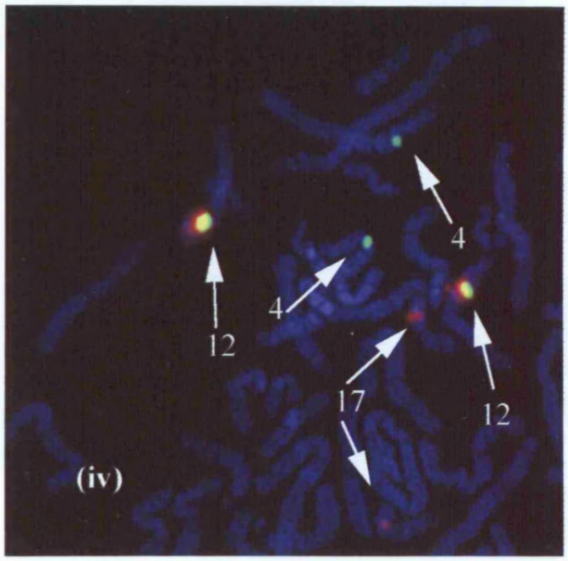
FISH of 46,XY control lymphocyte metaphase with centromeric probes for chromosomes 1 (green), 12 (orange), and X (red).



Chromosome 4- alpha-satellite (SG)

Chromosome 12- alpha-satellite (orange)

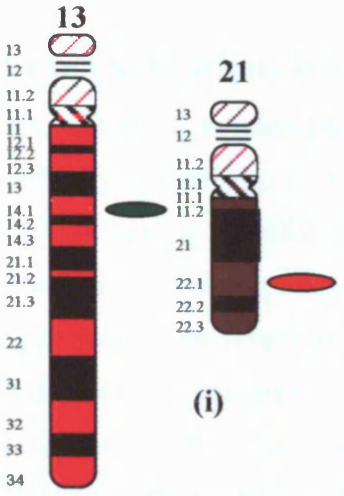
Chromosome 17- alpha-satellite (SO)



FISH of 46,XY control lymphocyte metaphase with centromeric probes for chromosomes 4 (green), 12 (orange), and 17 (red).

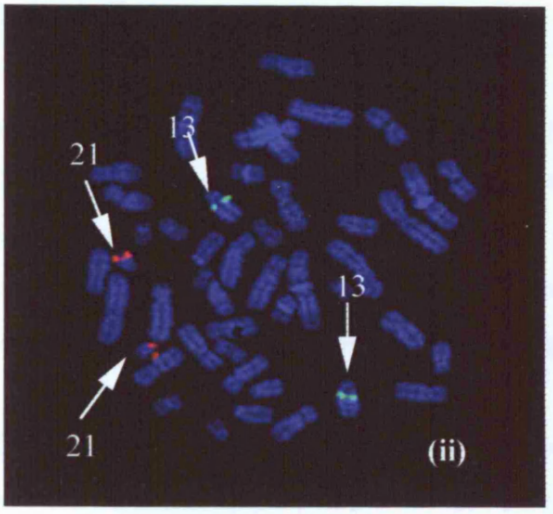
Fig. 4.1a: (i) Triple probe combination employed for the FISH analysis of oocytes and 1st PBs in Section 4.3.1. **(ii)** Three-colour FISH analysis of a metaphase spread coming from a 46,XY individual, using the probes from (i). **(iii)** Triple probe combination used for the FISH analysis of oocytes and 1st PBs in Section 4.3.4. The centromeric probes for 4 and 17 were also used for the examination of cells in Section 4.3.3. **(iv)** Three-colour FISH analysis of a metaphase spread coming from a 46,XY individual, with the probes from (iii).

Fig. 4.1b: Two sets of locus-specific probes employed for the second and third round FISH analysis of metaphase II oocytes and 1st PBs

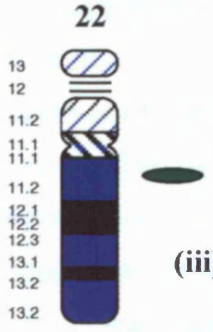


LSI 13 probe-13q14 (SG)

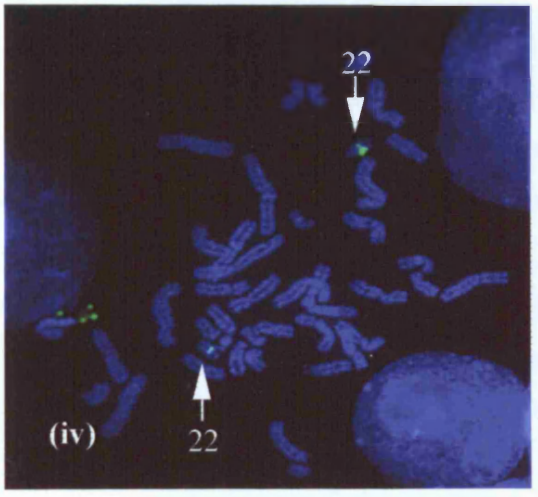
LSI 21 probe-21q22.13-q22.2 (SO)



FISH of 46,XX control lymphocyte metaphase spread with locus-specific probes for chromosomes 13 (green) and 21 (red).



LSI 22 probe- 22q11.2 (SG)



FISH of 46,XY control lymphocyte metaphase spread with locus-specific probe for chromosome 22 (green).

Fig. 4.1b: (i) Details of the two locus-specific probes employed for the FISH analysis of oocytes and 1st PBs during the second round (Section 4.4.2). **(ii)** Dual-colour FISH analysis of a metaphase spread coming from a 46,XX individual using the probes from (i). **(iii)** Details of the locus-specific probe employed for the FISH analysis of oocytes and 1st PBs during the third round (Section 4.5.1). **(iv)** FISH analysis of a metaphase spread from a 46,XY individual with the probe for 22.

4.3 First hybridisation round

Seven different chromosomes were assessed during this first round of FISH. More specifically, oocytes and corresponding 1st PBs were examined for chromosomes 1, 4, 12, 16, 17, 18, and X in different two or three-colour FISH combinations. All probes were centromeric, and were prepared in the laboratory, apart from the probe for chromosome 16, that was commercially available (Vysis, UK). These chromosomes were investigated in different combinations (Table 2.6).

An attempt to examine all 265 eggs, (124 patients), took place during this first round of FISH. Out of these, results were obtained for 51 oocytes and 11 PBs. The remaining 214 were lost during the FISH procedure. The results obtained during the first hybridisation round will be discussed below.

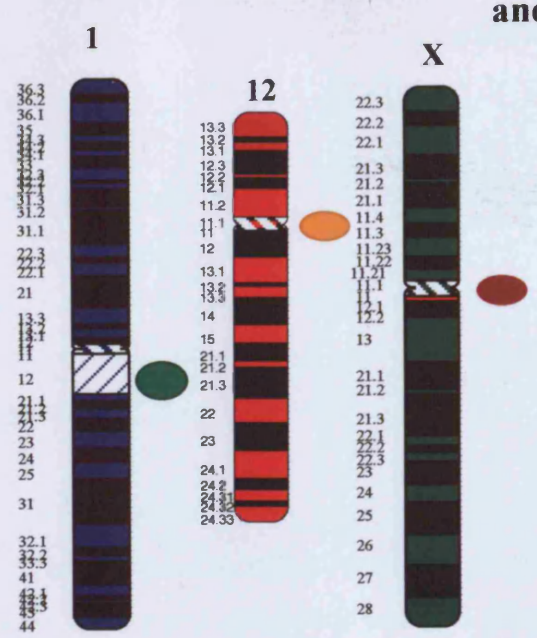
4.3.1 FISH with the centromeric probes for chromosomes 1, 12, and X

A total of 128 oocytes, some of which consisted of the 1st PB as well, underwent FISH with the centromeric probes for chromosomes 1, 12 and X. All cells were fixed on slides, normal or charged, using the modified Tarkowski protocol. Some were immersed in Acid Tyrodes prior to fixing to remove the zona pellucida. Ageing of cells occurred either by leaving them at room temperature for up to one month, or by placing them at 65°C for 12 hours, as was suggested in a report by Sandalinas and colleagues (2002). The oocytes and PBs were stained with DAPI after fixation, and identification took place under the fluorescent microscope. Some of the cells were also captured prior to FISH, with the use of the CCD camera.

The FISH procedure followed involved the standard initial incubation of cells in HCL/pepsin for 20 minutes, for cytoplasm removal. The probes used were the satellite III for chromosome 1 labelled in SG, the α-satellite for chromosome 12 labelled in orange (mix of SO+SG) and the α-satellite for chromosome X labelled in SO. Hybridisation time varied between 2-12 hours.

Analysis was feasible for eleven oocytes and two 1st PBs, from eight patients. All the remaining cells could not be detected after the FISH procedure. As all probes were complementary for centromeric regions of the chromosomes being investigated, they would either give rise to one large signal, or one doublet if sister chromatids were close to each other. Thus, premature division of chromatids would be seen as two distinct signals.

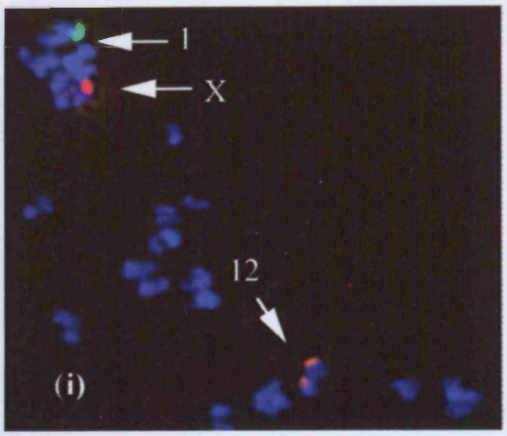
Fig.4.2: First hybridisation round- Centromeric probes for 1, 12, and X



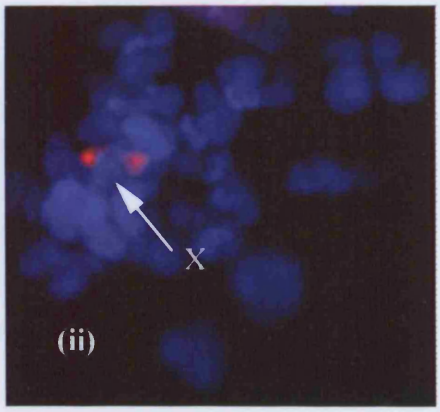
Chromosome 1- satellite III (SG)

Chromosome 12- alpha-satellite (orange)

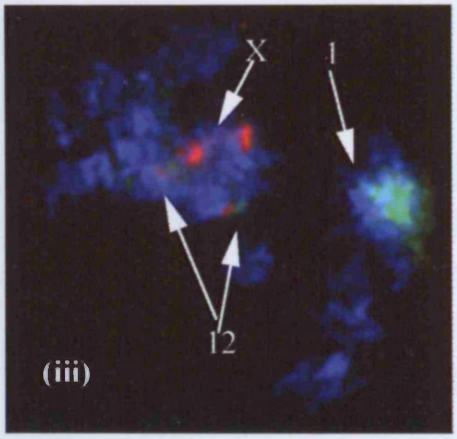
Chromosome X- alpha-satellite (SO)



(i) FISH analysis of oocyte 3782.1. The cell was classified as haploid normal for all examined chromosomes. One large signal was scored for 1 (green), and for X (red). The probe for 12 gave a doublet (orange).



(ii) FISH analysis of oocyte 3416.11. Premature division of chromosome X into its sister chromatids was apparent for this cell (2 separate red signals). No other signals were visible for chromosomes 1 and 12.



(iii) FISH analysis of PB 968.1. Two separate orange signals demonstrate the premature division of 12 to its sister chromatids. One large signal was scored for 1 (green) and a doublet for X (red), which also showed some degree of separation.

In the case of PBs, signal separation was sometimes attributed to their rapid degeneration. Oocytes and/or PBs were scored as abnormal only in the presence of extra signals, representing either a whole chromosome or a single chromatid. The absence of signals was either attributed to loss of chromosomes because of the spreading of cells or the FISH procedure or due to hybridisation failure.

Three oocytes (3782.1, 3783.2, 971.3) were characterised as being normal for chromosomes 1, 12 and X, showing clear and distinct signals for all probes. Three (5col1(t), 6col2(b), 1009.4) were normal for chromosomes 12 and X, having lost chromosome 1, two (968.1, 968.2) were normal for chromosomes 1 and X, having lost 12, and two (3855.1, 3416.12) were normal for chromosome X, having lost chromosomes 1 and 12.

Premature division of chromatids was observed in one oocyte and one PB, from two different patients. In the case of the oocyte no. 3416.11, two separate signals were visible for chromosome X only. Its corresponding 1st PB was also available, and was classified as normal for chromosome X. No other signals were observed in this pair of cells. In the case of the PB no. 968.1PB, two distinct signals were visible for chromosome 12, whereas one large signal was observed for chromosomes 1 and X. The corresponding oocyte was normal for chromosomes 1 and X, but had lost chromosome 12.

Tables 4.1 and 4.2 show the results obtained during this hybridisation round for metaphase II oocytes and corresponding 1st PBs respectively. FISH analysis of a normal oocyte, the oocyte showing the premature division of chromatids for X and the PB with the division of chromatids for 12 are seen in fig.4.2. Detailed cytogenetic analysis of all cells, using the ISCN nomenclature is given in Appendix C.

Abnormalities of either extra whole chromosomes or single chromatids were not observed in this group of cells. The high rate of chromosome loss (91.4%) that was experienced during this hybridisation round was notable. Affecting factors included the staining of the cells with DAPI, their extended exposure to fluorescent light prior to FISH and their extended incubation in pepsin during slide pre-treatment.

Table 4.1: FISH results for metaphase II oocytes investigated for chromosomes 1, 12 and X

Oocyte/patient no.	Chromosomes scored and further observations	Oocyte characterisation
5col1(t)	Two chromosomes scored Doublet for 12 Doublet for X	Normal for 12 and X
6col2(b)	Two chromosomes scored Doublet for 12 Doublet for X	Normal for 12 and X
3782.1	Three chromosomes Large signal for 1 Doublet for 12 Large signal for X	Normal for 1, 12 and X
3783.2	Three chromosomes Large signal for 1 Doublet for 12 Large signal for X	Normal for 1, 12 and X
3855.1	One chromosome Doublet for X	Normal for X
1009.4	Two chromosomes Doublet for 12 Doublet for X	Normal for 12 and X
971.3	Three chromosomes Large signal for 1 Large signal for 12 Large signal for X	Normal for 1, 12 and X
968.1	Two chromosomes Doublet for 1 Doublet for X	Normal for 1 and X
968.2	Two chromosomes Doublet for 1 Doublet for X	Normal for 1 and X

3416.11	One chromosome Two separate signals for X	Premature division of chromatids for X
3416.12	One chromosome Doublet for X	Normal for X

Table 4.2: FISH results for corresponding 1st PBs, investigated for chromosomes 1, 12, and X

PB/patient no.	**Chromosomes scored and further observations**	**PB characterisation**
968.1 PB	Three chromosomes Large signal for 1 Two separate signals for 12 Doublet for X	Separation of chromatids for chromosome X
3416.11 PB	Two chromosomes Doublet for 12 Doublet for X	Normal

Triple-colour FISH efficiency on lymphocytes: 90%

4.3.2 FISH with the centromeric probes for chromosomes 12, 16 and 18

Ten oocytes were investigated for chromosomes 12, 16 and 18, none of which contained a 1st PB. They came from a total of four patients. All cells were treated in exactly the same way as those in 4.3.1. The FISH procedure was also as described in 4.3.1, with the only difference being that the hybridisation time was just over one hour. Out of the three probes used in this round, the α-satellites for chromosomes 12 and 18 were prepared in the laboratory, labelled in orange (mix of SG+SO) and SG respectively. The centromeric probe for chromosome 16 was commercial (Vysis/Abbot, UK), mapped on satellite II of this chromosome and was labelled in SA.

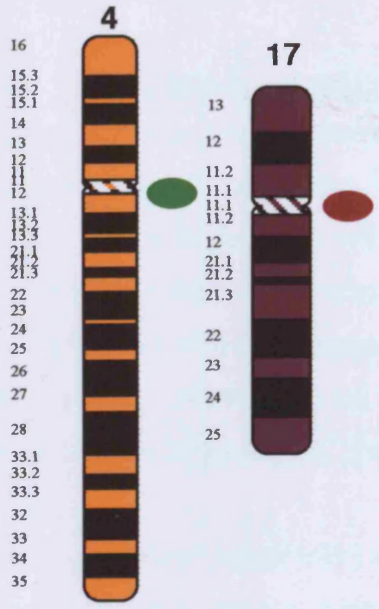
A total of four oocytes were detected on slides after this FISH round, while the remaining were all lost. No signals were visible for any of these oocytes, even though their chromosome numbers ranged between 18-20. The lymphocyte slide used as a positive control for this hybridisation round did not show signals on either metaphases or interphase nuclei for any of the probes. This observation led to the conclusion that the absence of signals was due to failure of the FISH, possibly because of the short hybridisation period.

4.3.3 FISH with the centromeric probes for chromosomes 4 and 17

Analysis of eleven eggs from three patients was attempted with the two centromeric probes for chromosomes 4 and 17. Cells were spread on slides according to either the modified Tarkowski protocol, or the gradual fixation protocol, and all were left to age at room temperature for up to 30 days. As it was suspected that prolonged exposure to fluorescent light could possibly affect the DNA quality of the oocytes and lead to them becoming loose from the slides and being lost after FISH, all cells in this group were stained with Giemsa and their identification occurred under the light microscope.

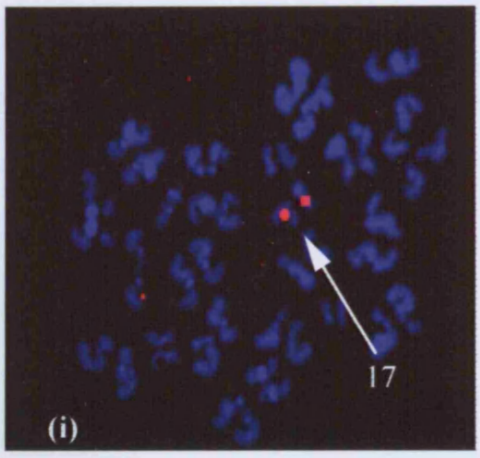
The FISH protocol was also modified and was carried out as suggested in a report by Clyde and colleagues (2001). More specifically, the time of slide incubation in HCL/pepsin was decreased to 5-8 minutes, and the concentration of pepsin was reduced from being 10mg/ml to 5mg/ml. Both probes employed in this round were prepared in the laboratory. The α-satellite for chromosome 4 was labelled in SG, whereas the one for

Fig.4.3: First hybridisation round- Centromeric probes for 4 and 17

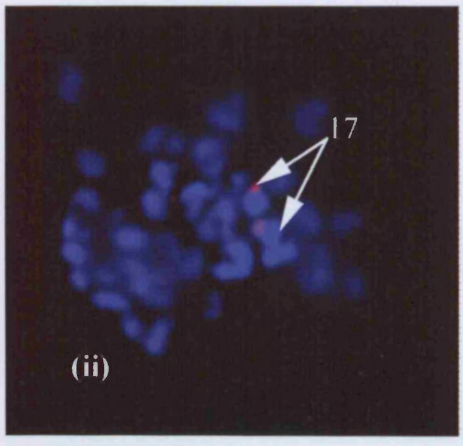


Chromosome 4- alpha-satellite (SG)

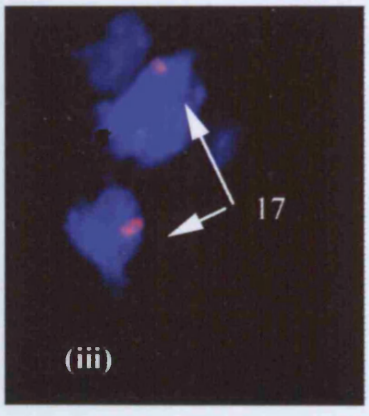
Chromosome 17- alpha-satellite (SO)



(i) FISH analysis of oocyte 3969.2. The cell showed a doublet for chromosome 17 (red), and no signals for chromosome 4.



(ii) FISH analysis of oocyte 3967.9. Premature division of chromosome 17 into its sister chromatids was observed for this cell (2 separate red signals). No signals were visible for chromosome 4.



(iii) FISH analysis of PB 3967.9. As with the corresponding oocyte, two separate red signals were visible, demonstrating the predivision of chromosome 17. Again, no signals were observed for chromosome 4.

chromosome 17 was labelled in SO. Hybridisation took place for 12 hours.

From the eleven cells being examined, FISH results were obtained for nine oocytes and four corresponding 1st PBs. Five oocytes (3969.2, 3967.8, 1084.4, 1084.6, 1084.8) from all three patients were characterised as being normal for chromosome 17, but no signals were visible for chromosome 4. Two oocytes (3969.3, 3967.3) did not show signals for any of the two probes. In both the above cases, oocyte chromosome number ranged between 10-12, the remaining possibly being lost during processing. The expected large signals for chromosome 17 were also visible for two PBs, while there was one that did not have signals for either 4 or 17.

Premature separation of chromatids was observed in two oocytes and one PB originating from two different patients. In the first case, the oocyte (3969.4) showed the expected doublet for chromosome 17 and two slightly separated signals for chromosome 4. In the second case, both oocyte (3967.9) and 1st PB were detected after FISH. Two separate signals corresponding to chromosome 17 were visible for the oocyte that did not show any signals for chromosome 4. The exact same signal pattern was observed for its 1st PB, i.e. the cell showed separation of sister chromatids for chromosome 17 and loss of chromosome 4. Two more oocytes (3967.3, 3967.8) were analysed from this patient, one of which did not show any signals, and the other was scored as normal for chromosome 17, having lost chromosome 4.
Tables 4.3 and 4.4 demonstrate the above results. FISH analysis of a normal oocyte, the oocyte and the PB showing the premature division of chromatids for chromosome 17 are seen in fig.4.3. Detailed cytogenetic analysis of all cells, using the ISCN nomenclature is given in Appendix C.

The rate of cell loss was significantly reduced in this hybridisation round, as out of the 11 oocytes examined only two (18%) could not be detected after FISH. The latter was attributed to the alterations made both in the staining and identification of the cells and the actual FISH procedure itself.

Table 4.3: FISH results for metaphase II oocytes, investigated for chromosomes 4 and 17

Oocyte/patient no.	**Chromosomes scored and further observations**	**Oocyte characterisation**
3969.2	One chromosome **Doublet for 17**	Normal for 17
3969.3	No signals	-
3969.4	Two chromosomes Two separate signals for 4 Large signal for 17	Premature division of chromatids for 4, normal for 17
3967.3	No signals	-
3967.8	One chromosome Doublet for 17	Normal for 17
3967.9	One chromosome Two separate signals for 17	Premature division of chromatids for 17
1084.4	One chromosome Doublet for 17	Normal for 17
1084.6	One chromosome Doublet for 17	Normal for 17
1084.8	One chromosome Doublet for 17	Normal for 17

Table 4.4: FISH results for corresponding 1st PBs, investigated for chromosomes 4 and 17

PB/patient no.	**Chromosomes scored and further observations**	**PB characterisation**
3969.4 PB	No signals	-
3967.3 PB	One chromosome Large signal for 17	Normal for 17
3967.9 PB	One chromosome Two separate signals for 17	Separation of chromatids for 17
1084.8 PB	One chromosome Doublet for 17	Normal for 17

Dual-colour FISH efficiency on lymphocytes: 85%

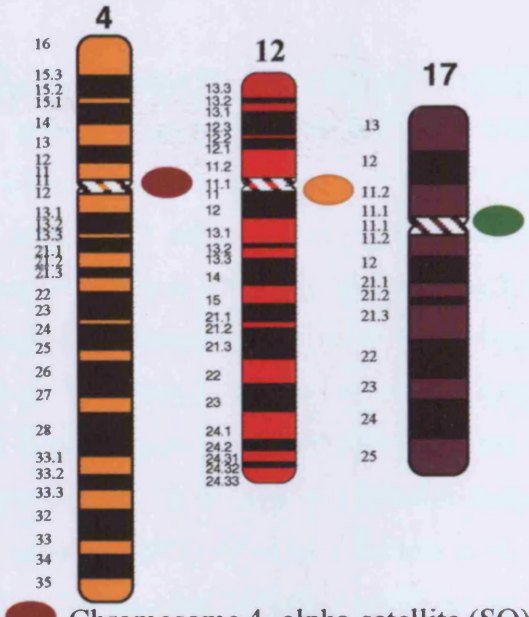
4.3.4 FISH with the centromeric probes for chromosomes 4, 12, and 17

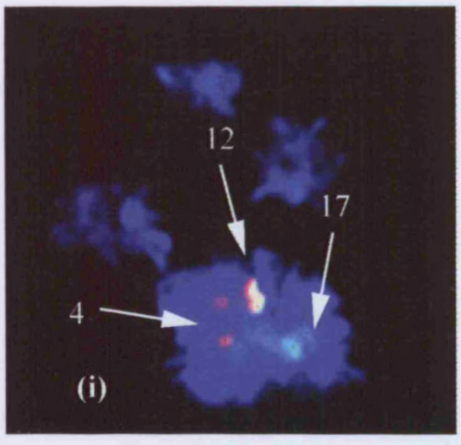
The combination consisting of the three centromeric probes for the examination of chromosomes 4, 12, and 17 was employed for the analysis of 115 oocytes, for some of which the corresponding PBs were also available. Cells were donated from sixty-one patients. All cells were fixed on slides using the modified Tarkowski protocol only, and were aged at room temperature for up to one month. Detection of oocytes took place under the light microscope. However, the chromosomes of eighty of them were stained with Giemsa, whereas the remaining thirty-five oocytes were not. The staining of the chromosomes was omitted towards the end, as it was observed that the Giemsa was affecting DNA quality in a similar way to the DAPI, leading to chromosomes either being of poor morphology, or being frequently lost after FISH. The FISH protocol itself was as described in 4.3.3 with no other modifications. The probes used were as for previous rounds, the α-satellite for chromosome 4 labelled in either SG or SO, the α-satellite for chromosome 12 labelled in orange (mix of SO+SG) and the α-satellite for chromosome 17 labelled in either SO or SG.

FISH analysis was feasible for 31 oocytes and 5 corresponding 1st PBs from a total of fourteen patients. All the remaining cells were lost during processing. Identification of either large signals or doublets for all three chromosome occurred for eight oocytes (3892.2, 4176.5, 4176.8, 4181.1, 4181.2, 4181.3, 4181.4, 4257.2), which were characterised as normal after this first hybridisation round. Two oocytes (4257.3, 4264.3) had signals for chromosomes 4 and 12 only, two (4077.1, 4292.9) for 4 and 17, two (3892.5, 4292.7) for 12 and 17, two (4091.2, 1152.1) for chromosome 4 only, five (1033.4, 3847.1, 3839.1, 4257.1, 4264.2) for chromosome 12 and two (1118.5, 1118.6) for chromosome 17 only. Absence of signals was attributed to chromosome loss during spreading or FISH.

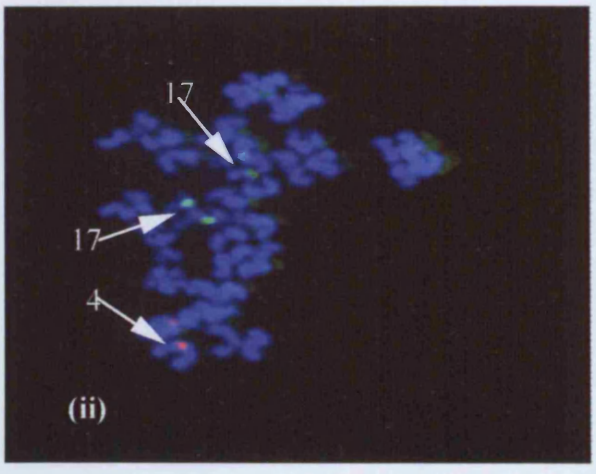
Out of the five PBs that were identified after the first hybridisation round, one (3892.1PB) showed the expected signals for chromosomes 4 and 12 and was characterised as normal for these chromosomes, and another (4181.4PB) showed the expected doublet for chromosome 4 only.

Fig.4.4: First hybridisation round- Centromeric probes for 4, 12, and 17





(i) FISH analysis of oocyte 4264.4. Premature division of chromosome 4 into its sister chromatids was observed for this cell (2 separate red signals). A double orange signal was visible for chromosome 12 and a double green signal was scored for 17. No separation was scored for the latter two.



(ii) FISH analysis of oocyte 4264.1. This cell was identified to contain an extra copy of chromosome 17 (two different double green signals). A double red signal was scored for chromosome 4, whereas chromosome 12 was absent.

Premature division of chromatids was observed in two oocytes originating from different patients, for one of which the corresponding PB was also available. For the first oocyte (4264.4), two distinct and separate signals were observed for chromosome 4, demonstrating its premature division to the two sister chromatids. This oocyte also showed the expected doublets for chromosomes 12 and 17. As far as the latter oocyte and 1st PB were concerned (1243.6), their chromosomes were clearly visible, but were mixed together. Some of the PB chromosomes could be distinguished due to their degenerate morphology. A doublet and an extra single signal were observed for chromosome 4, three distinctly separate signals were observed for chromosome 12 and four separate signals were observed for chromosome 17. Thus, for this oocyte/1st PB pair, premature division took place for one of the two chromosomes 4 with subsequent loss of the one sister chromatid, for both chromosomes 12 with similar loss of one of the chromatids and for both chromosomes 17. Division of chromatids for 4 and 17 were also observed in two different PBs.

An additional whole chromosome 17 was observed for another two oocytes, for which however, the PB was not available for analysis. In the first case (4264.1), the oocyte showed one doublet for chromosome 4, no signal for chromosome 12 and two doublets for chromosome 17, one of which demonstrated the presence of the extra chromosome. The other oocyte (1209.2) consisted of the expected doublets for chromosomes 4 and 12, but also had two doublets for chromosome 17.
These observations are shown in Tables 4.5 and 4.6. FISH analyses of an oocyte with premature division of chromatids of 4, an oocyte with an extra chromosome 17 and a PB are seen in fig.4.4. Detailed cytogenetic analysis of all cells is described in Appendix C.

The rate of chromosome loss for this hybridisation round was high, as out of 115 cells results were obtained for 31 only, while the remaining 84 (73%) could not be detected after FISH. Most of these cells had been initially stained with Giemsa, to aid in their identification under the microscope. Omitting this step meant that more cells remained on slides and were available for analysis.
Out of all the chromosomes investigated during the different first hybridisation rounds, chromosome 17 was the second smallest after 18. It was also the only chromosome from this group that appeared as an extra copy in two of the oocytes

examined, originating from two different patients. Even though the sample size is very small to draw any conclusions it is consistent with the observation of the preferential involvement of smaller chromosomes in maternally derived aneuploidy, as suggested by Cupisti and colleagues (2003). Premature division of chromatids was also observed for chromosome 4 in oocyte 4264.4 and for all the investigated chromosomes, in oocyte 1243.6 which, was spread with the corresponding 1st PB. In the latter case, the degenerate morphology of both the oocyte, and corresponding 1st PB, along with the fact that premature division was visible for all three chromosomes, could be attributed to cellular degeneration due to extended culture time.

Table 4.5: FISH results for metaphase II oocytes, investigated for chromosomes 4, 12, and 17

Oocyte/patient no.	Chromosomes scored and further observations	Oocyte characterisation
3892.1	Two chromosomes Large signal for 12 Large signal for 17	Normal for 12 and 17
3892.2	Three chromosomes Large signal for 4 Large signal for 12 Large signal for 17	Normal for 4, 12 and 17
3892.5	Two chromosomes Large signal for 12 Large signal for 17	Normal for 12 and 17
1033.4	One chromosome Doublet for 12	Normal for 12
1118.5	One chromosome Doublet for 17	Normal for 17
1118.6	One chromosome Doublet for 17	Normal for 17
3847.1	One chromosome Large signal for 12	Normal for 12
1054.1	Two chromosomes Doublet for 4 Doublet for 17	Normal for 4 and 17
3839.1	One chromosome Doublet for 12	Normal for 12
4077.1	Two chromosomes Doublet for 4 Large signal for 17	Normal for 4 and 17
4091.2	One chromosome Large signal for 4	Normal for 4
1169.3	Two chromosomes	Normal for 4 and 17

	Large signal for 4 Doublet for 17	
4130.6	Two chromosomes Doublet for 4 Doublet for 17	Normal for 4 and 17
1152.1	One chromosome Doublet for 4	Normal for 4
4176.5	Three chromosomes Doublets for 4, 12, 17	Normal for 4, 12, and 17
4176.8	Three chromosomes Doublets for 4, 12, 17	Normal for 4, 12, and 17
4181.1	Three chromosomes Doublets for 4, 12, 17	Normal for 4, 12, and 17
4181.3	Three chromosomes Doublets for 4, 12, 17	Normal for 4, 12 and 17
4181.4	Three chromosomes Doublets for 4, 12, 17	Normal for 4, 12 and 17
4184.2	Three chromosomes Doublets for 4, 12, 17	Normal for 4, 12 and 17
1209.2	Three chromosomes Doublet for 4 Doublet for 12 Two doublets for 17	Disomic for 17 No PB result
1243.6 oocyte and PB chromosomes were mixed	Three chromosomes Doublet for 4 or one signal for 4 Doublet for 12 or one signal for 12 Two separate signals for 17	Premature division of chromatids for 17 and either for 4 and 12 with loss of one of the two, or normal for 4 and 12
4257.1	One chromosome Large signal for 12	Normal for 12
4257.2	Three chromosomes Doublets for 4, 12, 17	Normal for 4, 12 and 17
4257.3	Two chromosomes	Normal for 4 and 12

	Doublets for 4, 12	
4292.7	Two chromosomes **Doublets for 12, 17**	Normal for 12 and 17
4292.9	Two chromosomes Doublets for 4, 17	Normal for 4 and 17
4264.1	Two chromosomes Doublet for 4 Two doublets for 17	Disomy for 17 No PB result
4264.2	One chromosome Doublet for 12	Normal for 12
4264.3	Two chromosomes Doublets for 4, 12	Normal for 4 and 12
4264.4	Three chromosomes Two separate signals for 4 Doublet for 12 and 17	Premature division of chromatids for 4, normal for 12 and 17

Table 4.6: FISH results for corresponding 1st PBs, investigated for chromosomes 4, 12, and 17

PB/patient no.	Chromosomes scored and further observations	PB characterisation
3892.1 PB	Two chromosomes Large signal for 12 Large signal for 17	Normal for 12 and 17
4181.3 PB	Two chromosomes Two separate signals for 4 Large signal for 17	Separation of sister chromatids for 4, normal for 17
4181.4 PB	One chromosome Doublet for 4	Normal for 4
4184.2	Two chromosomes Doublet for 12 Two separate signals for 17	Separation of sister chromatids for 17, normal for 12
1243.6 PB oocyte and PB chromosomes were mixed	Three chromosomes Doublet for 4 or one signal for 4 Doublet for 12 or one signal for 12 Two separate signals for 17	Premature division of chromatids for 17 and either for 4 and 12 with loss of one of the two, or normal for 4 and 12

Triple-colour FISH efficiency on lymphocytes: 95%

4.4 Second hybridisation round

The second hybridisation round comprised the assessment of chromosomes 13 and 21. Further examination of the status of chromosome 16 was also attempted. Investigation of all oocytes and PBs that yielded results after the first hybridisation round was attempted. In total, 85 oocytes, some including PBs, donated from 47 patients underwent this second hybridisation round. FISH analysis was feasible for 29 oocytes and 8 corresponding 1st PBs, while the remaining 56 cells were lost.

Two probe combinations were employed, the first using the commercial (Vysis/Abbott,UK) β-satellite probe for chromosome 16 labelled in SA, while the second one involved the application of two commercial (Vysis/Abbott, UK) locus-specific probes for chromosome 13 labelled in SG, and chromosome 21 labelled in SO. Probe details are shown in Table 2.5. As far as the oocytes and PBs were concerned, no further treatment took place. Removal of the first round probes took place as described in 2.2.6.5 and denaturation followed. Hybridisation time was 12 hours.

4.4.1 FISH with the centromeric probe for chromosome 16

This probe was applied to examine the chromosome complement of the oocytes that had failed to show any signals during the first hybridisation round, that took place with the centromeric probes for chromosomes 12, 16 and 18. That failure was attributed to inadequate hybridisation time. Fifteen oocytes were to be analysed during this second hybridisation round. Upon visualisation at the fluorescent microscope, it was observed that all cells had been lost.

Thus, no results were obtained during this hybridisation round. All oocytes had been stained with DAPI twice, once in order to locate them prior to FISH, and once to identify them after the first hybridisation round. Both times they were exposed to fluorescent light for a prolonged period. The above could explain why all oocytes were lost after the second hybridisation round.

4.4.2 FISH with the locus-specific probes for chromosomes 13 and 21

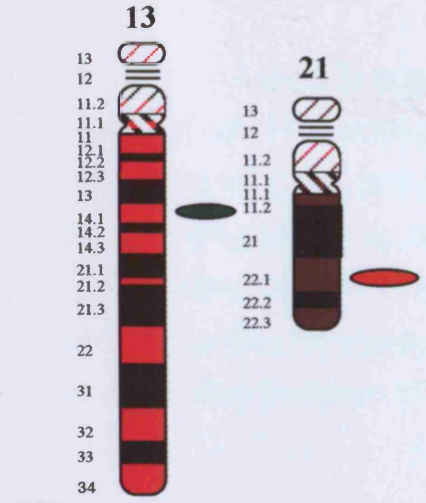
Analysis of seventy cells originating from 39 patients was attempted in this hybridisation round. The probes applied both mapped on regions of the long arms of chromosomes 13 and 21. Therefore, their signals would always appear as doublets showing slight separation, corresponding to each of the two sister chromatids comprising the chromosomes. A total of 29 oocytes and 8 1st PBs yielded results after this round, leading in this way to the investigation of eggs from 22 patients. The remaining cells could not be detected after FISH.

Nine oocytes (1005.2, 1084.2, 1084.6, 3967.3, 1033.4, 1147.1, 4081.1, 1243.6, 4257.1) showed the expected doublets for chromosomes 13 and 21, and were characterised as being normal. Five oocytes (3782.1, 3967.9, 3839.,1, 3887.6, Hutchinson-1) showed a double signal for 13 and no signals for 21 and six oocytes (3982.,1 3892.5, 1001.5, 4176.5, 4297.7, 5coll(t)) had the expected complement for 21, but no chromosome 13. Loss of chromosomes was attributed either to the spreading of the cells or the FISH procedure. Signals for both chromosomes were scored in three 1st PBs (1084.6PB, 3967.9PB, 3892.5PB), one (3967.3PB) was identified as having the expected double signal for 13, but no signal for 21 and one (3982.1PB) consisting of chromosome 21, but having lost chromosome 13. All the above cells came from fifteen patients.

Premature division of chromatids was observed for both chromosomes, but was more frequent for chromosome 21 in this small sample of cells. Thus, four oocytes (1001.5, 4181.3, 4184.2, 4257.1) and two PBs (4184.1 PB, 1243.6 PB) were scored as having undergone premature division of chromosome 21 into its two sister chromatids. Predivision of chromatids for chromosome 13 was observed in two oocytes (4257.3, and 3969.3) only.

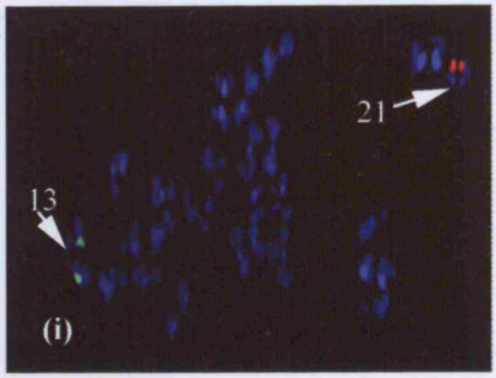
FISH analysis of oocyte 1084.4 revealed two double SG signals corresponding to two chromosomes 13. The oocyte had probably lost chromosome 21 during processing. The corresponding PB was also available, but did not show any signals for either chromosome probably indicating whole chromosome non-disjunction for 13. Technical chromosome loss may have affected chromosome 21. An extra copy of chromosome 13 was scored for

Fig.4.5: Second hybridisation round- Locus-specific probes for 13 and 21

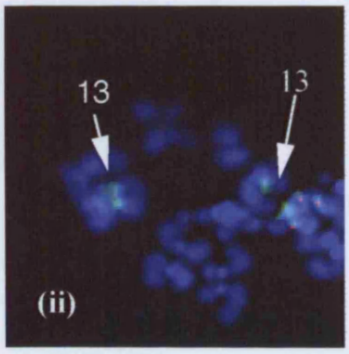


LSI 13 probe-13q14 (SG)

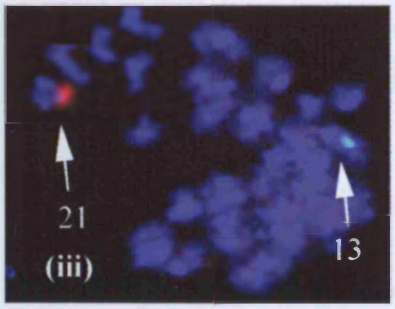
LSI 21 probe-21q22.13-q22.2 (SO)



(i) FISH analysis of oocyte 1147.1. The cell was characterised as normal haploid for both chromosomes 13 and 21. Two sligtly separated green signals were scored for 13, whilst a red doublet was visiblefor 21.



(ii) FISH analysis of oocyte 1084.4. An extra copy of chromosome 13 was observed in this cell (2 green doublets). Chromosome 21 was missing.



(iii) FISH analysis of PB 4181.4. The cell was characterised as normal haploid for both chromosomes 13 and 21. A green doublet was scored for 13 and a red for 21.

oocyte 3969.3. Both copies of this chromosome had prematurely divided into their sister chromatids. The cell had a normal complement for chromosome 21. The corresponding 1st PB was identified with the oocyte. The latter had two double signals, each one demonstrating the presence of one chromosome 13 and one chromosome 21. Thus, the PB was characterised as normal for both chromosomes. These observations led to the conclusion that this patient could have been a germinal mosaic for trisomy 13 with the extra chromosome ending up in the oocyte.

These results are summarised in Tables 4.7, and 4.8. Signal patterns for a normal oocyte, an oocyte disomic for 13 and a PB normal for 13 and 21 are shown in fig.4.5. Cytogenetic analysis of all oocytes and PBs using the ISCN nomenclature is given in Appendix C.

In summary, seven patients appeared to have abnormalities after oocyte analysis during this second hybridisation round. A case of whole chromosome non-disjunction was detected for one oocyte that contained an extra copy of 13, whilst its corresponding PB did not have any signals for this chromosome. Evidence of germinal mosaicism for a trisomic cell line was also obvious after this FISH analysis in one patient. Extra copies were observed for chromosome 13, but not for chromosome 21, as would be expected, considering the preferential involvement of smaller chromosomes in oocyte aneuploidy (Cupisti *et al.*,, 2003, Sandalinas *et al.*, 2002). The latter could be attributed to the relatively high chromosome loss (60%) experienced during this hybridisation round. Effectively only a few oocytes were available for analysis and most of them were either considered to be normal for chromosome 21, or had lost it altogether.

Table 4.7: FISH results for metaphase II oocytes investigated for chromosomes 13 and 21

Oocyte/patient no.	**Chromosomes scored and further observations**	**Oocyte characterisation**
5col1(t)	One chromosome Double signal for 21	Normal for 21
3782.1	One chromosome Double signal for 13	Normal for 13
1005.2	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
1084.2	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
1084.4	One chromosome Two double signals for chromosome 13	Disomic for 13
1084.6	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
3892.1	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
3967.3	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
3969.3	Two chromosomes Four separated signals for 13 Double signal for 21	Premature division of chromatids for 13 and disomic for 13, normal for 21
3967.9	One chromosome Double signal for 13	Normal for 13
3892.5	One chromosome Double signal for 21	Normal for 21
1033.4	Two chromosomes	Normal for 13 and 21

	Double signals for 13 and 21	
3839.1	One chromosome Double signal for 13	Normal for 13
3887.6	One chromosome Double signal for 13	Normal for 13
1001.5	One chromosome Single signal for 21	Premature division of 21, loss of other chromatid
H-1	One chromosome Double signal for 13	Normal for 13
1147.1	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
4081.1	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
4176.5	One chromosome Double signal for 21	Normal for 21
4181.3	Two chromosomes. Double signal for 13. Two separate signals for 21	Premature division of sister chromatids for 21, normal for 13
4181.4	No signals	-
4184.2	Two chromosomes Double signal for 13 Two separate signals for 21	Premature division of sister chromatids for 21
1243.6	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
4257.1	One chromosome Two separate signals for 21	Premature division of sister chromatids for 21
4257.2	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
4257.3	Two chromosomes Two separate signals for 13	Premature division of sister chromatids for 13,

	Double signal for 21	normal for 21
4292.7	One chromosome Double signal for 21	Normal for 21

Table 4.8: FISH results for corresponding 1st PBs investigated for chromosomes 13 and 21

PB/patient no.	**Chromosomes scored and further observations**	**PB characterisation**
1084.4 PB	No signals	-
1084.6 PB	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
3892.1 PB	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
3967.3 PB	One chromosome Two separate signals for 21	Division of sister chromatids for 21
3969.3 PB	Two chromosomes Double signals for 13 and 21	Normal for 13 and 21
3892.5 PB	Two chromosomes Double signal for 13 and 21	Normal for 13 and 21
4181.4 PB	Two chromosomes Double signal for 13 Two separate signals for 21	Division of sister chromatids for 21, normal for 21
1243.6 PB	Two chromosomes Double signal for 13 Two separate signals for 21	Division of sister chromatids for 21, normal for 13

Two-colour FISH efficiency on lymphocytes: 90%

4.5 Third hybridisation round

During this hybridisation step the status of chromosome 22 was assessed. Analysis was attempted for the 29 oocytes and 8 corresponding 1st PBs that yielded results during the second hybridisation round. Out of these, signals were visible for seven oocytes and two PBs. The remaining were lost after FISH. Eggs from 22 patients were investigated in this final part of the study.

The probe used for chromosome 22 investigation was commercial (Vysis/Abbott, UK), locus-specific and was labelled in SG. Details are seen in Table 2.5. Similar to the second hybridisation round, no further pre-treatment of the cells took place. They were denatured after removal of the probes for chromosomes 13 and 21 and were left to hybridise for 12 hours.

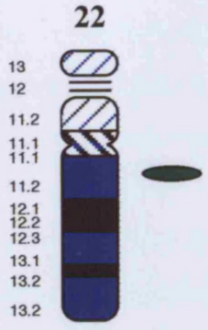
4.5.1 FISH with the locus-specific probe for chromosome 22

The probe employed for the investigation of chromosome 22, hybridised to the long arms, leading to the observation of slightly separated signals on oocytes, as for the locus-specific probes for chromosomes 13 and 21.

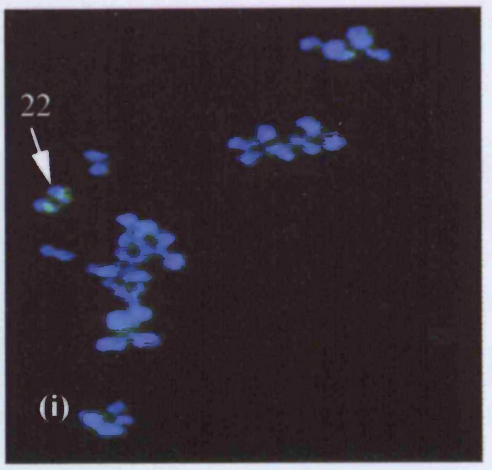
Analysis of seven oocytes from seven different patients was feasible with the application of this probe. The oocytes/patients examined were: 5coll(t), 1084.4, 3839.1, 4176.5, 4181.4, 1243.6, and 4292.6. All appeared to have a normal constitution for chromosome 22. Neither premature division of chromatids or extra copies of this chromosome were detected in any of these cells.

Corresponding 1st PBs were investigated for two oocytes, 1243.6 and 4181.4. In the first case, the 1243.6 PB appeared to be a mirror image of the corresponding oocyte, being normal for chromosome 22. However, premature separation of chromatids was evident for this chromosome. In the case of the 4181.4 PB, three distinct and separate signals were observed upon visualisation of the cell under the fluorescent microscope. The latter meant that there was an extra chromatid 22 in the PB, in addition to the prematurely separated chromosome 22. This observation, combined with the fact that the actual oocyte was scored as normal for chromosome 22, led to the conclusion that this patient was possibly a germinal mosaic for trisomy 22, with the extra chromosome 22 ending up

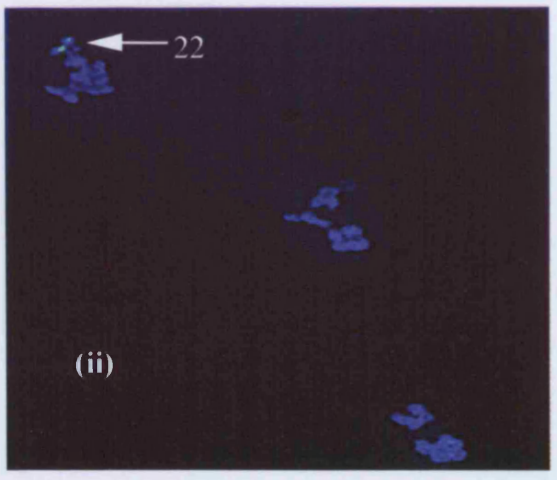
Fig.4.6: Third hybridisation round- Locus-specific probe for chromosome 22



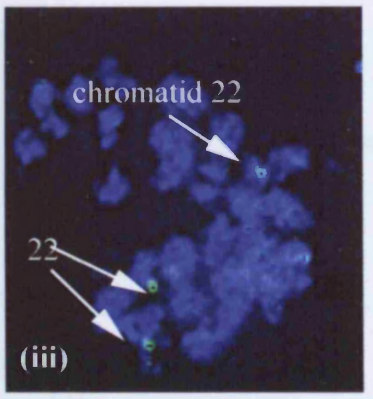
LSI 22 probe- 22q11.2 (SG)



(i) FISH analysis of oocyte 4176.5. The cell was scored to have the normal complement for chromosome 22. The expected green doublet was observed.



(ii) FISH analysis of oocyte 4181.4. The expected green doublet was osberved for chromosome 22. The latter meant that this cell was normal for 22.



(iii) FISH analysis of PB 4181.4. The cell was identified to contain an extra chromatid 22, as a single green signal was visible in addition to the expected doublet. Signal separation was attributed to PB dgeneration.

in the 1st PB, and then separating to its sister chromatids either prematurely, or due to rapid PB degeneration. One of the two chromatids could have been lost during processing.

Tables 4.9 and 4.10 demonstrate all the above observations. FISH analysis of a normal oocyte for chromosome 22 and of the oocyte and corresponding 1st PB from patient 4181.4 are illustrated in fig.4.6. Detailed cytogenetic analysis of all oocytes and PBs with the ISCN nomenclature is given in Appendix C.

The chromosome loss rate after this final hybridisation round was calculated to be 76%. Consequently, the size of the studied sample was very small for more abnormalities to be identified. The morphology of the chromosomes from the oocytes and PBs that remained after the third round of FISH appeared to be much poorer compared to their morphology after the first round of FISH. The latter was a result of the repeated staining of the chromosomal DNA with DAPI in combination to the extended exposure to fluorescent light. Oocytes and PBs, which appeared to be more degenerate after the second round of FISH, were not detected after the third round.

Table 4.9: FISH results for metaphase II oocytes, investigated for chromosome 22

Oocyte/patient no.	Observations	Oocyte characterisation
5coll(t)	Double signal for 22	Normal for 22
1084.4	Double signal for 22	Normal for 22
3839.1	Double signal for 22	Normal for 22
1243.6	Double signal for 22	Normal for 22
4292.7	Double signal for 22	Normal for 22
4176.5	Double signal for 22	Normal for 22
4181.4	Double signal for 22	Normal for 22

Table 4.10: FISH results for corresponding 1st PBs investigated for chromosome 22

PB/patient no.	Observations	PB characterisation
12436. PB	Two separate signals for 22	Division of sister chromatids for 22
4181.4 PB	Double signal for 22 Single signal for 22	Extra chromatid 22

Single-colour FISH efficiency on lymphocytes: 85%

4.6 Aneuploidy in human metaphase II oocytes and corresponding 1st polar bodies

Three consecutive hybridisation rounds took place for the analysis of 265 metaphase II oocytes and their corresponding 1st PBs, when these were available. Investigation of chromosomes 1, 4, 12, 13, 17, 21, 22, and X, belonging to different groups occurred, in an attempt to establish how frequent each of these was present as an extra whole univalent or a single chromatid. Absence of chromosomes was not scored as an anomaly, as this loss could have resulted from overspreading or the FISH procedure. FISH analysis for chromosomes 1, 4, 12, 17, and X (1st round) was feasible on 51 oocytes and 11 PBs. Chromosomes 13 and 21 (2nd round) were examined in a total of 29 oocytes and 8 PBs, whereas seven oocytes and 2 PBs yielded results for chromosome 22. All the remaining cells were lost either before the first hybridisation or during one of the subsequent rounds. Data on the chromosome status of the female gametes was obtained from thirty-three patients.

Hyperhaploid eggs were recorded for five patients (average age 32.2), with the anomalies scored affecting one chromosome only. In four cases one extra chromosome was observed in the oocyte. Chromosomes that presented as extra copies included 17 in two patients (4264, 1209), and 13 in another two patients (1084, 3969). The fifth patient (4181) appeared to have an extra chromatid 22 in the 1st PB, with the corresponding oocyte being normal. These results are shown in Table 4.11.

The presence of germinal mosaicism was evident in two out of the five patients, whose eggs were scored as abnormal. More specifically, an oocyte and its corresponding 1st PB were investigated for patient 3969. An extra whole copy of chromosome 13 was scored in the oocyte, whereas the PB had a normal complement for this chromosome. An extra chromatid 22 was present in the 1st PB coming from patient 4181, while the corresponding oocyte had the expected double signal for this chromosome. The remaining two oocytes from this patient were lost during FISH and no further information on the complement of 22 could be obtained. It is possible that one or more of the primary oocytes from these two patients were trisomic for

chromosomes 13 and 22 respectively, with the extra copy ending up in the oocyte for the first case and the 1st PB for the second.

Balanced predivision of chromatids affected nearly all the chromosomes investigated. This phenomenon was first reported by Angell (1991) and is thought to take place prior to anaphase I. The separated chromatids run the risk of segregating randomly to either pole, during meiosis II. In this study, it was observed for 11 oocytes (4181.3, 4184.2, 4257.1, 4257.3, 3416.11, 3969.3, 3969.4, 3967.9, 1243.6, 4264.1, 1001.5) and six PBs (968.1PB, 3967.9PB, 1243.6PB, 4181.3PB, 4181.4PB, 3967.3PB). As far as the PBs are concerned, this phenomenon could be the result of their rapid degeneration, during prolonged culture. The chromosome, which most frequently was identified as having precociously separated into its sister chromatids, was 21, with seven such events being scored. The latter could be attributed to the small size of this chromosome, which has an effect the formation of very few or no chiasmata during meiosis I in the female gamete (Antonarakis *et al.,* 1993). The frequency with which each of the examined chromosomes was affected by premature division into its sister chromatids is demonstrated in Table 4.12.

Five abnormalities were identified in total, four of which involved additional chromosomes, and just one an additional chromatid. Anomalies were observed for chromosomes 13, 17, and 22, with a frequency ranging between 9-14.3%. The highest frequency corresponded to chromosome 22, which was the smallest presenting with an extra chromatid in one PB. No abnormalities were observed for the larger chromosomes 1, 4, 12 and X. Similar results have been obtained from other studies from our group (Cupisti *et al,* 2003, Mahmood *et al.,* 2000), which postulated the preferential involvement of smaller chromosomes in oocyte aneuploidy. Table 4.13 demonstrates the frequency of hyperploidy found in oocytes and 1st PBs for all the chromosomes that were examined.

The number of oocytes and corresponding 1st PBs available for investigation in this part of the research project was very small, and the latter could explain why no abnormalities were observed for chromosome 21, which was one of the two smallest in the group. High cell loss was experienced during processing. Attributing factors

included the actual spreading and FISH procedures, the staining of the oocyte DNA with DAPI or Giemsa, and the prolonged exposure to fluorescent light, which also affected chromosome quality. Even though many cells were lost, certain anomalies were scored for the ones that remained and went through all three rounds of FISH. The data obtained provided evidence for different mechanisms leading to maternal aneuploidy, which was the first hypothesis being tested. These include the classical non-disjunction of a whole univalent chromosome, the precocious separation of chromosomes into their sister chromatids prior to anaphase I with the potential risk of random segregation during meiosis II, the preferential involvement of smaller chromosomes in oocyte aneuploidy (second hypothesis being tested), and the presence of germinal mosaicism for a trisomic cell line, involving again smaller chromosomes. The effect of advanced maternal age could not be established because of the small sample size.

Table 4.11: Patients with extra chromosomes/chromatids in one or more oocytes or PBs. Chromosomes investigated are indicated

Patient no.	Age	Infertility cause	Egg[a] number/ status	Examined chromosomes[f]								Abnormalities observed
				1	4	12	13	17	21	22	X	
4264	35	Unknown	4 unfertilised		*	*		*				Extra chromosome 17 in oocyte 4264.1 (no PB). Remaining oocytes normal for 17
1209	33	MF	3 unfertilised/ 3 immature		*	*		*				Extra chromosome 17 in oocyte 1209.2 (no PB)
1084	31	MF	3 unfertilised/ 5 immature			*	*		*			Extra chromosome 13 in oocyte 1084.4; no PB result. Remaining oocytes normal
3969	31	MF	12 unfertilised				*		*			Extra chromosome 13 in oocyte 3969.3; PB normal. Remaining oocytes lost[b]
4181	31	Tubal	3 unfertilised		*	*		*		*		Extra chromatid 22 in 1st PB (4181.4PB), corresponding oocyte normal[b]

[f] Indicated chromosomes examined. MF, male factor

[a] An egg consists of an oocyte and a first polar body, but each may be present alone without the other

[b] Evidence of trisomic precursor cell and germinal mosaicism

Table 4.12: Patients showing precocious separation of chromosomes into their sister chromatids in oocytes and/or PBs.

Patient no.	Oocyte no.	PB no.	Predivision of chromatids							
			1	4	12	13	17	21	22	X
3416	3416.11	-								*
968	-	968.1 PB			*					
3969	3969.3	-				*				
	3969.4	-		*						
3967	3967.9	3967.9 PB					*			
	-	3967.3 PB						*		
1243	1243.6	1243.6 PB		*	*		*	* (PB)		
4181	4181.3	4181.3 PB		*(PB)				*		
	-	4181.4 PB						*	*	
1001	1001.5	-						*		
4184	4184.2	-						*		
4257	4257.1	-						*		
	4257.3	-				*				

[f] Chromosomes showing predivision to sister chromatids are indicated

Table 4.13: Hyperploidy frequency identified in oocytes or 1st PBs per chromosome

Chromosome	Eggs scored	Whole chromosome	Single chromatids	% of abnormalities
1	5	0	0	0
4	22	0	0	0
12	28	0	0	0
13	22	2	0	9
17	29	2	0	7
21	21	0	0	0
22	7	0	1	14.3
X	10	0	0	0

Chapter 5- Results

The analysis of human metaphase II oocytes and corresponding polar bodies with CGH

5.1 CGH investigation of human metaphase II oocytes and corresponding 1st PBs

The final part of the study involved the analysis of unfertilised human oocytes, arrested at metaphase II, and their corresponding 1st PBs with the application of Comparative Genomic Hybridisation (CGH). As mentioned, CGH enables the screening of an entire genome in a single hybridisation (Kallioniemi *et al.*, 1992). Thus, the application of this technique for the analysis of oocytes and 1st PBs would provide data on the whole of the maternal genome. The aims of this part of the study were the following:

- To examine chromosomes not generally targeted in oocyte examination by FISH.
- To investigate how and whether chromosomes of larger size than 13 affect maternal aneuploidy.
- To accurately estimate hypohaploidy and hence the aneuploidy rate in the examined cells.

All investigated oocytes had either failed to fertilise after their exposure to sperm, either via IVF or ICSI or were immature and were not exposed to sperm. Three differently prepared groups of cells were examined:

Group 1: Oocyte and PB were separated after removal of the zona pellucida (ZP) using Acid Tyrode's solution, and were suspended in 100µl of sterile PBS. Removal of excess PBS was essential before further treatment of these cells. Thirteen oocytes and 13 PBs from this group underwent the CGH procedure, out of which 12 were pairs.

Group 2: Similar to the first group, oocytes and PBs were separated after treatment with Acid Tyrode's, but each cell was suspended in approximately 2µl of sterile PBS. This difference in preparation occurred to avoid the risk of losing the cell during the initial removal of the excess PBS. CGH analysis was attempted for 12 oocytes and 14 PBs, nine of which were pairs.

Group 3: Pronase was employed for the removal of the ZP and the oocytes and their corresponding PBs were separated by agitation. Similar to the second group each cell was suspended in 2µl of sterile PBS, and overlaid with a drop of oil to prevent possible

contamination by external DNA agents. CGH analysis of 15 oocytes and 17 PBs was attempted, 15 of which were pairs.

All mature unfertilised oocytes and especially those that underwent ICSI were considered to contain sperm DNA. Hence, analysis of a potential zygote occurred with CGH. In other words, data may have been obtained on both the maternal and the paternal genomes in some cases. PCR analysis to identify maternal and paternal polymorphisms would detect the presence of sperm. However, this did not take place, due to lack of time available for this study. The situation was different for the corresponding 1st PBs, as they were extruded during the first meiotic division, and sperm contamination was unlikely. Therefore, the results obtained from these cells were considered to represent solely the maternal genome. The rapid degeneration of first PBs was considered a potential source of DNA loss. For this reason, their morphology was always checked prior to them being placed in tubes.

The patients that took part in this study were assumed to be of normal karyotype (46 XX, 46 XY), with maternal ages between 22 and 39 years (mean 31.9). Treatment for infertility took place for various reasons, including male factor, anovulation and polycystic ovary syndrome. All couples underwent treatment at the Assisted Conception Unit at Tayside University Hospitals, NHS Trust, Dundee, Scotland. All cells arrived in London frozen in a special container, and were stored at -80°C until they were processed.

5.2 Evaluation of the CGH protocol

Analysis of single cells with the application of CGH was achieved in three sequential stages, as suggested by Wells and colleagues (2002). More specifically, during the first stage the entire genome of the single cell (6-10pg) was amplified to provide enough DNA for subsequent investigations. The amplified samples were then fluorescently labelled, combined and hybridised onto target lymphocyte metaphases, coming from a normal male. Evaluation of the CGH protocol prior to its application on the oocytes and PBs occurred by carrying out seven different positive control experiments. These were the following:

1. Normal female (46,XX) lymphocyte genomic DNA labelled in SG against normal male (46,XY) lymphocyte genomic DNA labelled in SR.
2. Fibroblast DNA trisomic for chromosome 13 labelled in SG against 46,XY genomic DNA labelled in SR.
3. Fibroblast DNA trisomic for chromosome 21 labelled in SG against 46,XX genomic DNA labelled in SR.
4. Fibroblast DNA trisomic for chromosome 22 labelled in SG against 46,XY genomic DNA labelled in SR.
5. Clump of buccal cells 46,XX labelled in SG against clump of buccal cells 46,XY labelled in SR.
6. Clump of buccal cells 46,XX labelled in SG against clump of buccal cells 46,XX labelled in SR.
7. Single fibroblast trisomic for chromosome 18 labelled in SG against 46,XY genomic DNA labelled in SR.

Initial amplification for all the above samples was achieved with the application of the Degenerate Oligonucleotide Primed (DOP) PCR. This reaction employed a semi-degenerate primer that annealed at many sites throughout the genome. DOP-PCR was shown to provide a good coverage of the genome, allowing the subsequent amplification of approximately 223 loci, out of a total of 250 (Wells *et al.,* 1999). Some preferential amplification of certain parts of the genome exists. In this study, evaluation of the DOP PCR, was achieved by firstly attempting the amplification of the above DNAs, which were all of low initial concentrations ranging from 1-800 ng/μl, and then of the clumps (3-5 cells) or single buccal cells, and fibroblasts. Stringent precautions against contamination took place for all experiments, as described in the Methods section.

The incorporation of fluorescent labels into the amplified products occurred enzymatically, with the use of a kit (Vysis/Abbot, UK). For all experiments, the DNAs requiring examination (test), were labelled in green, and were hybridised against DNA known to be normal (46,XX or 46,XY). The latter was considered to be the reference sample, and was labelled in red.

The above positive control experiments were set up to ensure firstly that small concentrations of DNA were accurately amplified, the nick translation was efficiently

incorporating the fluorescent labels in the amplified products, and that the remainder of the protocol would result in target metaphases showing bright and clear signals for both test and reference DNAs. Moreover, these experiments enabled the assessment of the accuracy and sensitivity of the computer software employed for the analysis and interpretation of the captured images. All samples were of known karyotypes, prior to analysis. The aim was to check whether the interpretation of the analysed images would demonstrate the difference in fluorescence in the sex chromosomes when female samples were hybridised against male, and also the trisomies of the different chromosomes, in the analysis of the fibroblast DNAs. Description of the results obtained from these positive control experiments will take place in the following sections.

5.2.1 CGH analysis of 46,XX genomic DNA against 46,XY genomic DNA

The DNA concentration of both samples was 1000pg/μl. This experiment was carried out to assess the ability of the DOP PCR to amplify DNA of a low concentration and to demonstrate the fluorescence pattern observed when a normal female DNA sample was hybridised against a normal male DNA sample. During CGH analysis of DNAs of a normal diploid karyotype, it was essential that the autosome regions showed minimal or no deviation from 1. The ratio profile obtained for chromosome X could be considered as the positive internal control for the assessment of the dynamic range of the particular hybridisation (Karhu *et al.,* 1997).

Amplified products were initially analysed by agarose gel electrophoresis. Visualisation of the gel showed bright smears of sizes between 300-1500bp in length for both samples. Smears of such sizes were observed by Wells and colleagues (1999) and Voullaire and colleagues (1999), in their attempt to assess the efficiency of the DOP-PCR. No smear was seen for the contamination control PCR sample, consisting of all the reaction components, apart from DNA. These observations led to the conclusion that the DOP-PCR had effectively amplified both samples, without any contamination being present. Incorporation of fluorescent tags followed, with the female genomic DNA being labelled in green, and the male in red. Both samples were subsequently combined and hybridised onto normal male target metaphases. Examination of these metaphases under the

Fig. 5.1: Positive control experiment: CGH analysis of 46,XX genomic DNA against 46,XY genomic DNA

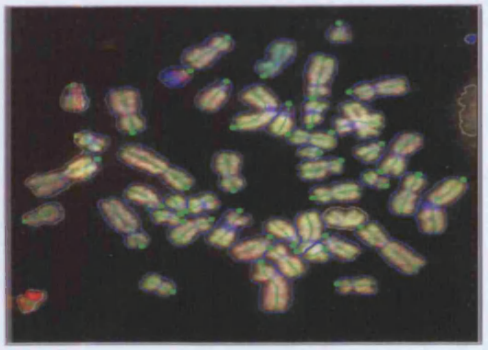


Fig.5.1a: Normal 46,XY metaphase chromosomes hybridised with 46,XX genomic DNA *(green)* and 46,XY genomic DNA *(red)*.

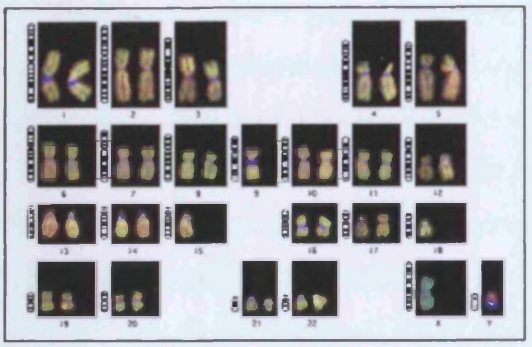


Fig.5.1b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. All 23 autosomes had an even orange/yellow colouration due to the combination of the green and red fluorochromes. Chromosome X was excessively green, as an extra copy was present in the green 46,XX DNA, whilst chromosome Y was excessively red, as it was present in the red 46,XY DNA.

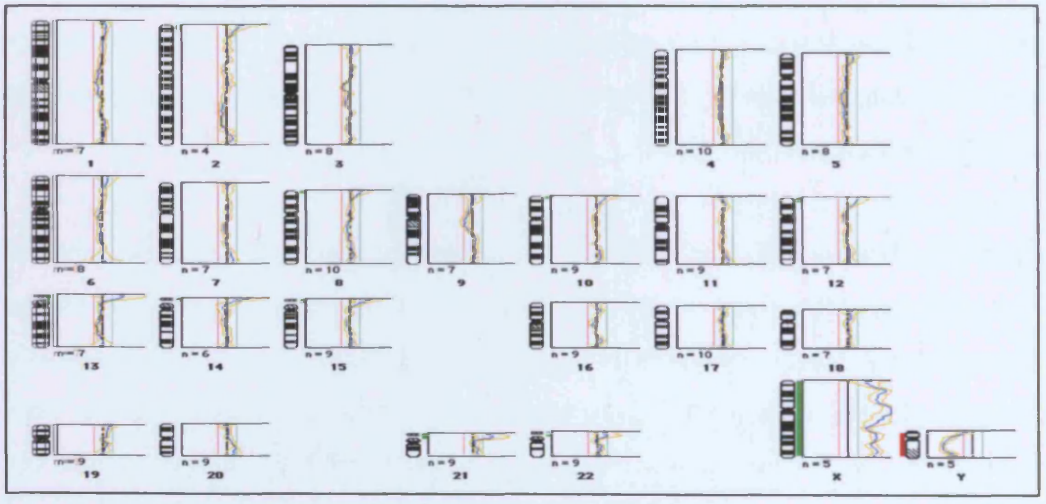


Fig.5.1c: Interpretation of the positive control CGH experiment described in section 5.2.1. No difference in fluorescence intensities was detected for any of the 23 autosomes, as they were present in equal numbers in the "test" (46,XX- green) and "reference" (46,XY- red). DNAs. Excess green fluorescence and the expected deviation of the ratio profile towards the right threshold (more than 1.20) was detected for the X chromosome, indicating its presence in two copies in the green "test" DNA. Excess red fluorescence and the expected deviation of the ratio profile towards the left threshold (less than 0.80) was identified for the Y chromosome, indicating its presence in the "reference" DNA and its absence from the "test" DNA.

fluorescent microscope revealed that both the green and the red fluorochromes gave intense and clear signals, without any background. Combination of both led to an even orange coloration on all the chromosomes, apart from the X and Y. More specifically, chromosome X was excessively green, whereas chromosome Y was excessively red. This was expected, as the female sample containing two chromosomes X was labelled in green, whereas the male sample with an X and a Y was labelled in red.

Five metaphases were analysed and 188 chromosomes were included in the interpretation. As was expected, almost no deviation from 1 was observed for the ratio profiles of all autosomes. Some regions of the karyotype, including the centromeres of chromosomes 13, 14, 15, 21 and 22 and the short arm telomeres of chromosomes 2, 10 and 12 showed deviations from 1. These were attributed to known hybridisation artefacts and were not included in the analysis of the images. Excess green fluorescence was consistently observed on the X chromosome (Xp22.3-q28), leading to a deviation of the ratio profile towards the right threshold (more than 1.2). The latter was expected, as the "test" sample came from a female. Consistent excess red fluorescence was observed for the Y chromosome (Yp11.3-q12), resulting to a deviation of the ratio profile towards the left threshold (less than 0.8). This was also expected, as the "reference" DNA came from a male. Thus, confirmation of both karyotypes was achieved. A metaphase showing the fluorescent signals on the chromosomes can be seen in fig. 5.1a and b, whereas the interpretation of this experiment is shown in fig. 5.1c.

5.2.2 CGH analysis of fibroblast DNA trisomic for chromosome 13 against 46,XY genomic DNA

The concentration of both DNA samples was of the range of 80ng/μl. The karyotype of the test fibroblast DNA was 47,XY, +13. This experiment was carried out to assess whether the CGH was sensitive enough to identify the trisomy for chromosome 13 in the test sample. In addition, it would serve as a reference for the detection of comparable abnormalities in oocytes and/or PBs.

Both test and reference DNAs were amplified with the use of the DOP-PCR. Agarose gel analysis demonstrated smears of similar intensities and fragment sizes with those

Fig. 5.2: Positive control experiment: CGH analysis of 47,XY+13 fibroblast DNA against 46,XY genomic DNA

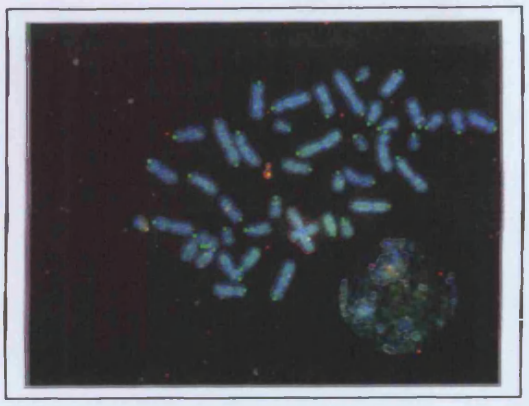


Fig.5.2a: Normal 46,XY metaphase chromosomes hybridised with 47,XY,+13 fibroblast DNA (*green*) and 46,XY genomic DNA (*red*).

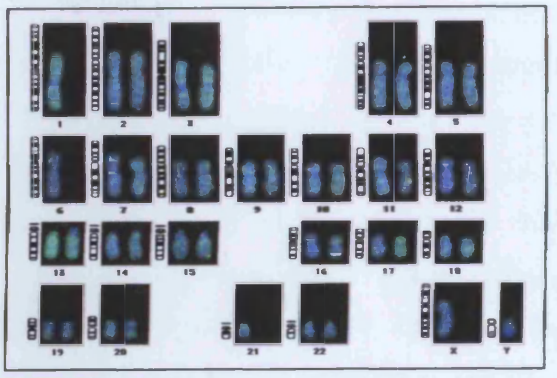


Fig.5.2b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome 13 was excessively green, the latter indicating the presence of the extra copy for 13 in the test sample (47,XY,+13). Both test and reference DNAs were XY. Hence no difference in fluorescence was seen for the sex chromosomes.

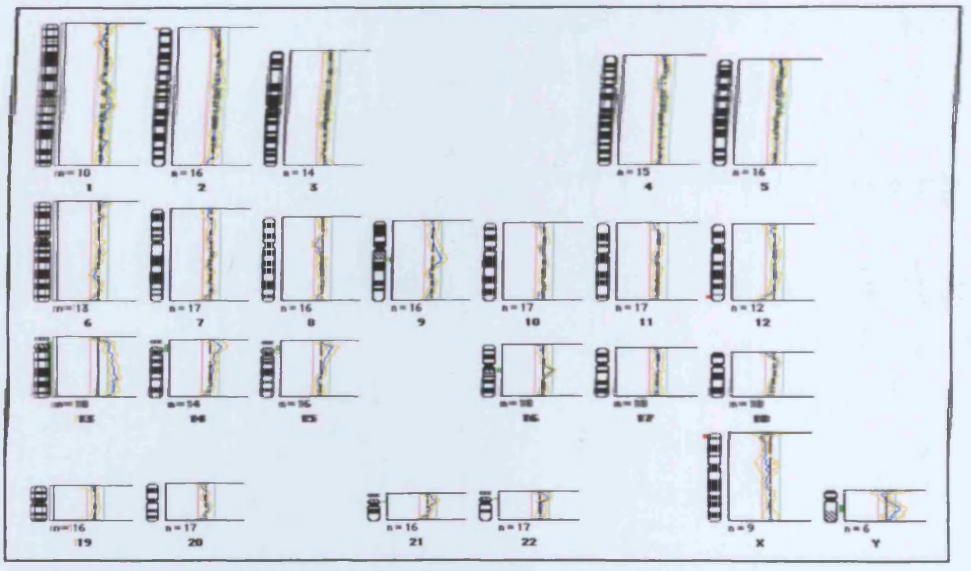


Fig. 5.1c: Interpretation of the positive control CGH experiment described in section 5.2.2. The ratio profile for chromosome 13 deviated towards the right threshold (more than 1.20), the latter indicating the presence of an extra copy of this chromosome in the green test DNA compared to the red reference DNA. No other deviations of ratio profiles were observed either for the autosomes or the sex chromosomes. Accurate identification of the karyotype of the test sample as 47,XY,+13 was achieved.

observed for the previous experiment. The trisomic fibroblast DNA was considered to be the test and was labelled in green, whereas the reference normal male DNA was labelled in red. Both test and reference DNAs were combined and hybridised onto target male metaphases.

Visualisation of the slide under the fluorescent microscope revealed that both fluorochromes gave clear and even signals on the chromosomes. The green fluorescently labelled fibroblast DNA was relatively more intense, compared to the red, resulting in the chromosomes having a slightly more green rather than orange colouration. Chromosome 13 was easily distinguished, as it was even greener, due to the trisomy of the test sample. There was no fluorescence difference in the sex chromosomes.

Analysis took place on ten metaphases and 362 chromosomes were included in the interpretation. Hybridisation artefacts were observed for the long arm telomeres of chromosomes 1, 9, 12, 15, 16, and Y, and for the short arm telomeres of chromosomes X and 14. These were anticipated and were not included in the analysis. The ratio profiles for all autosomes apart from 13 did not deviate from 1, as expected. Chromosome 13 was consistently excessively green (regions: 13p12-q34). Its ratio profile deviated to the right (more than 1.2). This demonstrated the trisomy in the test sample. There was no fluorescence difference in the sex chromosomes, as both test and reference DNAs were male. Hence, the karyotype of the test sample was correctly identified as male trisomy 13. A metaphase demonstrating the fluorescent signals on the chromosomes is shown in fig. 5.2a and b, whereas the interpretation of this experiment is demonstrated in fig. 5.2c.

5.2.3 CGH analysis of fibroblast DNA trisomic for chromosome 21 against 46,XX genomic DNA

The concentration of the fibroblast DNA sample was 70ng/μl, whereas that of the female genomic DNA was 200ng/μl. The karyotype of the test fibroblast DNA was 47,XY, +21. This experiment was performed to evaluate CGH sensitivity for the detection of the trisomy for the smaller chromosome 21 in the test sample. It would serve as a reference

Fig. 5.3: Positive control experiment: CGH analysis of 47,XY+21 fibroblast DNA against 46,XX genomic DNA

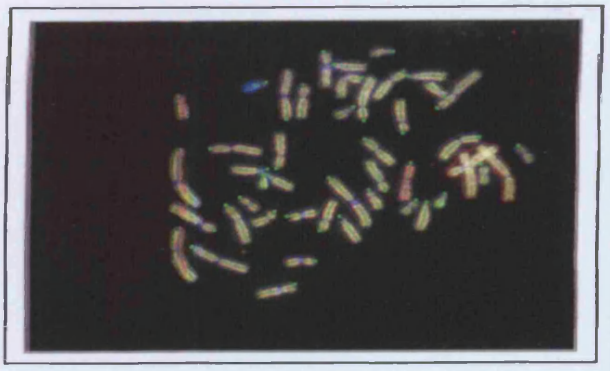


Fig.5.3a: Normal 46,XY metaphase chromosomes hybridised with 47,XY,+21 fibroblast DNA (*green*) and 46,XX genomic DNA (*red*).

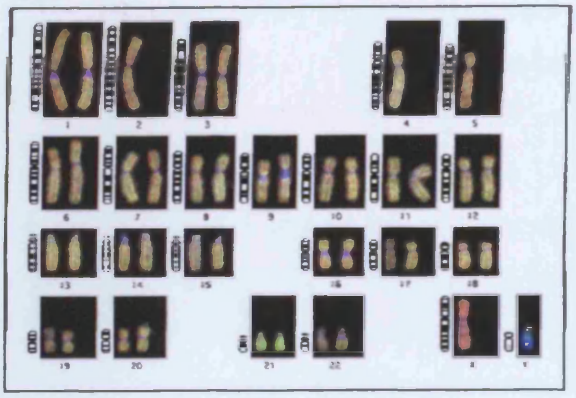


Fig. 5.3b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome 21 was excessively green, the latter indicating the presence of the extra copy for 21 in the test sample (47,XY,+21). Chromosome X was excessively red, indicating the presence of this chromosome in 2 copies in the female red reference DNA, while chromosome Y was excessively green, as the green test was XY.

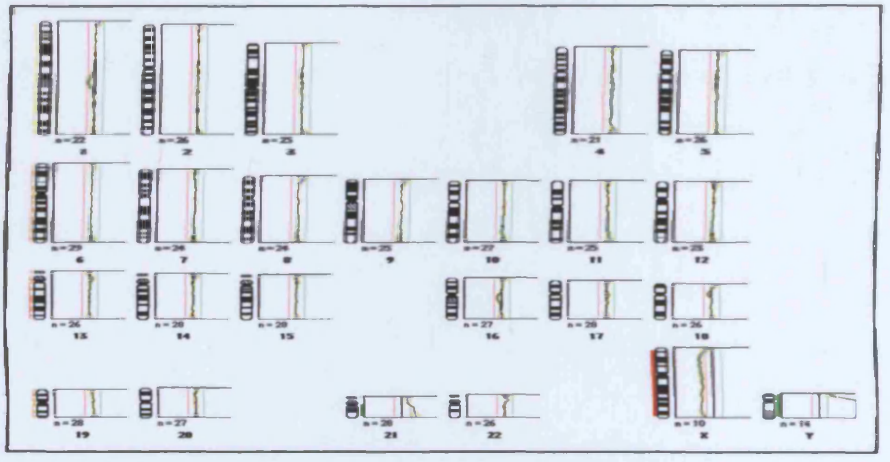


Fig. 5.3c: Interpretation of the positive control CGH experiment described in section 5.2.3. The ratio profile for chromosome 21 deviated towards the right threshold (more than 1.20), the latter indicating the presence of an extra copy of this chromosome in the green test DNA compared to the red reference DNA. A deviation of the ratio profile of chromosome X towards the left threshold (less than 0.80) and of chromosome Y towards the right threshold (more than 1.20) indicates that the reference DNA was XX, while the test DNA was XY. Accurate identification of the karyotype of the test sample as 47,XY,+21 was achieved.

for the identification of similar abnormalities in oocytes and/or PBs.

Analysis of the amplified products with agarose gel electrophoresis revealed smears similar to those observed for the previous experiments, and as above, the fibroblast DNA was labelled in green, while the female genomic DNA was labelled in red.

Visualisation of the target metaphase chromosomes, under the fluorescent microscope showed that they had a clear, intense and even orange coloration. Both the green and the red fluorochromes were very bright, and did not give any background. Chromosome 21 was distinct from the others, as it was visibly greener, due to the trisomy in the test sample (regions 21p13-q22). Deviation of its ratio profile towards the right threshold (more than 1.2) was also observed. Chromosome X was relatively more red (regions Xp22.3-q28), as the reference DNA was female. The latter resulted in the ratio profile of this chromosome deviating towards the left threshold (less than 0.8). Chromosome Y, on the other hand, appeared green (regions Yp11.3-q12) with its ratio profile also deviating to the right. The above confirmed the karyotype of the test sample, as male trisomy 21. Analysis was carried out on 15 metaphases and 593 chromosomes were included in the interpretation. Hybridisation artefacts were not observed for this experiment. The latter was attributed to the good quality of the hybridisation for both the test and reference DNA probes. A metaphase showing the fluorescent signals on the chromosomes can be seen in fig. 5.3a and b, whereas the interpretation of this experiment is shown in fig. 5.3c.

5.2.4 CGH analysis of fibroblast DNA trisomic for chromosome 22 against 46,XY genomic DNA

The concentration of the fibroblast DNA sample was 100ng/μl and the karyotype was 47,XY, +22. The male genomic DNA had a concentration of 200ng/μl. This experiment would also serve as a reference for the oocyte and PB CGH results. DOP- PCR amplification and agarose gel analysis resulted in smears of similar intensities and fragment sizes as those observed for the previous experiments. During the nick

Fig. 5.4: Positive control experiment: CGH analysis of 47,XY+22 fibroblast DNA against 46,XY genomic DNA

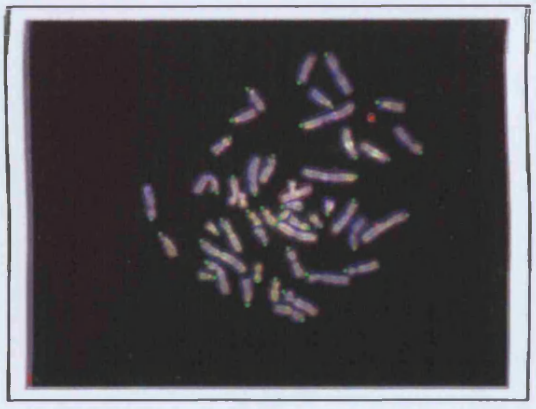


Fig.5.4a: Normal 46,XY metaphase chromosomes hybridised with 47,XY,+22 fibroblast DNA (*green*) and 46,XY genomic DNA (*red*).

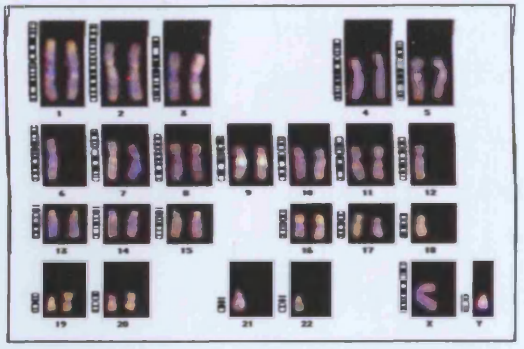


Fig. 5.4b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome 22 was excessively green, the latter indicating the presence of the extra copy for 22 in the test sample (47,XY,+22). Both test and reference DNAs were XY. Hence no difference in fluorescence was seen for the sex chromosomes.

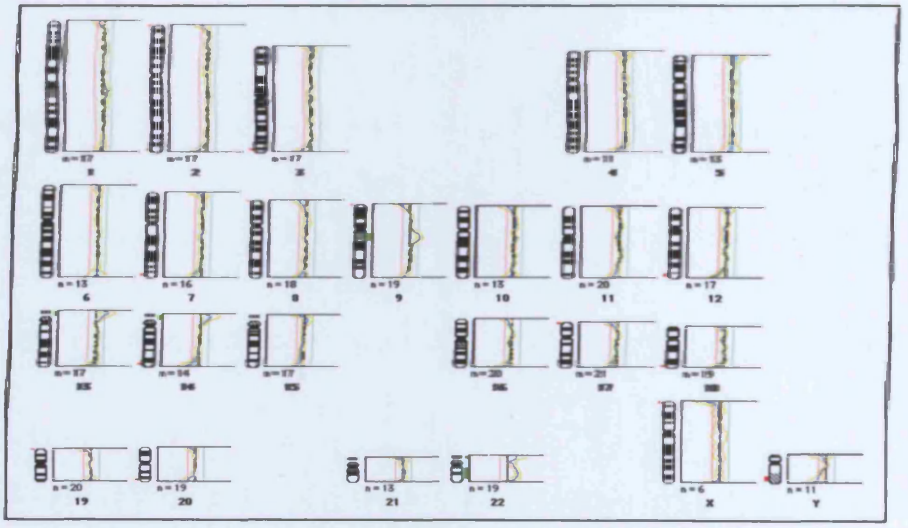


Fig. 5.4c: Interpretation of the positive control CGH experiment described in section 5.2.4. The ratio profile for chromosome 22 deviated towards the right threshold (more than 1.20), the latter indicating the presence of an extra copy of this chromosome in the green test DNA compared to the red reference DNA. No other deviations of ratio profiles were observed either for the autosomes or the sex chromosomes. Accurate identification of the karyotype of the test sample as 47,XY,+22 was achieved.

translation of both samples, the fibroblast DNA was labelled in green, whilst the male genomic DNA was labelled in red.

Observation of the slide revealed that both green and red fluorochromes were clearly visible, but not as bright as in previous experiments. Chromosome 22 was identified easily, as it was greener, compared to the rest of the autosomes, which were orange, while there was no difference in the fluorescent intensities of the sex chromosomes, as both test and reference DNAs were male.
Analysis occurred in eleven metaphases and 387 chromosomes were included in the interpretation. Hybridisation artefacts were observed for the short arm telomeres of chromosomes 13, 14, and 17, the centromere of 9, and the long arm telomeres of chromosomes 12, 18, 20, X and Y. These were excluded from the analysis. Chromosome 22 was consistently greener (regions: 22p13-q13). Its ratio profile deviated to the right (more than 1.2). There was no fluorescence difference on the sex chromosomes, as both test and reference samples were male. Thus, the karyotype of the test DNA was confirmed as male trisomy 22. A metaphase showing the fluorescent signals on the chromosomes is illustrated in fig. 5.4a and b, whereas the interpretation of this experiment can be seen in fig. 5.4c.

5.2.5 CGH analysis of 46,XX buccal cell clump against 46,XY buccal cell clump

All the above experiments confirmed that the CGH protocol was functional at the genomic DNA level, and was sensitive enough to detect trisomies for chromosomes ranging in size, and the difference in the sex chromosomes when sex mismatched test and reference DNAs co-hybridised. The same protocol was then employed for the analysis of much lower concentrations of DNA, to test its efficiency in the examination of cell clumps and single cells. Clumps of buccal cells consisted of 3-5 cells. Hence DNA concentrations were estimated to be between 18-30pg of starting material. This experiment would also provide an initial indication of the fluorescence pattern that would be obtained when either an oocyte or a PB were hybridised against male DNA. Agarose gel analysis of the DOP-amplified products demonstrated that the obtained smears did not vary in either brightness, or fragment sizes from the smears given by the

Fig. 5.5: Positive control experiment: CGH analysis of 46,XX buccal cell clump DNA against 46,XY buccal cell clump DNA

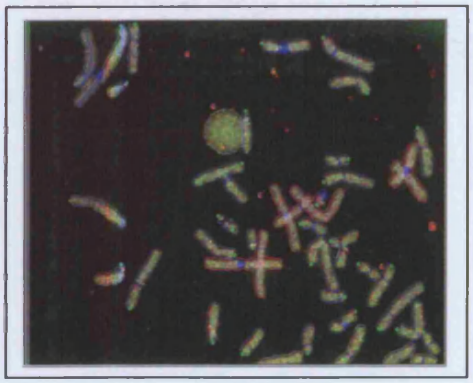


Fig.5.5a: Normal 46,XY metaphase chromosomes hybridised with 46,XX buccal cell DNA (*green)* and 46,XY buccal cell DNA (*red)*.

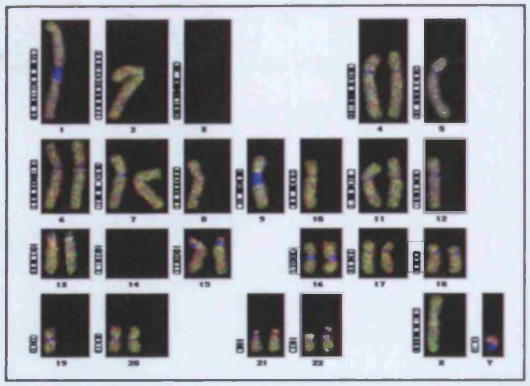


Fig.5.5b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome X was excessively green, as the "test" sample was female, whilst chromosome Y was excessively red, as the "reference" sample was male.

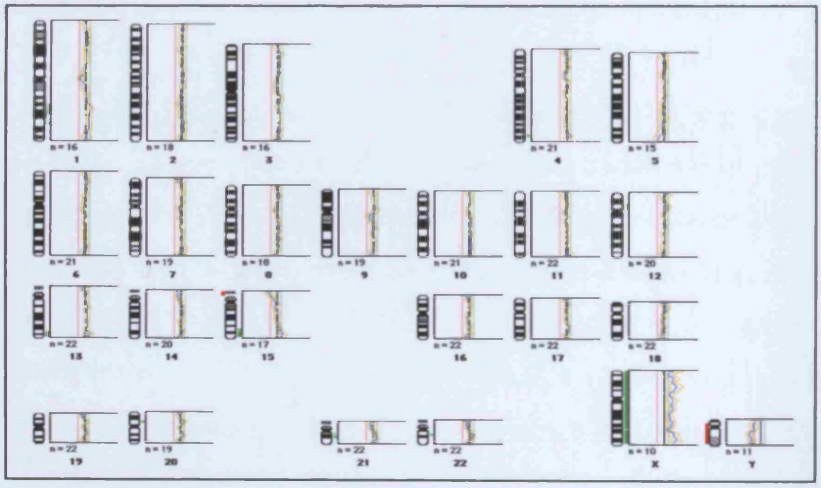


Fig.5.5c: Interpretation of the positive control CGH experiment described in section 5.2.5. No difference in fluorescence intensities was detected for any of the 23 autosomes, as they were present in equal numbers in the "test" (46,XX- green) and "reference" (46,XY- red). DNAs. The fluorescence pattern and the shifts of the ratio profiles observed for the sex chromosomes were identical to those observed when 46,XX genomic DNA (green) was hybridised against 46,XY genomic DNA (red), as described in fig. 5.1c.

genomic DNA. The observation of smears led to the conclusion that the DOP PCR had the ability to effectively amplify clumps of cells. The remainder of the CGH protocol was identical to the one followed for the genomic DNA samples. Hence, enzymatic incorporation of fluorescent labels took place, resulting in the labelling of the female clump in green ("test"), and the male clump in red ("reference"). Combination of both and their hybridisation on normal male metaphase target slides followed.

Upon visualisation of the hybridisation area under the fluorescent microscope it was observed that all chromosomes had a very bright and even orange colouration, resulting from the combination of the red and green fluorochromes. The X and Y chromosomes were clearly distinguished, due to their difference in intensities between the red and the green. The above demonstrated that the nick translation and remainder of the CGH protocol were successful.

Analysis of 13 metaphases took place, and 457 chromosomes were included in the interpretation. Hybridisation artefacts were observed for the long arm telomere of chromosome 15. The ratio profiles for all autosomes did not deviate from 1, as was expected because both test and reference DNAs were normal. Excess green fluorescence was observed on almost the whole of chromosome X (Xp22.3-q27), leading to a deviation of its ratio profile to the right (more than 1.2). This demonstrated that the test DNA was of a female karyotype. Excess red fluorescence was observed for the Y chromosome (regions: Yp11.2-Yq12). Its ratio profile deviated to the left (less than 0.8). This showed that the reference DNA was of male karyotype. Thus, both karyotypes were confirmed. A metaphase showing the fluorescent signals on the chromosomes is illustrated in fig. 5.5a and b, whereas the interpretation of this experiment can be seen in fig. 5.5c.

5.2.6 CGH analysis of 46,XX buccal cell clump against 46,XX buccal cell clump

The previous experiment established that the CGH protocol was applicable at the single cell level. Co-hybridisation of two clumps of female buccal cells would indicate how the fluorescent pattern for a normal oocyte or PB would be if they hybridised against female DNA. Similar to the above, DOP-PCR was carried out for the amplification of the buccal cell DNA. One clump of cells was labelled in green and the other in red and

Fig. 5.6: Positive control experiment: CGH analysis of 46,XX buccal cell clump DNA against 46,XX buccal cell clump DNA

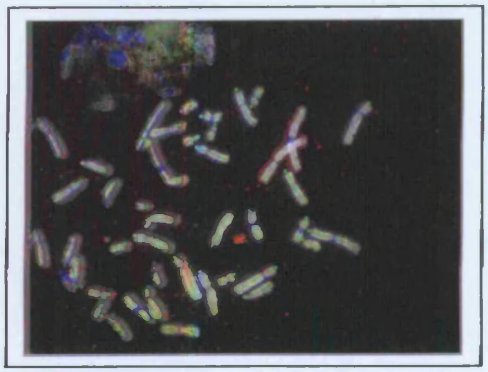


Fig.5.6a: Normal 46,XY metaphase chromosomes hybridised with 46,XX buccal cell DNA *(green)* and 46,XX buccal cell DNA *(red)*.

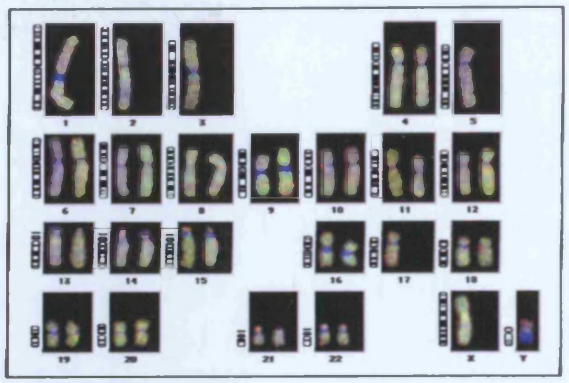


Fig.5.6b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. All 23 autosomes and chromosome X had even fluorescence intensities due to the combination of the green and red fluorochromes. No fluorescence was visible for the Y chromosome, as there was no DNA present either in test or the reference samples.

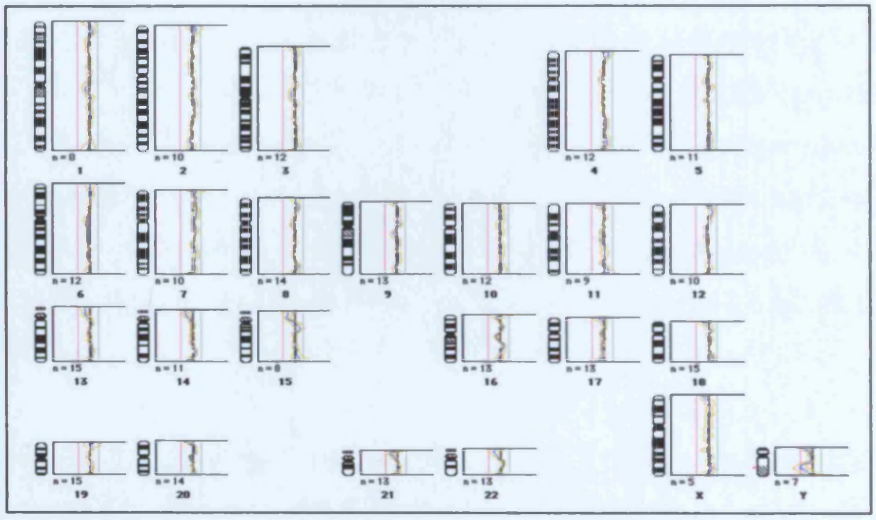


Fig.5.6c: Interpretation of the positive control CGH experiment described in section 5.2.6. No difference in fluorescence intensities was detected for any of the 23 autosomes and the X chromosome, as they were present in equal numbers in the "test" (46,XX- green) and "reference" (46,XX- red) samples.

co-hybridisation took place on male metaphases as before. Since there was no male DNA, it was expected that the Y chromosome would not show any fluorescence, whereas no difference in fluorescence intensity would be observed for the X chromosome.

Observation of the slide revealed that all chromosomes had a bright and even colouration, resulting from the combination of the red and green fluorochromes. The Y chromosome was visible with the DAPI filter only, as there was no hybridising DNA, whilst the X chromosome had equal amounts of both red and green, as expected.

Analysis was carried out on eight metaphases, and 275 chromosomes were included in the interpretation. Hybridisation artefacts were visible for the centromeres of chromosomes 14 and 15 and the long arm of chromosome Y. No deviation from 1 was observed for the ratio profiles of all autosomes and the X chromosome. Thus, the 46,XX karyotype was confirmed. Fig. 5.6a and b demonstrates the appearance of the fluorescent signals on a metaphase, whereas the interpretation of this experiment is shown in fig. 5.6c.

5.2.7 CGH analysis of a single fibroblast cell trisomic for chromosome 18 against 46,XY genomic DNA

The karyotype of the fibroblast cell was 47,XX, +18. The concentration of the genomic DNA was of the range of 1000pg/μl. This experiment took place to investigate whether the CGH profiles would show notable fluctuations, due to the difference of the initial DNA concentrations (6pg versus 1000pg) of the two samples. In addition, it would serve as a reference for the identification of similar abnormalities in oocytes and/or PBs.
Both samples were amplified with the use of the DOP-PCR, resulting in smears very similar to those observed previously. The fibroblast was labelled in green, whereas the male genomic DNA was labelled in red. Their combination and hybridisation occurred as previously described.

Upon visualisation of the target metaphases, it was obvious that both the red and the green fluorochromes were equally intense and clear, giving an even orange coloration

Fig. 5.7: Positive control experiment: CGH analysis of 47,XX+18 fibroblast cell DNA against 46,XY genomic DNA

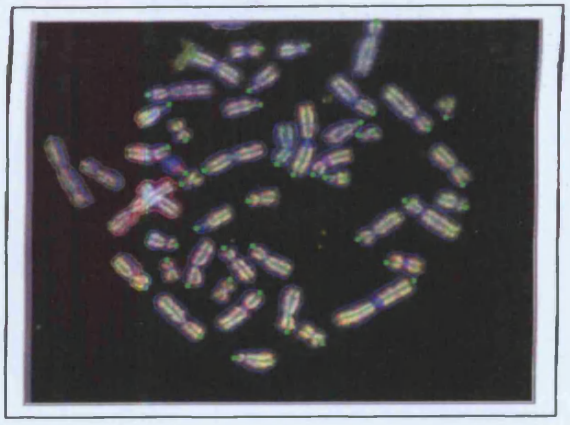


Fig.5.7a: Normal 46,XY metaphase chromosomes hybridised with 47,XX,+18 fibroblast cell DNA (*green*) and 46,XY genomic DNA (*red*).

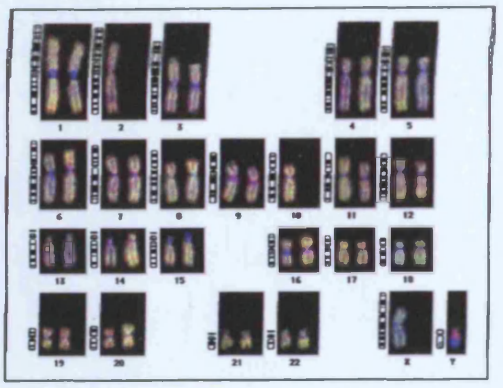


Fig. 5.7b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome 18 was excessively green, the latter indicating the trisomy of this chromosome in the test DNA. Chromosome X was also excessively green, indicating the female karyotype of the test DNA, while chromosome Y was excessively red, as the reference DNA was XY.

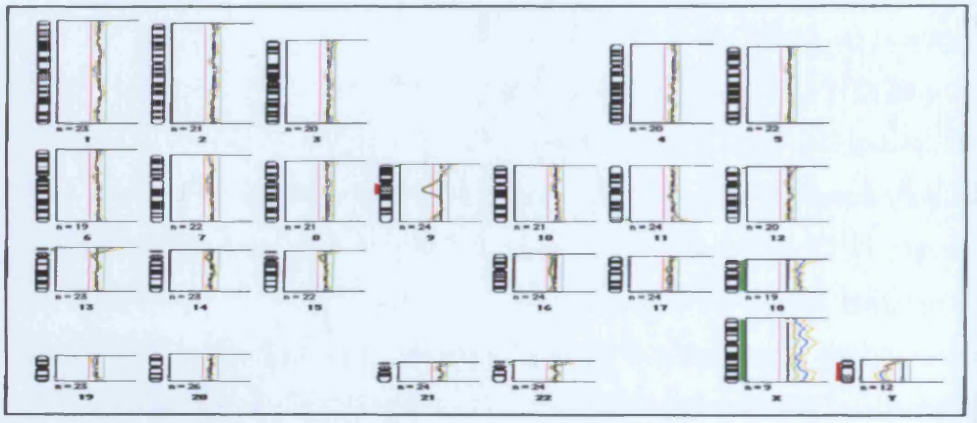


Fig. 5.7c: Interpretation of the positive control CGH experiment described in section 5.2.7. The deviation of the ratio profile for chromosomes 18 and X towards the right threshold (more than 1.20) demonstrated that the test DNA was female and trisomic for chromosome 18. The male karyotype of the reference DNA was obvious due to the deviation of the ratio profile of chromosome Y towards the left threshold (less than 0.8), which also demonstrated its absence from the test DNA. Accurate identification of the karyotype of the test sample as 47,XX,+18 was achieved.

throughout the chromosomes. Chromosome 18 was easily distinguished, as it was relatively more green than red (regions 18p11.3-q23). Its ratio profile deviated towards the right threshold (more than 1.2), demonstrating the trisomy of this chromosome in the test sample. A similar green colouration was visible for chromosome X (regions Xp22.30q28), leading to its ratio profile deviating to the right and the identification of the female karyotype in the test sample. Chromosome Y was easily scored as well, due to its excessive red colouration (regions Yp11.2-q12). Its ratio profile deviated to the left (less than 0.8). This showed that the reference DNA was of male karyotype. Thus, the karyotype of the test sample was confirmed as female trisomy 18.
Analysis took place on 13 metaphases and 510 chromosomes were included in the interpretation. Hybridisation artefacts were observed for the centromere and short arm telomere of chromosome 9, and the centromere of chromosome 13. A metaphase showing the fluorescent signals on the chromosomes can be seen in fig. 5.7a and b. The interpretation of this experiment can be seen in fig. 5.7c.

Optimisation of the CGH protocol was essential during the positive control experiments described above, especially at the single cell level. The latter meant that some of these experiments took place more than once, in order for the desired results to be obtained. Various modifications in the initial CGH protocol occurred. Thus, the extension temperature in the DOP-PCR conditions was decreased from the 72°C that was initially used to 68°C. This temperature was considered to be optimal for the highest performance of the Super Taq Plus™ Enzyme. Moreover, the incubation time for the labelling of the CGH probes varied between 1-2 hours, depending on the sizes of the generated DNA fragments. Ideally these should be between 500bp- 1Kb. During initial CGH experiments, the target metaphase chromosomes were pre-treated with the application of Proteinase K (PK), in order for the surrounding cytoplasm to be removed. The latter was achieved by incubating the slides in PK buffer for 10 minutes at 37°C. This slide pre-treatment was omitted in subsequent experiments, as it was determined to be too aggressive and to affect chromosome morphology, and hence the quality of hybridisation of the CGH probes.

5.3 CGH analysis of human metaphase II oocytes

CGH analysis was attempted for 40 human oocytes that were arrested at the metaphase II stage of meiosis. Eleven cells yielded results, coming from nine patients. Out of these, two oocytes belonged to the first group, four to the second group, and five to the third group with reference to the method of preparation. Different types of reference DNA were used, against which these cells hybridised. Oocytes were initially co-hybridised with 46,XY genomic DNA or clumps of buccal cells. However, the analysis and interpretation of an oocyte (1243.2-green) that was not exposed to sperm and was hybridised against a 46,XY clump of buccal cells (red) did not result in the expected fluorescence difference on the sex chromosomes (excess green on the X, excess red on the Y), indicating the possible contamination of the cell with male DNA, most likely from the technician responsible for cell isolation. It was perceived that the use of female DNA as reference would have allowed the contamination to be more readily detected, as the Y chromosome would have appeared to be green. In addition, the use of 46,XX DNA as reference would enable the detection of X chromosome hyperhaploidy. The latter was not feasible when 46,XY DNA was used as reference, as the test DNA came from female sources, and the X would always appear overrepresented relative the 46,XY control DNA as CGH is not capable of accurately distinguishing chromosome excess (e.g. trisomy versus tetrasomy). For these reasons, the majority of oocytes were hybridised against 46,XX DNA, either genomic or derived from cell clumps. In the latter case, clumps were preferred to single cells, in order to avoid the possibility of an anucleate or degenerate buccal cell, not yielding any results.

All oocytes, clumps of buccal cells, and genomic DNA were initially amplified with the use of the DOP-PCR. Agarose gel analysis revealed smears of DNA fragments whose sizes were between 300-1500bp, as previously. The smears obtained for the oocytes were usually not as bright (i.e. less DNA was present) compared to those from the clumps or the genomic DNA. This observation was attributed to the fact that fewer copies of the genome were present in oocytes than in clumps or genomic DNA samples and that the quality of the oocyte DNA may have been inferior due to prolonged *in vitro* culture. However, the sizes of the amplified fragments from the oocytes, demonstrated that their

Fig.5.8: Agarose gel analysis demonstrating the DOP-PCR amplification of oocyte and PB 1134.3, and two buccal cell clumps

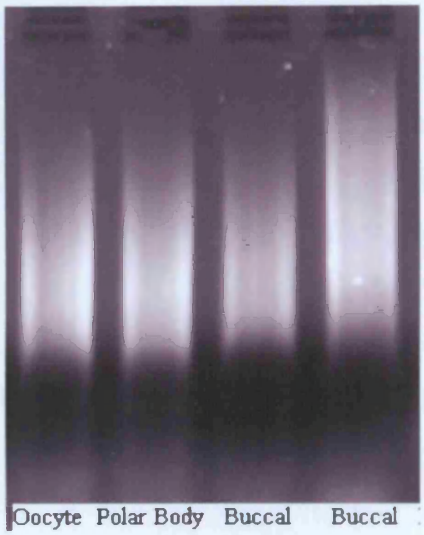


Fig.5.8: Agarose gel analysis demonstrating the DOP-PCR amplification of oocyte no. 1134.3, its corresponding 1st PB and two clumps of buccal cells, which were used as reference DNA against which the oocyte and PB were hybridised. Both the amplified products from the oocyte and its corresponding PB produced smears of similar intensities and fragment sizes with the two clumps of buccal cells. Fragment sizes were between 300-1500bp in length for all samples. The intensity of the smears that were produced from the amplified oocytes and/or PBs varied, being usually fainter compared to those observed for the buccal cell clumps.

DNA was adequately amplified with the use of the DOP-PCR. A gel showing the smears representing an oocyte, a PB, and two clumps of buccal cells can be seen in fig. 5.8.
Oocytes were considered to behave in the same way as a diploid female cell. Out of the eleven oocytes analysed with CGH, eight were considered to be normal 23,X. These were the following: oocyte no. 1141.2 from the first group, oocytes 1172.2, 1173.1, 1243.2, 1174.1 from the second group, and oocytes 4412.6, 1355.4 and 4405.3 from the third group. Fertilisation was attempted with ICSI for five of them (1141.2, 1172.2, 1173.1, 1174.1, 1355.4) and with IVF for the remaining two, whilst oocyte 1243.2 was immature at the time of retrieval and was not exposed to sperm.

Oocytes 1141.2, 1172.2, 1173.1, 1174.1, 1243.2 were all hybridised against normal male DNA. After visualisation of the target metaphase areas under the fluorescent microscope, it was observed that the red fluorescence being emitted from the reference DNA was almost always intense, clear and even on the chromosomes, without giving any background. The green fluorescence corresponding to the oocyte DNA was almost always much fainter and background was frequently observed. The latter was attributed to the DNA quality of the oocytes. Analysis was carried out on 7-12 metaphases each time.

In the case of oocyte 1174.1, which was exposed to sperm via ICSI, and hybridised against a clump of 46,XY buccal cells, the interpretation of the captured images did not detect any marked fluorescence difference on the sex chromosomes, and no obvious deviation of their ratio profiles from 1. This observation led to the conclusion that this oocyte was penetrated with Y-bearing sperm. The karyotype of the actual oocyte was considered to be normal 23,X.
For the remaining four oocytes that co-hybridised with 46,XY DNA, interpretation of the captured metaphase images would demonstrate autosomes with ratio profiles not deviating from 1, and difference in the fluorescence intensities on the sex chromosomes. Hence, chromosome X was consistently excessively green (areas: Xp22.3-Xq27) leading to a deviation of its ratio profile towards the right threshold (more than 1.2). This showed that the test DNA was of a female karyotype.

Fig. 5.9: CGH analysis of oocyte no. 1173.1, 23,X against 46,XY buccal cell clump DNA

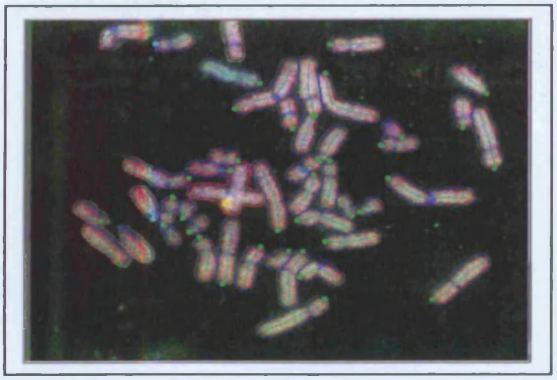


Fig.5.9a: Normal 46,XY metaphase chromosomes hybridised with DNA from oocyte 1173.1 *(green)* and 46,XY buccal cell DNA *(red)*.

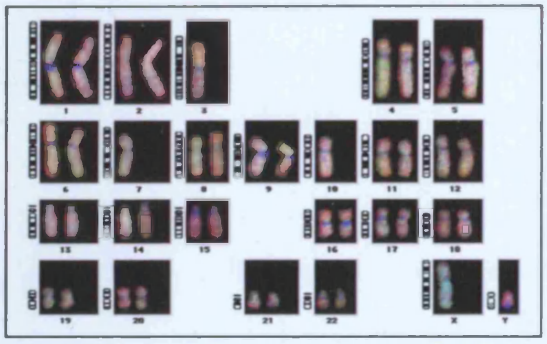


Fig.5.9b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. All 23 autosomes had an even orange colour resulting from the combination of the green oocyte DNA and the red buccal clump DNA. We considered that a haploid oocyte behaved as a diploid female cell. Thus, chromosome X was excessively green indicating the female test DNA, whilst chromosome Y was excessively red, due to the male reference DNA.

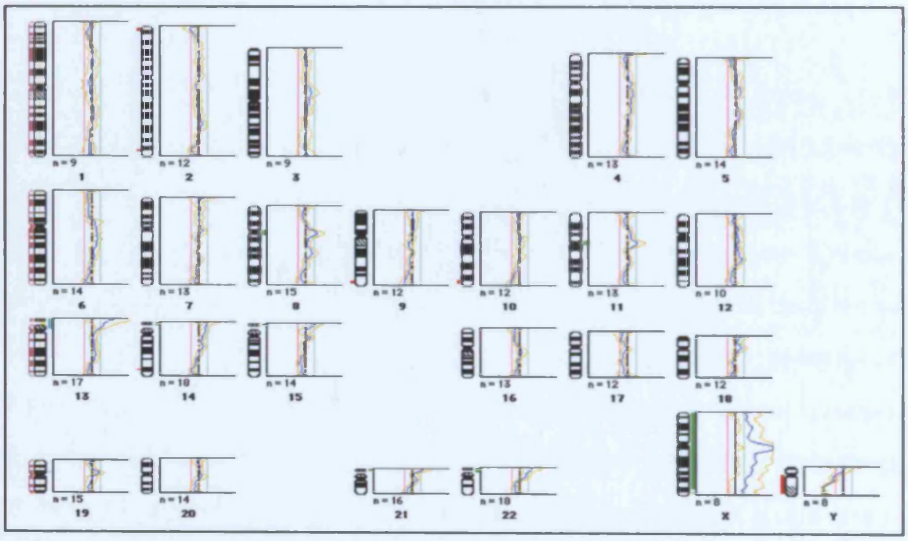


Fig.5.9c: Interpretation of the CGH experiment for oocyte 1173.1. The oocyte was characterised as normal haploid (23,X), as no difference in fluorescence intensities was detected for any of the 23 autosomes, meaning that they were present in equal numbers in the "test" (green) and "reference" (red) DNAs. The fluorescence pattern and the shifts of the ratio profiles observed for the sex chromosomes were identical to those observed when sex mismatched DNAs are hybridised against each other, as described in fig.5.1 and fig.5.5.

Chromosome Y was excessively red (areas: Yp11.1-q12). Its ratio profile deviated towards the left threshold (less than 0.8), demonstrating the male karyotype of the reference DNA. All these oocytes, apart from oocyte 1243.2, were injected with sperm. Thus, it was considered that the fluorescence difference observed on the sex chromosomes was attributed to the fact that the sperm was carrying an X chromosome as well. In all cases the oocytes were considered to be normal 23,X. A metaphase showing the appearance of the fluorescent signals on the chromosomes for oocyte 1173.1 is shown in fig.5.9a and b, whereas its corresponding interpretation is illustrated in fig.5.9c.

Oocytes 4412.6, 1355.4, and 4405.3 were all hybridised against 46,XX clumps of buccal cells. Observation of the hybridisation target areas led to similar findings to the above, i.e., the clumps of buccal cells always gave bright and clear red fluorescent signals, whereas fainter green signals were visible for the oocyte DNA, combined with some background green fluorescence. Chromosome Y did not show any fluorescent signals, in all cases apart from one (oocyte no. 4405.3). The fluorescent signal absence for chromosome Y was expected and was also demonstrated during the analysis of the positive control experiment, involving the co-hybridisation of 46,XX buccal cells, labelled in red and green.

Analysis and interpretation of the metaphase images for oocytes 4412.6, and 1355.4 gave very similar results. All autosomes and the X chromosome showed an orange colouration from the combination of the red and the green fluorochromes. The latter was interpreted as no deviation of ratio profiles from 1. In all these cases, the karyotype of the test oocyte was considered to be normal 23,X, possibly having been penetrated with an X-bearing sperm.

The situation was different for oocyte 4405.3. Fertilisation of this cell was attempted via IVF. Interpretation of the metaphase images did not show any deviation from 1 for any of the autosomes. A very small part towards the bottom of the long arm of chromosome X (Xq25-q27) showed excess green. This was considered to be attributable to hybridisation artefacts that often affect telomeric regions, and was excluded from the analysis. Excess red fluorescence, was observed for the whole of chromosome Y (Yp11.3-q12), leading to a deviation of the ratio towards the left threshold (less than 0.8). The latter was unexpected, as the reference DNA used for

Fig. 5.10: CGH analysis of oocyte no. 1355.4, 23,X against 46,XX buccal cell clump DNA

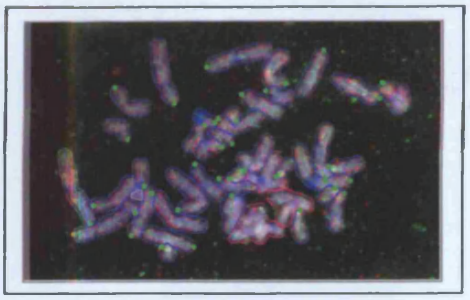


Fig.5.10a: Normal 46,XY metaphase chromosomes hybridised with DNA from oocyte 1355.4 (*green)* and 46,XX buccal cell DNA (*red).*

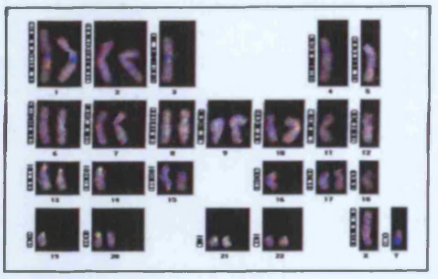


Fig.5.10b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Even fluorescence intensities were observed for all 23 autosomes and the X chromosome, as both the test oocyte DNA and the reference DNA were female.

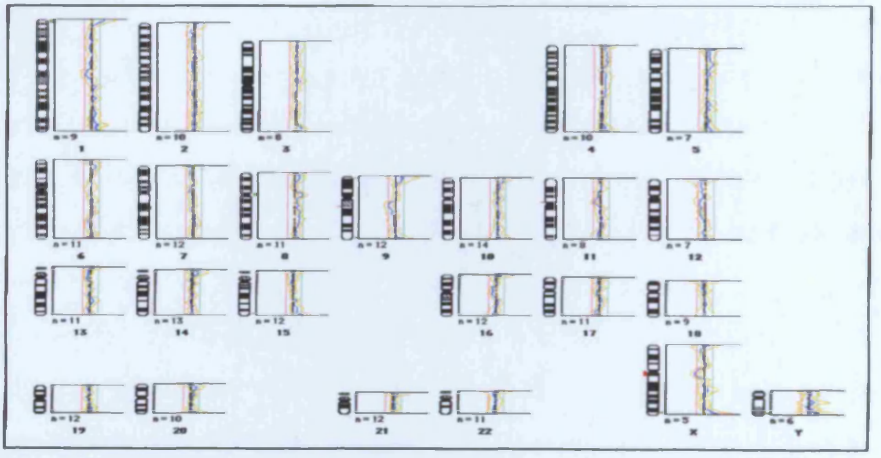


Fig.5.10c: Interpretation of the CGH experiment for oocyte 1355.4. The oocyte was characterised as normal haploid (23,X), as no difference in fluorescence intensities was detected for any of the 23 autosomes, meaning that they were present in equal numbers in the "test" (green) and "reference" (red) DNAs. No shift was observed for the ratio profile of chromosome X, as the latter was also present in equal amounts in both test and reference (46,XX) DNAs. The fluorescence pattern for the sex chromosomes in this experiment was similar to the one described in fig.5.6, when a 46,XX buccal cell clump (green) was hybridised against another 46,XX buccal cell clump (red).

this experiment was 46,XX. It was very likely that the clump of buccal cells was contaminated. The oocyte was considered to be normal 23,X. A metaphase demonstrating the appearance of the fluorescent signals for oocyte 1355.4 is shown in fig.5.10a and b. Its corresponding interpretation can be seen in fig.5.10c.

Details of the above observations for all the oocytes that were characterised as normal are shown in Table 5.1.

Abnormalities were observed for oocytes 1134.3, 4412.4 and 4412.5, the first belonging to the first group, whilst the other two the third one. As was mentioned in the previous chapter, non-disjunction of whole chromosomes and premature separation of a chromosome into its sister chromatids prior to anaphase I, can both lead to aneuploidy in oocytes. Distinction between extra or missing chromosomes and chromatids was not feasible in most cases due to the relative detection insensitivity of the CGH and the suboptimal condition of the oocyte DNA. In addition, the abnormalities observed in oocytes 4412.4 and 4412.5 described below could have been due to sperm presence. Confirmation that such abnormalities were the result of non-disjunction would only occur if the reciprocal loss or gain were scored in the corresponding 1st PB. This occurred for the oocyte no. 4412.5 and will be discussed in the following section. Oocyte 1134.3 was at the germinal vesicle stage when collected, was not injected due to its immaturity and was left to mature to metaphase II *in vitro*.

Oocyte 1134.3 was hybridised against a clump of 46,XY buccal cells. Upon visualisation of the target metaphase chromosomes the following were observed: the red fluorochrome corresponding to the reference DNA was very intense and even on the chromosomes. The green fluorescence for the oocyte DNA was even on the chromosomes as well, but fainter. No difference in fluorescence could be seen for the sex chromosomes, which was not expected. Analysis of 12 metaphases took place, and 412 chromosomes were included in the interpretation. Chromosome 13 appeared to be excessively green (areas: 13p13-q21). Its ratio profile deviated towards the right threshold (more than 1.2). This indicated the presence of extra chromosomal material for 13 in the test DNA. In the positive control experiment involving the CGH analysis of fibroblast DNA that was trisomic for chromosome 13, the areas of the chromosome that showed the excess green were 13p12-q34. Comparison with the results obtained for this oocyte confirmed the

Fig.5.11: CGH analysis of oocyte no.1134.3 against 46,XY buccal cell clump DNA

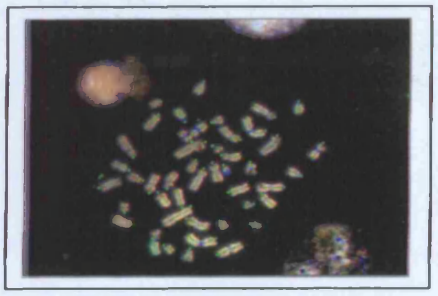


Fig.5.11a: Normal 46,XY metaphase chromosomes hybridised with DNA from oocyte 1134.3 (*green*) and 46,XY buccal cell DNA (*red*).

Fig.5.11b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome 13 appeared to be excessively green, compared to the rest of the autosomes, the latter indicating the presence of extra DNA for this chromosome in the test sample.

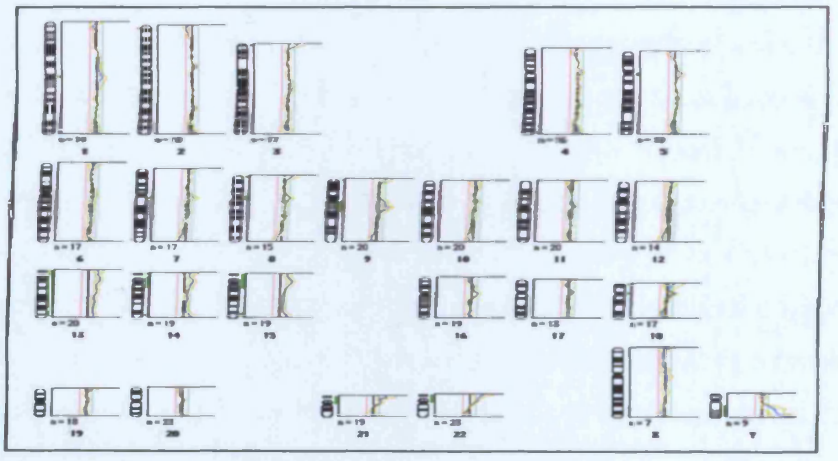


Fig.5.11c: Interpretation of the CGH experiment for oocyte 1134.3. The ratio profile of chromosome 13 deviated towards the right threshold (more than 1.20), demonstrating the presence of extra DNA material for this chromosome in the green oocyte sample. CGH analysis of the corresponding PB did not demonstrate the expected reciprocal loss of chromosome 13. It is possible that the extra DNA for 13 was due to the presence of an additional chromatid in the oocyte, with the CGH software not detecting its loss in the corresponding PB. Another possibility was that this patient was a germinal mosaic for trisomy 13, with the extra copy ending up in the oocyte. The oocyte was not exposed to sperm, as it was immature at the time of collection. Hence the karyotype of this oocyte was thought to be either 24,X,+13 or 23X,+1/2 13cht.

presence of the extra DNA for this chromosome in the cell. CGH analysis of the corresponding 1st PB revealed that the latter had a normal 23,X karyotype as will be described in the following section. Thus, the presence of the extra chromosome 13 in oocyte 1134.3 could be attributed to the fact that the patient was a germinal mosaic for trisomy 13. Another possibility could be that the oocyte contained an extra chromatid 13. In this case, the CGH analysis may have had insufficient sensitivity to detect the loss of the chromatid 13 in the corresponding PB. It has been shown that CGH is less efficient in detecting chromosome loss than chromosome gain (Malmgrem *et al.*, 2002; D. Wells personal communication). As far as the remaining autosomes and the sex chromosomes were concerned, there was no difference in fluorescence intensities between the two fluorochromes, and ratio profiles did not deviate from 1. Since the reference DNA was male and the oocyte uninjected, it was concluded that the cell was contaminated by the male handler during its tubing. Thus, CGH analysis of the test DNA demonstrated that the karyotype of this oocyte was 24,X,+13 or 23,X,+1/213cht in the case of an extra chromatid. Appearance of fluorescent signals on a metaphase is shown in fig.5.11a and b, whereas the interpretation of this oocyte is illustrated in fig.5.11c.

Fertilisation was attempted with IVF for oocyte 4412.4. The reference DNA used was a clump of 46,XX buccal cells. Observation of the slide under the fluorescent microscope showed chromosomes that had an even orange colouration, with the green fluorochrome corresponding to the oocyte DNA being relatively more faint compared to the red fluorochrome for the reference DNA. Analysis was carried out on seven metaphases and 224 chromosomes were included in the interpretation. Excess green fluorescence was observed for chromosome 13 (areas: 13q14-q32). The ratio profile for this chromosome deviated towards the right threshold (more than 1.2), indicating the presence of extra chromosomal material for 13 in the test DNA. Excess red fluorescence was scored for chromosome 22 (areas: 22p13-p11.1, 22q11.2-q13), leading to a deviation towards the left threshold (less than 0.8) This deviation indicated that the test DNA was missing chromosomal material for 22. Comparison with the positive control experiment involving the CGH analysis of fibroblast DNA, trisomic for 22 showed the presence of excess green fluorescence on the exact same regions of this chromosome, and confirmed the results for the oocyte.

Fig.5.12: CGH analysis of oocyte no. 4412.4 against 46,XX buccal cell clump DNA

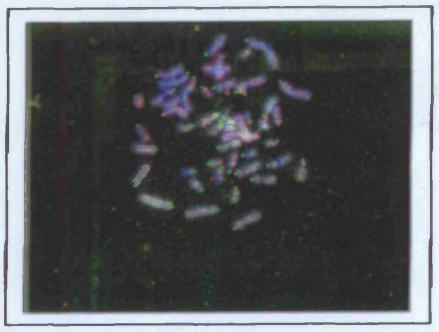


Fig.5.12a: Normal 46,XY metaphase chromosomes hybridised with DNA from oocyte 4412.4 *(green)* and 46,XX buccal cell DNA *(red)*.

Fig.5.12b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome 13 appeared to be excessively green, whilst chromosome 22 was excessively red, compared to the rest of the autosomes. These differences in fluorescence intensities demonstrated that the test oocyte sample contained extra DNA material for 13 and was also missing DNA material for 22.

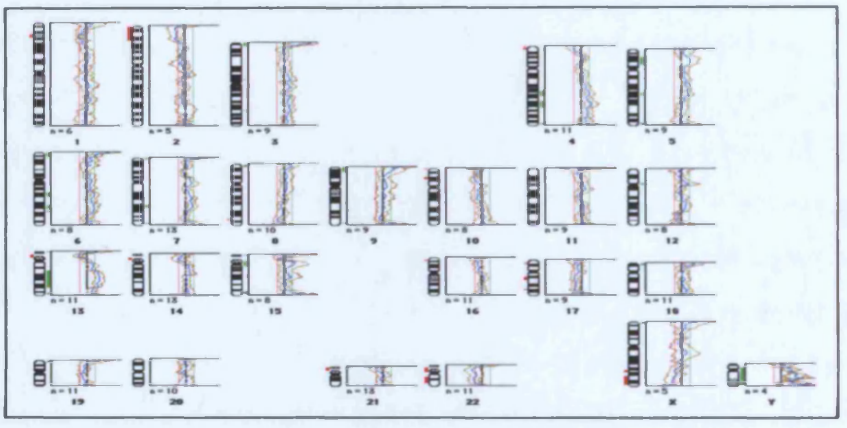


Fig.5.12c: Interpretation of the CGH experiment for oocyte 4412.4. The ratio profile of chromosome 13 deviated towards the right threshold (more than 1.20), whereas the ratio profile for chromosome 22 deviated towards the left (less than 0.80). These shifts demonstrated the presence of extra DNA material for 13 and the absence for 22 in the oocyte sample. CGH analysis of the corresponding PB did not demonstrate the reciprocal loss and gain for these chromosomes. Sperm contamination was evident, as chromosome Y was excessively green, and the reference DNA used in this experiment was 46,XX. It was possible that these abnormalities could have been caused by an aneuploid sperm. Another possibility was that the abnormalities observed were caused by single chromatids. In the case that these anomalies were of maternal origin, then the karyotype of the oocyte was considered to be either 23,X,+13,-22 or 23,X,+1/2 13cht,-1/2 22cht.

Chromosome Y was consistently excessively green (areas: Yp11.2-q12), with its ratio profile deviating to the right. This observation made the presence of sperm DNA in the oocyte evident. CGH analysis of its corresponding 1st PB showed the latter to be normal 23,X. The abnormalities observed in this cell could either be attributed to the sperm, and thus, oocyte 4412.4 had a normal 23,X karyotype and the sperm had a 23,Y+13,-22 karyotype, or they were all caused by meiotic errors taking place in the oocyte. The fact that the reciprocal gain and loss were not detected in the corresponding 1st PB argues for an origin in the sperm. However, it is also possible that the CGH failed to detect the imbalance in the corresponding 1st PB due to poor hybridisation. In the case that these chromosome abnormalities were of maternal origin, the karyotype of the oocyte would be either 23,X,+13,-22 if whole chromosomes were involved or 23,X,+½13cht,-½22cht if single chromatids were participating. A metaphase from the CGH of this oocyte is seen in fig.5.12a and b, while the interpretation for this experiment is shown in fig.5.12c.

Analysis took place on nine metaphases and 352 chromosomes were included in the interpretation for oocyte 4412.5. Excess red fluorescence was observed for chromosome X (areas: Xp21-Xq28). This was accompanied by a shift of the ratio profile for this chromosome towards the left threshold (less than 0.8), indicating the loss of this chromosome from the test sample. A slight shift towards the right threshold (more than 1.2) was observed for the ratio profile of chromosome 21. Some hybridisation artefacts were observed on chromosome 9 (p and q telomeres, and heterochromatic region), at the bottom part of chromosome Y (Yq11.2-q12) and a small part of the short arm of chromosome 2. These well described CGH artefacts were excluded from the interpretation. The above observations led to the conclusion that oocyte 4412.5 was missing chromosome X and could contain extra chromosome material for 21, possibly an extra chromatid. The corresponding PB was also investigated with CGH and the reciprocal gain of DNA for chromosome X and loss of DNA for chromosome 21 were both identified, as discussed in the following section. Hence, the karyotype of oocyte 4412.5 according to the obtained CGH results was 22,-X,(possibly +1/221cht). A metaphase demonstrating the hybridisation signals for this oocyte is shown in fig. 5.13a and b, whilst the interpretation for this experiment can be seen in fig.5.13c. Oocyte 4412.6 coming from the same patient was characterised to be normal haploid 23,X. The patient that donated these oocytes was

Fig.5.13: CGH analysis of oocyte no. 4412.5 against 46,XX buccal cell clump DNA

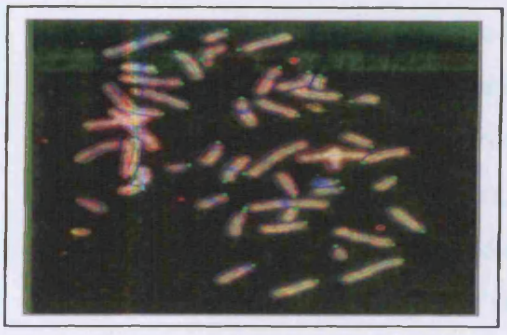


Fig.5.13a: Normal 46,XY metaphase chromosomes hybridised with DNA from oocyte 4412.5 *(green)* and 46,XX buccal cell DNA *(red)*.

Fig.5.13b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome X appeared to be excessively red. The latter indicated that the test oocyte sample was missing DNA for this chromosome.

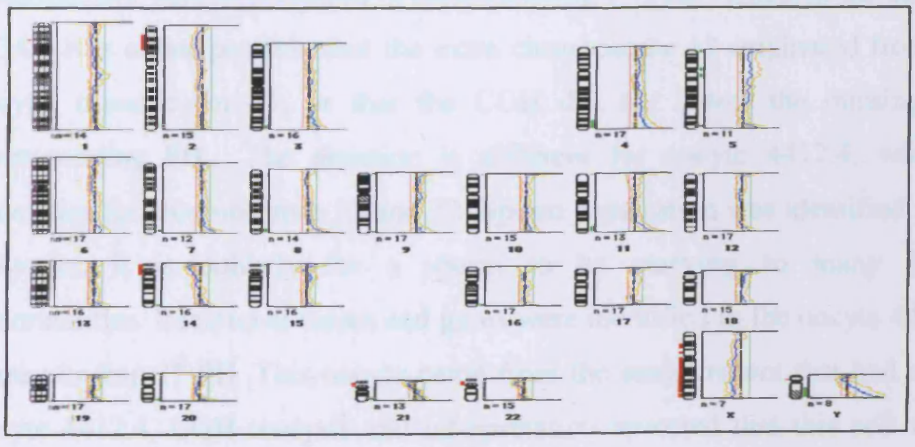


Fig.5.13c: Interpretation of the CGH experiment for oocyte 4412.5. The ratio profile of chromosome X deviated towards the left (less than 0.80). This shift demonstrated the absence of this chromosome in the oocyte sample. A slight shift of the ratio profile for 21 towards the right (more than 1.20) was also seen, indicating the possible presence of extra DNA for this chromosome in the test sample. The reciprocal gain of X and loss of chromosome 21 was evident from the CGH analysis of the corresponding PB (see fig.5.16). These anomalies were most likely of maternal origin, as they were confirmed in the corresponding PB. The karyotype of the oocyte was 22,-X, possibly +1/2 21cht.

treated for infertility due to polycystic ovary syndrome. Such patients frequently produce oocytes with multiple chromosome anomalies (Sengoku *et al.*, 1997; Clyde *et al.*, 2001). So it was very likely that the abnormalities observed in the oocyte no. 4412.5 were of maternal origin. Details of the observations from the CGH analysis of the oocytes are summarised in Table 5.1.

Amplified DNA from the oocytes that did not yield results after they underwent CGH was analysed by agarose gel electrophoresis. Out of the 29 samples analysed, only two oocytes gave very faint smears, demonstrating that there was amplification of their DNA. The fact that the CGH did not work for these samples could be attributed to very poor DNA quality. None of the other samples gave smears. It was very likely that these oocytes were either lost or lysed at a stage during their processing.

In summary, CGH results were obtained for 11 oocytes out of a total of 40, for which analysis was attempted. Nine patients were investigated. From these 11 oocytes eight could be characterised as normal 23,X. Abnormalities were identified for three oocytes coming from two different patients. In two of the three cases the reciprocal abnormalities were not detected in corresponding 1st PBs. Hence, in the case of oocyte 1134.3 it is either possible that the extra chromosome 13 originated from a primary oocyte, trisomic for 13, or that the CGH did not detect the missing 13 in the corresponding PB. The situation is different for oocyte 4412.4, which showed anomalies for chromosomes 13 and 22. Sperm penetration was identified for this cell. However, it is unlikely for a sperm to be carrying so many chromosome abnormalities. Reciprocal losses and gains were identified in the oocyte 4412.5 and its corresponding 1st PB. This oocyte came from the same patient that had also donated oocyte 4412.4. CGH analysis and interpretation revealed that this cell was missing chromosome X and could possibly contain extra DNA for chromosome 21. The reciprocal gain of chromosome X was identified in the corresponding 1st PB that was also identified to be missing chromosome 21. Hence in this case, the abnormalities scored in the oocyte were most likely due to meiotic non-disjunction taking place in the female.

Confirmation that the above chromosome abnormalities were of maternal origin also comes from the fact, that the female patient that donated these cells was being treated

for infertility due to polycystic ovary syndrome. Such patients could tend to produce gametes carrying multiple chromosome abnormalities (Sengoku *et al.*, 1997).

Table 5.1: CGH analysis of human metaphase II oocytes

Oocyte/patient no.	ART method	Reference DNA	CGH interpretation
1141.2	ICSI	46,XY clump of buccal cells	23,X
1134.3	Uninjected	46,XY clump of buccal cells	24,X+13 or 23,X+1/2 13cht
1172.2	ICSI	46,XY clump of buccal cells	23,X
1173.1	ICSI	46,XY clump of buccal cells	23,X
1174.1	ICSI	46,XY clump of buccal cells	23,X
1243.2	Uninjected	46,XY clump of buccal cells	23,X
1355.4	ICSI	46,XX clump of buccal cells	23,X
4412.4	IVF	46,XX clump of buccal cells	23,X+13,-22 or 23,X+1/2 13cht,– ½ 22cht
4412.5	IVF	46,XX clump of buccal cells	22 –X, or 22,-X +1/2 21cht
4412.6	IVF	46,XX clump of buccal cells	23,X
4405.3	IVF	46,XX clump of buccal cells	23,X

5.4 CGH analysis of 1st Polar Bodies

A total of 45 first PBs underwent CGH analysis, 37 of which had their corresponding oocyte also investigated. Results were obtained for 15 PBs, three belonging to group 1, four belonging to group 2, and eight belonging to group 3, from fourteen different patients. It was thought that sperm contamination was unlikely for the PBs, and thus the obtained CGH results were representative of the maternal genome only. The reference DNA used was as for the oocytes, i.e. genomic or clumps of buccals of 46,XY or 46,XX DNA. All PBs and reference DNAs were amplified with the use of the DOP-PCR as previously described. Agarose gel analysis demonstrated the same difference in smear intensities between PBs and reference DNAs, as that observed for the oocyte DNA. Average fragment sizes were in the range of 1500bp.

Similar to the oocytes, the PBs were also considered to behave as diploid female cells. Eleven PBs were characterised as being normal 23,X after their CGH analysis. These were the following: 1141.2PB, 1134.3PB, 4047.3PB from the first group, 1173.1, 4125.1PB, 1243.1PB, 4069.1PB from the second group, and 4412.4PB, 4412.6PB, 1355.5PB, 4405.3PB from the third group.

Normal 46,XY DNA in the form of clumps of buccal cells was used as reference during the CGH analysis of 1173.1PB, 1134.3PB, 4047.3PB and 4125.1PB. Upon visualisation of the hybridisation target areas under the fluorescent microscope, the metaphase chromosomes were seen to have a uniform orange colour from the combination of the red and green fluorochromes. As before, the red fluorescence emitted from the reference DNA was very intense and sharp, without any background. The green fluorescence, corresponding to the PB was much brighter and clearer compared to that observed for the oocyte DNA. Chromosomes X and Y could easily be distinguished as the former was more green and the latter more red, demonstrating the difference in DNA quantities between the test and reference DNAs for these chromosomes.

Analysis was carried out on 15 metaphases for all four PBs. Interpretation of the images showed no marked fluorescence difference between test and reference DNAs in any of the autosomes for all these. Their ratio profiles did not deviate from 1.

Fig. 5.14: CGH analysis of PB no. 4125.1, 23,X against 46,XY buccal cell clump DNA

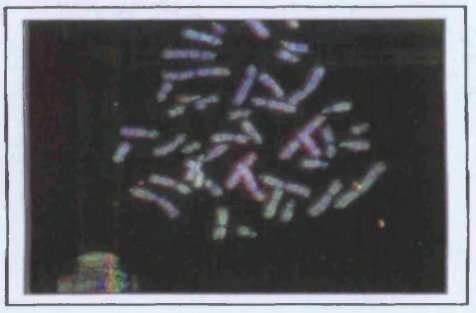


Fig.5.14a: Normal 46,XY metaphase chromosomes hybridised with DNA from PB no. 4125.1 (*green)* and 46,XY buccal cell DNA (*red).*

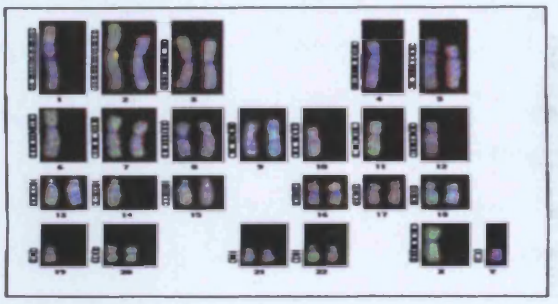


Fig.5.14b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. As with the oocytes, we considered that PBs behaved as diploid female cells. Chromosome X was excessively green indicating the female test DNA, whilst chromosome Y was excessively red, due to the male reference DNA.

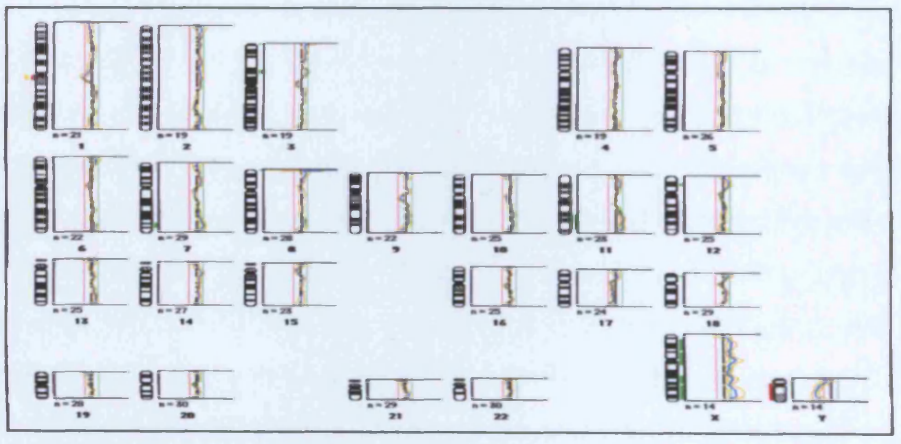


Fig.5.14c: Interpretation of the CGH experiment for PB 4125.1. This PB was characterised as normal haploid (23,X), as no difference in fluorescence intensities was detected for any of the 23 autosomes, meaning that they were present in equal numbers in the "test" (green) and "reference" (red) DNAs. The fluorescence pattern and the shifts of the ratio profiles observed for the sex chromosomes were identical to those observed when sex mismatched DNAs are hybridised against each other, as described in fig.5.1, 5.5 and 5.9.

Chromosome X was consistently excessively more green (areas: Xp22.1-Xq27), with its ratio profile deviating towards the right threshold (more than 1.2). Chromosome Y was more red (areas Yp11.2-q12), with its ratio profile deviating towards the left threshold (less than 0.8). The above showed that the karyotype of all four PBs was normal 23,X. A metaphase showing the appearance of the fluorescent signals obtained for 4125.1PB can be seen in fig.5.14a and b. Its corresponding interpretation is shown in fig.5.14c.

In the case of 1134.3PB, CGH results were obtained for its corresponding oocyte, which appeared to be carrying extra chromosome material for 13. The reciprocal loss of 13 was not observed in the 1st PB. This could either be attributed to this patient being a germinal mosaic for trisomy 13, or to inability of the CGH software to identify a missing chromatid for 13 in the PB, as mentioned in the previous section.

Normal 46,XX DNA either genomic or in the form of buccal cell clumps was used as reference for the 1141.2PB, 1243.1PB, 4069.1PB, 4412.4PB, 4412.6PB, 1355.5PB and 4405.3PB. The appearance of fluorescent signals on the target metaphase chromosomes was as previously for both reference and test DNA, with the only difference being that no fluorescence was visible for the Y chromosome. The latter was expected, as neither the test nor the reference DNAs were male. Analysis was carried out on 7-15 metaphases. Hybridisation artefacts were sometimes visible for the centromeres and/or telomeres of chromosomes 1, 2, 5, 6, 7, 8, 9, 13, 15, 21, and 22. These were excluded from the analysis. Interpretation of the metaphase images demonstrated that the ratio profiles of all autosomes and the X chromosome did not deviate from 1. In the case of 4412.6PB excess green fluorescence was consistently observed on the Y chromosome, leading to a deviation to the right. The latter indicated the presence of male DNA contamination in this PB, most likely from the handler. The karyotype of all the above analysed PBs was considered to be normal 23,X. Normal reciprocal results were obtained for the following pairs of cells: 1141.2 oocyte and PB, 1173.1 oocyte and PB, 4405.3 oocyte and PB, and 4412.6 oocyte and PB. All were characterised as haploid 23,X. A metaphase from 4412.6 PB is shown in fig.5.15a and b, whereas its interpretation can be seen in fig.5.15c. Details of the observations made in all the above PBs are summarised in Table 5.2.

Fig. 5.15: CGH analysis of PB no. 4412.6, 23,X against 46,XX buccal cell clump DNA

Fig.5.15a: Normal 46,XY metaphase chromosomes hybridised with DNA from PB no. 4412.6 *(green)* and 46,XX buccal cell DNA *(red)*.

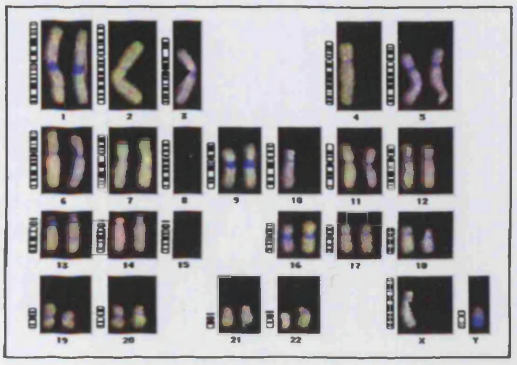


Fig.5.15b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Even fluorescence intensity was observed for all 23 autosomes and the X chromosome, as both the test PB DNA and the reference DNA were female. No fluorescence was visible for chromosome Y, due to its absence from both samples.

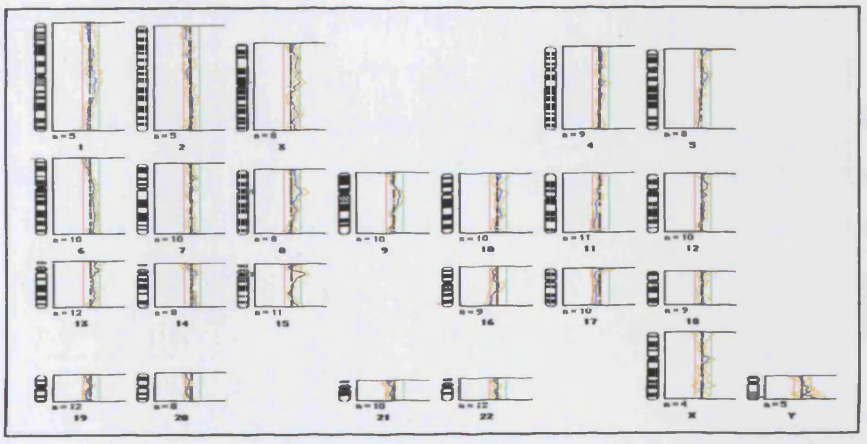


Fig.5.15c: Interpretation of the CGH experiment for PB 4412.6. The PB was characterised as normal haploid (23,X), as no difference in fluorescence intensities was detected for any of the 23 autosomes and chromosome X. These were present in equal numbers in the "test" (green) and "reference" (red) DNAs. The fluorescence pattern for the sex chromosomes in this experiment is similar to the one described in fig.5.6 and 5.10.

Fig.5.16: CGH analysis of PB no. 4412.5 against 46,XX buccal cell clump DNA

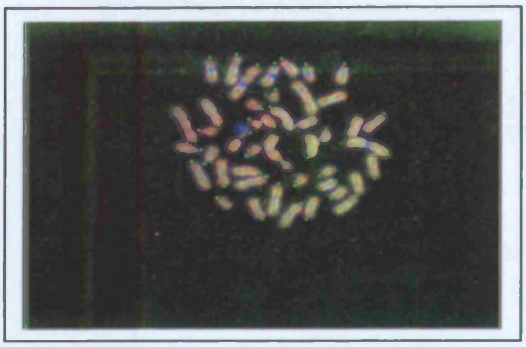


Fig.5.16a: Normal 46,XY metaphase chromosomes hybridised with DNA from PB 4412.5 *(green)* and 46,XX buccal cell DNA *(red)*.

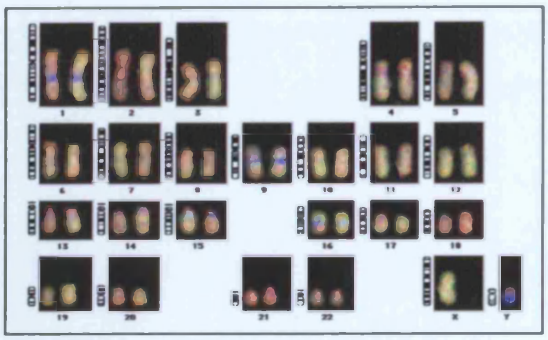


Fig.5.16b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome X appeared to be excessively green and chromosome 21 excessively red. The latter indicated that the test PB sample contained extra DNA material for X and was missing DNA material for 21.

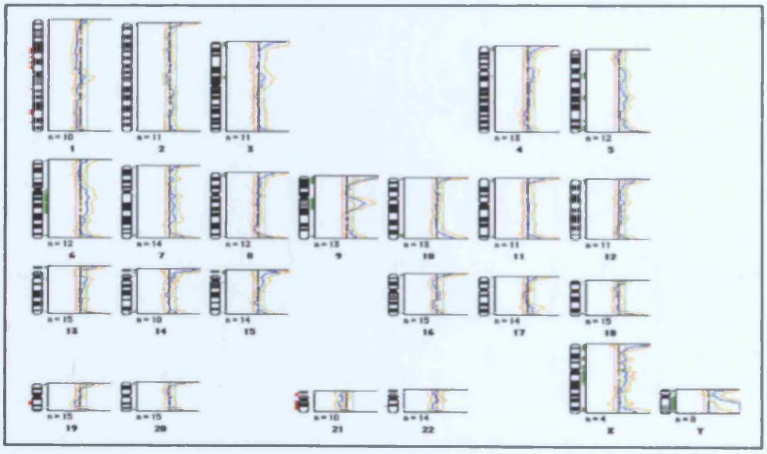


Fig.5.16c: Interpretation of the CGH experiment for PB 4412.5. The ratio profile of chromosome X deviated towards the right threshold (more than 1.20). This shift demonstrated the presence of extra chromosomal material in the PB sample. A shift of the ratio profile for 21 towards the left (less than 0.80) was observed as well, indicating the absence of chromosomal material for 21 in the test. The reciprocal loss of X and possible gain of chromosome 21 were evident from the CGH analysis of the corresponding oocyte (see fig.5.13). Contamination by the male handler was seen in this PB. These anomalies were of maternal origin, as they were confirmed in the corresponding oocyte. The karyotype of this PB was 23,X,+X,-21(or -1/2 21cht).

Chromosome abnormalities were scored in four PBs: 1143.4PB, 1254.6PB, 4412.5PB and 1355.1PB. Similar to the oocytes, anomalies involving extra or missing chromosomes could not usually be distinguished from those that involved single chromatids.

The most pronounced case was again that of patient 4412, who was suffering from polycystic ovaries. CGH analysis was carried out on four PBs (4412.1PB, 4412.4PB, 4412.5PB, and 4412.6PB) from this patient, out of which three yielded results.

The 4412.5PB was hybridised against a clump of 46,XX buccal cells. Analysis took place on eight metaphases, and 292 chromosomes were included in the interpretation. Excess red fluorescence was observed on chromosome 21 (areas: 21p12-p11.2, 21q11.1-q22). The ratio profile of this chromosome deviated towards the left threshold (less than 0.8), something, which demonstrated that the PB was missing DNA material for 21. The latter was confirmed by comparing the fluorescence pattern with the positive control experiment involving the CGH analysis of fibroblast DNA trisomic for chromosome 21. Excess green was observed on the exact same areas of this chromosome. Chromosome X was excessively green, as well (areas: Xp22.3-p22, Xp21-p11.2, Xq21, Xq27). The deviation of its ratio profile towards the right threshold, demonstrated that the 4412.5PB consisted of extra chromosomal material for X. Excess green was also observed for chromosome Y (areas Yq11.1-12), indicating the presence of male DNA contamination, possibly from the handler, as it was unlikely for the actual PB cell to be penetrated by sperm. The reciprocal loss of DNA for chromosome X was detected in the corresponding oocyte, but not the gain of DNA for chromosome 21, as described in the previous section. According to these observations, it is possible that this PB contained an extra whole chromosome X and was missing a single chromatid 21, its karyotype being 24,X,+X,-½21cht. The appearance of the fluorescent signals on a metaphase coming from the CGH analysis of this PB can be seen in fig.5.16a and b, whereas the interpretation results are shown in fig.5.16c.

In total three oocytes and four PBs from patient 4412 were investigated, and abnormalities were seen for two oocytes and one PB, the latter corresponding to one

Fig.5.17: CGH analysis of PB no. 1143.4 against 46,XY buccal cell clump DNA

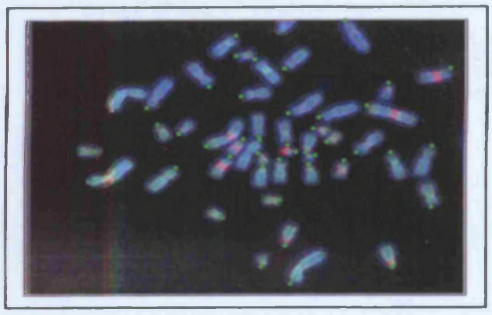


Fig.5.17a: Normal 46,XY metaphase chromosomes hybridised with DNA from PB 1143.4 *(green)* and 46,XY buccal cell DNA *(red)*.

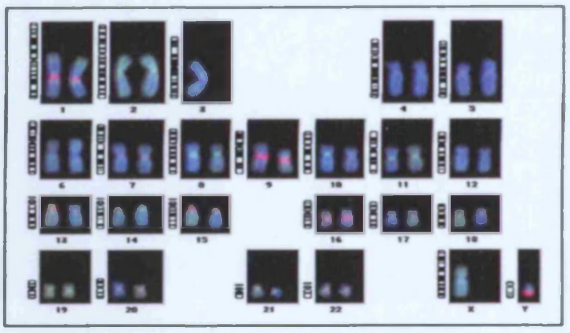


Fig.5.17b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome 8 appeared to be excessively green. The latter indicated that the test PB sample contained extra DNA material for this chromosome.

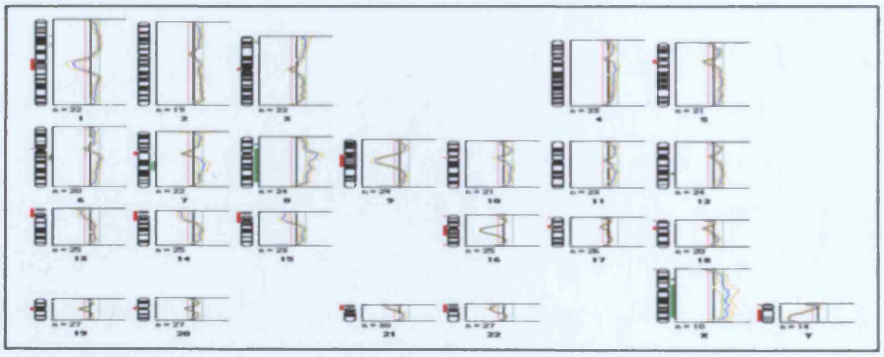


Fig.5.17c: Interpretation of the CGH experiment for PB 1143.4. The ratio profile of chromosome 8 deviated towards the right threshold (more than 1.20). This shift demonstrated the presence of extra chromosomal material in the PB sample. The fluorescence pattern and the shifts of the ratio profiles towards the right threshold for chromosome X and towards the left threshold for chromosome Y were seen previously when sex mismatched DNAs co-hybridised. The karyotype of this PB was thought to be 24,X,+8. The corresponding oocyte was not investigated.

of the two oocytes. Different chromosomes were involved, including 13 and X, which appeared to be present in extra copies, and the smaller 21 and 22, which appeared to be missing. One oocyte and PB pair (4412.6) appeared to be normal haploid, 23,X. The maternal age was 22 years.

The PB no. 1143.4 was hybridised against 46,XY genomic DNA. Analysis took place on 15 metaphases and 544 chromosomes were included in the interpretation. Hybridisation artefacts were observed for the centromeres of chromosomes 1, 5, 7, 9, 13, 14, 15, 16, 17, 18, 19, 20, 21, and 22. These were not included in the analysis. Chromosome 8 appeared to be consistently excessively green (areas 8p22-q24.1), with its ratio profile deviating towards the right threshold (more than 1.2). The latter indicated the presence of extra chromosome material for 8 in this PB. The sex chromosomes showed the expected fluorescence pattern, obtained when a female test DNA was hybridised against male reference DNA. Thus, the X chromosome was greener (areas: Xp22.2-q27), and its ratio profile deviated to the right, whereas the Y chromosome was more red (areas: Yp11.1-q12), with its ratio profile deviating towards the left. Hence, the karyotype of this PB was considered to be 24,X,+8. No results were obtained from the corresponding oocyte. The reason of infertility treatment for this patient was due to severe asthenoligospermia of the male partner. The maternal age was 32 years. A metaphase from the CGH analysis is shown in fig.5.17a and b and the interpretation in fig.5.17c.

PB no.1254.6 was hybridised against a clump of 46,XX buccal cells. Nine metaphases were analysed, and 338 chromosomes were included in the interpretation. The ratio profiles of chromosomes 4 and 5 deviated towards the right threshold (more than 1.2), as they were both excessively green (areas: 4p15.3-p12, 4q13-q26, 4q28-q31.3, 5p14-p12, 5q11.2-q23, 5q31-q33). Excess green was also observed for a part of the long arm of chromosome 12 (12q14-q24.1). The above indicated that the PB consisted of extra DNA for chromosomes 4 and 5 and possibly for chromosome 12. No fluorescence difference was visible for chromosome X, as both test and reference DNA were female. This cell was identified to be 26,X,+4,+5,+12. The corresponding oocyte was not investigated, while the maternal age of this patient was 39 years. Figures 5.18a and b and 5.18c demonstrate the fluorescent signals of a metaphase and the interpretation results obtained for this PB respectively.

Fig.5.18: CGH analysis of PB no. 1254.6 against 46,XX buccal cell clump DNA

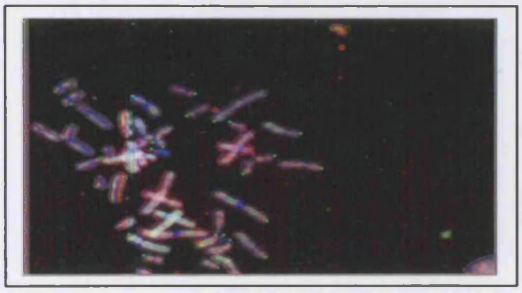


Fig.5.18a: Normal 46,XY metaphase chromosomes hybridised with DNA from PB 1254.6 *(green)* and 46,XX buccal cell DNA *(red)*.

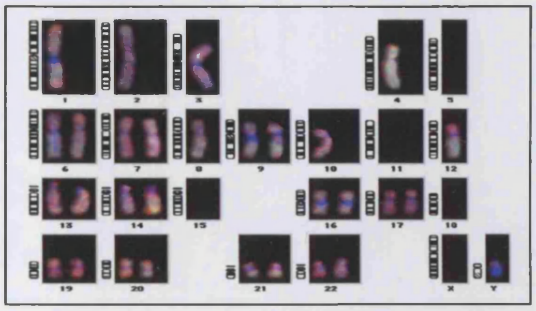


Fig.5.18b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosomes 4, 5, and the long arm of 12 appeared to be excessively green, compared to the rest of the autosomes. The latter indicated that the test PB sample contained extra DNA material for these chromosomes. Only chromosomes 4 and 12 are shown in this metaphase.

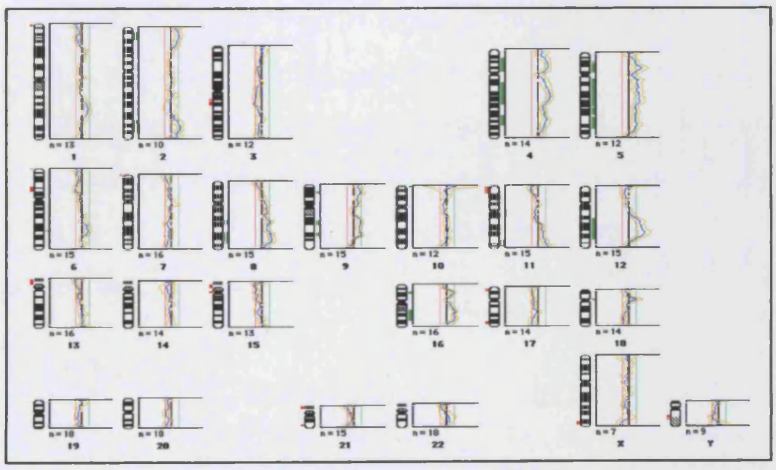


Fig.5.18c: Interpretation of the CGH experiment for PB 1254.6. The ratio profile of chromosomes 4, 5 and 12q14-q24.1 deviated towards the right threshold (more than 1.20). The shifts demonstrated the presence of extra DNA material for these chromosomes in the PB sample. As far as chromosome 12 was concerned, we considered that extra DNA was present for the whole chromosome and that the software did not identify it in its entirety. The karyotype of this PB was thought to be 26,X,+4,+5,+12. The corresponding oocyte was not investigated.

Fig.5.19: CGH analysis of PB no. 1355.1 against 46,XX buccal cell clump DNA

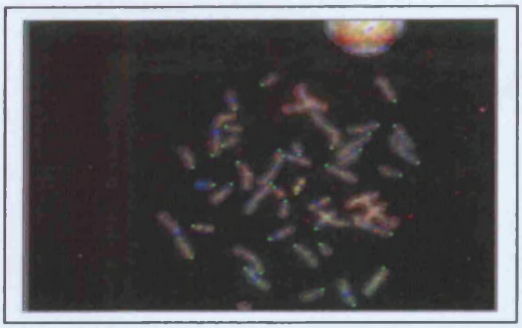


Fig.5.19a: Normal 46,XY metaphase chromosomes hybridised with DNA from PB 1355.1 *(green)* and 46,XX buccal cell DNA *(red)*.

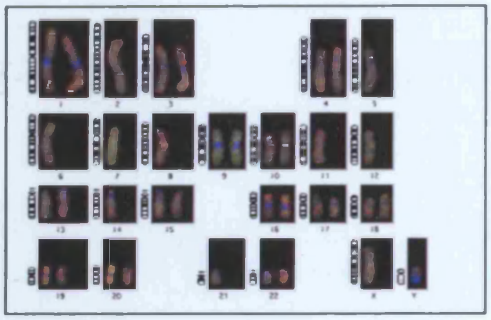


Fig.5.19b: Normal 46,XY metaphase chromosomes classified according to their banding patterns. Chromosome 9 was observed to be excessively green. The latter indicated that the test PB sample contained extra DNA material for this chromosome.

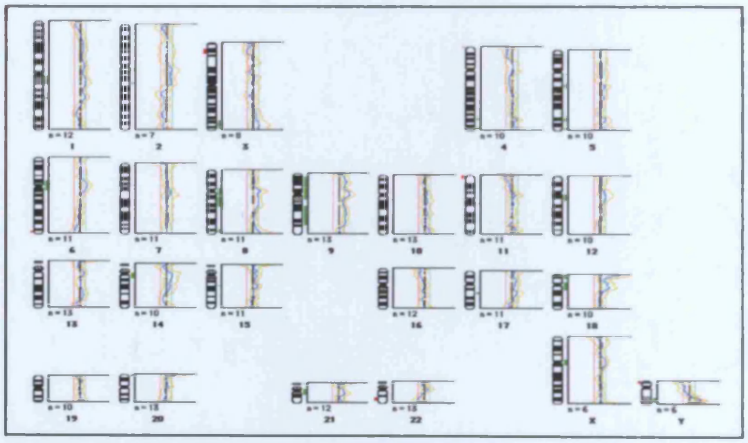


Fig.5.19c: Interpretation of the CGH experiment for PB 1355.1. The ratio profile of chromosome 9 deviated towards the right threshold (more than 1.20), the shift demonstrating the presence of extra chromosomal material in this PB sample. The karyotype of this PB was thought to be 24,X,+9. The corresponding oocyte underwent CGH as well, but it failed to yield any results. Hence the possible reciprocal loss of chromosome 9 in the oocyte was not confirmed.

PB CGH Results

A clump of 46,XX buccal cells was used as reference DNA for the CGH investigation of 1355.1PB. Analysis was carried out on seven metaphases and 254 chromosomes were included in the interpretation. The ratio profiles for all the autosomes apart from chromosome 9, and for chromosome X did not deviate from 1. No fluorescence was observed for chromosome Y, which was expected, as both test and reference DNAs were female. Chromosome 9 showed excess green fluorescence (areas: 9p23-p21, 9p12-q12, 9q21-q33), with its ratio profile deviating towards the right threshold. This observation led to the conclusion that this PB contained extra chromosomal material for 9. Its karyotype was 24,X,+9. There were no results obtained from the corresponding oocyte, due to CGH failure. However, oocyte 1355.4 and the 1355.5PB coming from the same patient, were characterised as being normal 23,X. The latter could indicate possible germinal mosaicism for trisomy 9 for this patient. The couple were being treated for unexplained infertility, and the maternal age was 32. Fig.5.19a and b show the appearance of the fluorescent signals of a metaphase, while fig.5.19c illustrates the interpretation of this PB.
Details of all the abnormalities scored in these PBs are shown in Table 5.2.

Similar to the oocytes, all the DOP-PCR products from the 30 PBs that failed to yield results with CGH were analysed on agarose gels. Only one of these cells was shown to give a very faint smear after amplification with the DOP-PCR. Failure of CGH for this cell was attributed to inferior DNA quality. The remaining cells did not show smears, which meant that they were either lost or lysed during processing, or the DNA degraded due to prolonged storage in the -80°C.

In summary, fifteen PBs yielded results and four of those were considered to be abnormal, corresponding to four different patients. All analysed PBs were thought to be representing solely the maternal genome. Male contamination was detected for one cell and was attributed to the handler. The obtained results provide preliminary data concerning the effect of polycystic ovary syndrome on maternal meiosis and identified the presence of possible germinal mosaicism for a trisomic cell line for two patients, although chromatid errors are also possible. Two PBs appeared to have abnormalities

affecting at least two different chromosomes. Extra copies of larger chromosomes, including 4, 5, 8, 9, and 12 were identified. Abnormalities of these chromosomes have not as yet been observed during FISH studies of PBs, even if the actual chromosomes are being investigated (e.g. Cupisti *et al.,* 2003). One PB was also identified to be missing chromosomal material for 21. Both the above observations illustrate the ability of CGH in detecting abnormalities of chromosomes that would not generally be targeted for examination during similar FISH studies, and in reliably identifying missing chromosome material as well.

Table 5.2: CGH analysis of corresponding 1st PBs

PB/patient no.	ART method	Reference DNA	CGH interpretation
1141.2PB	ICSI	46,XX genomic DNA	23,X
1134.3PB	Uninjected	46,XY genomic DNA	23,X
1173.1 PB	ICSI	46,XY genomic DNA	23,X
4047.3PB	IVF	46,XY buccal cell clump	23,X
4125.1PB	IVF	46,XY buccal cell clump	23,X
1143.4PB	ICSI	46,XY buccal cell clump	24,X+8
1243.1PB	Uninjected	46,XX buccal cell clump	23,X
4069.1PB	IVF	46,XX genomic DNA	23,X
4412.4PB	IVF	46,XX buccal cell clump	23,X
4412.5PB	IVF	46,XX buccal cell clump	24,X, +X,- ½ 21cht
4412.6 PB	IVF	46,XX buccal cell clump	23,X
1355.1PB	ICSI	46,XX buccal cell clump	24,X,+9
1355.5PB	ICSI	46,XX buccal cell clump	23,X
1254.6PB	Uninjected	46,XX buccal cell clump	26,X,+4,+5,+12
4405.3PB	IVF	46,XX buccal cell clump	23,X

5.5 Aneuploidy in human metaphase II oocytes and corresponding 1st polar bodies analysed by CGH

Comparative genomic hybridisation was employed for the study of 40 oocytes and 45 polar bodies. The majority of the oocytes had failed to fertilise after their exposure to sperm either via IVF or ICSI, and they were all arrested at metaphase II, having extruded the 1st PB. Out of the 45 PBs examined, 37 corresponded to oocytes. CGH was applied in an attempt:

1. To gain information for the entire maternal genome.
2. To investigate the involvement of larger autosomes in meiotic errors leading to female aneuploidy.
3. To establish the possible hypohaploidy rate for all chromosomes. The latter was not feasible with the application of FISH as artefactual loss of chromosomes could occur due to the spreading procedure.

Seven different positive control experiments were carried out prior to the application of CGH on oocytes and PBs. These took place for the following reasons:

1. To demonstrated that the protocol used was able to amplify minute amounts of DNA.
2. The analysis and interpretation were sensitive enough to detect trisomies of chromosomes ranging in size.
3. To be used as reference in cases where comparable results were obtained from the CGH analysis of the oocytes and PBs.

Oocytes and PBs were categorised into three groups, according to the way they were prepared. In total results were obtained from 26 cells (11 oocytes and 15 PBs). From these, 6 belonged to the first group, 8 to the second group, and 12 to the third group. Fourteen different patients were investigated. Five of these were treated via IVF, six via ICSI, while for three no ART procedure took place, as the oocytes were immature at the time of retrieval. As far as the oocytes were concerned, we considered that the presence of sperm DNA to be likely. Thus, the CGH results yielded from these cells were thought to demonstrate the karyotypes of both the paternal and the maternal

genomes (i.e. a potential zygote genome). This was not the case for the CGH analysis of 1st PBs. The latter were considered to be free of sperm and the data obtained from them was representing the maternal genome only. During analysis and interpretation of CGH metaphase images it was not always feasible to distinguish between extra or missing chromosomes or chromatids.

Nine oocytes and eleven PBs were identified to contain a normal number of chromosomes. Out of these four were corresponding: 1141.2 oocyte+PB, 1173.1 oocyte+PB, 4405.3 oocyte+PB, and 4412.6 oocyte+PB. For the remaining, results were obtained either for the PB with CGH having failed for the oocyte (4125.1PB, 4069.1PB, 4047.3PB, 1355.5PB), or the opposite (1172.2, 1174.1, 1243.2, 1174.1, 1355.4). The corresponding oocyte was not investigated for 1243.1PB and 1254.6PB. PBs 1134.3PB and 4412.4PB were characterised as being normal 23,X with anomalies being scored in the corresponding oocytes.

Abnormalities in the form of chromosome gains were identified in oocytes 1134.3 and 4412.4, and the following PBs: 4412.5PB, 1143.4PB, 1254.6PB,and 1355.1PB. Losses were detected for oocyte 4412.4, 4412.5 and 4412.5PB. Reciprocal gains and losses were seen in one pair of corresponding cells only, with oocyte 4412.5 having lost DNA material for chromosome X and 4412.5PB having gained DNA material for the same chromosome. The inability to identify reciprocal gains and losses in the case of the remaining 2 abnormal oocytes, was attributed either due to germinal mosaicism for a trisomic cell line resulting to the extra chromosome copy ending up in the oocyte, or due to the actual female cell having been penetrated by an abnormal sperm. As far as the PBs were concerned, 1143.4PB, and 1254.6PB did not have their oocytes analysed. No result was obtained for the oocyte 1355.1, whose PB appeared to contain extra chromosomal material for 9. Data was obtained for another oocyte and another PB originating from the same patient. Both these were normal. The possibility of the CGH technique being unable to detect the expected abnormalities in the corresponding cells was always considered.

Oocyte 4412.4, and two PBs: 4412.5PB, and 1254.6PB appeared to have anomalies affecting more than one chromosomes. Interpretation of the CGH results obtained for

oocyte 4412.4 led to the conclusion that the cell contained extra chromosome material for 13 and was missing 22. The PB no.4412.5 from the same patient contained extra chromosome material for X and was missing 21. Detection of extra DNA for chromosomes 4, 5, and 12 was observed after the interpretation of the CGH results for the 1254.6PB.

Abnormalities were observed for several chromosomes. Gains of the larger autosomes 4, 5, 8, 9, 12, and X were evident in four PBs. Since the PBs were thought to represent solely the maternal genome, it was very likely that these gains were true and were not an artefact of the hybridisation process. Extra copies for these autosomes have not been reported in any oocyte and PB FISH study so far. Meiotic errors affecting larger autosomes were evident in three other CGH studies, one carried out on first PBs and the other two on embryonic blastomeres (Wells *et al.*, 2002; Wells and Delhanty, 2000; Voullaire *et al.*, 2000), and one study that applied SKY on fresh non-inseminated oocytes (Sandalinas *et al.*, 2002). Gains of chromosome 13 were identified in two oocytes, whereas one oocyte and one PB were detected to be missing chromosomes 22 and 21 respectively. These chromosomes have been shown in the FISH study described in the previous section, and in others from our group (Mahmood *et al.*, 2000, Cupisti *et al.*, 2003) to be frequently involved in meiotic errors leading to maternal aneuploidy. The abnormalities observed for all the oocytes and corresponding 1st PBs, along with patient details are summarised in Table 5.3.

A total of five patients were recognised to be carrying chromosome anomalies in one or more of their examined cells. Maternal ages varied between 22-39 (mean 25). Patient no. 4412 was the youngest in the group, and the one whose cells appeared to be the most abnormal. Four oocytes and their corresponding PBs were investigated and only one pair (4412.6 oocyte+PB) was characterised to be fully normal 23,X. The remaining cells appeared to have gains and losses for different chromosomes, some consisting of more than one abnormality. This patient was being treated due to polycystic ovary syndrome. It has been postulated that polycystic ovaries could lead to oocytes with multiple abnormalities (Clyde *et al.*, 2001). This could explain the observations made in the oocytes and PBs from this patient. Multiple abnormalities were also observed in the 1254.6PB. This patient was the oldest in the group, 39 years

of age, and the reason for infertility treatment was unknown. A case of possible germinal mosaicism for trisomy 13 was detected. Patient 1134 was 32 years old and was being treated for male factor infertility.

Out of a total of 85 cells (oocytes and PBs) for which CGH analysis was attempted, results were obtained for 26 cells (30.6%). Seven of those appeared to have abnormalities for one or more chromosomes, resulting to an aneuploidy rate of nearly 27%. If patient 4412 is excluded from this group, then the aneuploidy rate becomes 15.4%.

In summary, although the sample size of oocytes and PBs analysed with CGH was relatively small, chromosome anomalies were observed, even for larger autosomes. Non-disjunction of chromosomes was identified, along with the possibility of germinal mosaicism for a trisomic cell line. We were not able to distinguish with certainty aneuploidy associated with premature division of chromosomes into their sister chromatids, due to the relative insensitivity of the single cell CGH and the suboptimal condition of the oocyte and PB DNA. Chromosome loss was identified in two different cases. The majority of the abnormalities were observed for the younger patients in the group. The above show the advantage of CGH over FISH, in its ability to detect anomalies for chromosomes other than the ones commonly expected to be involved in maternal aneuploidy. Estimation of hypohaploidy, and which chromosomes could be affected is feasible, but a larger sample would be essential for definite conclusions to be drawn.

Table 5.3: Summary of CGH analysis of human metaphase II oocytes and their corresponding 1st PBs. Patient details and ART method are indicated

Patient no.	Maternal age	Reason for infertility treatment	ART method	Oocyte no.	Oocyte CGH result	PB no.	PB CGH result
1141	31	Not known	ICSI	1141.2	23,X	1141.2PB	23,X
1134	32	Azoospermia	Uninjected	1134.3 GV	24,X+13 or 23,X,+1/2 13cht	1134.3PB	23,X
1172	38	Vas reversal failure	ICSI	1172.2	23,X	1172.2PB	CGH failure
1173	30	Azoospermia	ICSI	1173.1	23,X	1173.1PB	23,X
1174	33	Asthenooligospermia	ICSI	1174.1	23,X	1174.1PB	CGH failure
1243	31	Male factor	Uninjected	1243.1	Not investigated	1243.1PB	23,X
				1243.2	23,X	1243.2PB	CGH failure
1355	32	Unexplained	ICSI	1355.1	CGH failure	1355.1PB	24,X,+9
				1355.4	23,X	1355.4PB	CGH failure
				1355.5	CGH failure	1355.5PB	23,X
4412	22	Polycystic ovaries	IVF	4412.1	CGH failure	4412.1PB	CGH failure
				4412.4	23,X,+13,-22 or 23,X,+1/2 13cht,– ½ 22cht	4412.4PB	23,X
				4412.5	22,-X, possibly +1/2 21cht	4412.5PB	24,X,+X, –1/2 21cht
				4412.6	23,X	4412.6PB	23,X
4405	37	Bicornuate uterus, recurrent miscarriages	IVF	4405.3	23,X	4405.3PB	23,X
4047	35	Unexplained	IVF	4047.3	CGH failure	4047.3PB	23,X

Oocyte and PB CGH Results Summary Table

Patient no.	Maternal age	Reason for infertility treatment	ART method	Oocyte no.	Oocyte CGH result	PB no.	PB CGH result
4125	30	Anovulation	IVF	4125.1	CGH failure	4125.1PB	23,X
1143	32	Asthenooligospermia	ICSI	1143.4	Not investigated	1143.4PB	24,X,+8
4069	28	Idiopathic	IVF	4069.1	CGH failure	4069.1PB	23,X
1254	39	Male factor	Uninjected	1254.6	Not investigated	1254.6PB	26,X,+4,+5,+12

Chapter 6

Discussion

6.1 The application of PGD for the detection of chromosomal abnormalities

The main aim of Preimplantation Genetic Diagnosis (PGD) is to ensure the initiation of a healthy pregnancy (Munne and Wells, 2002). The first application of PGD took place in 1989, and involved the sex selection of embryos generated by couples who were at risk of transmitting an X-linked recessive disorder (Handyside *et al.,* 1990). Since then this procedure has progressed rapidly and is currently being offered at more than fifty centres all over the world (ESHRE PGD Consortium, 2002). Data collected on the obstetric and neonatal outcome of pregnancies achieved after PGD did not demonstrate any increase in major congenital malformations, compared to spontaneous pregnancies, or those that ensued after IVF or ICSI (ESHRE PGD Consortium, 2000; 2002). The latter confirmed the safety and efficacy of this procedure. Effectively, PGD was developed as a very early form of prenatal diagnosis. It is advantageous over prenatal diagnosis, as it avoids the physical and psychological trauma that could be caused by the termination of an affected pregnancy.

Patients requesting PGD fall into two large categories: those that are at risk of transmitting a single gene disorder to their offspring, and those that are carriers of a chromosome abnormality (Delhanty, 1998). The patients of the second group can be further subdivided into those that are carriers of a structural chromosome abnormality (usually reciprocal or Robertsonian translocation), and those whose karyotype is normal, but are unable either to establish or maintain a pregnancy, including women over the age of 35. Pregnancy loss in such cases is most likely attributed to one or more numerical abnormalities. The identification of both structural and numerical abnormalities in single embryonic blastomeres is accomplished with the application of FISH. Different strategies are used for the diagnosis of structural and numerical chromosome abnormalities.

The frequency of balanced translocations in the neonatal population has been estimated to be between 1 and 2 per 1,000 (Nielsen and Silessen, 1975; Hamerton *et al.,* 1975, Jacobs *et al.,* 1974). Half of these are Robertsonian, and the remaining

reciprocal translocations (Jacobs, 1977). Reciprocal translocations were also identified in 0.6% of infertile couples, 3.2% of couples with over 10 failed IVF attempts, 9.2% of fertile couples that suffered more than three sequential first-trimester miscarriages (Stern *et al.*, 1999), and 2-3.2% of males needing ICSI (Testart *et al.*, 1996; Meschede *et al.*, 1998; Van der Ven *et al.*, 1998). The scope of PGD for such couples is to decrease the rate of spontaneous abortions, and reduce the risk of an unbalanced conception (Munne and Wells, 2002). Several factors including the chromosomes involved, the position of the breakpoints and the sex of the translocation carrier affect the risk of conceiving an unbalanced baby (Goldman and Hulten, 1993; Faraut *et al.*, 2000). The application of FISH for the diagnosis of reciprocal and Robertsonian translocations differs, due to the different nature of these two structural chromosome abnormalities.

Reciprocal translocations can theoretically take place between any two chromosomes at any position. Thus, each case is considered to be unique, with the exception of the most common reciprocal translocation t(11;22). Two FISH approaches have been established for the detection of such abnormalities in interphase blastomeres. The strategy employed currently at the UCL centre for PGD uses two probes that flank the breakpoint on one of the two chromosomes involved in the translocation, with a third probe mapping at any position on the other chromosome. This FISH approach has the ability to detect the products of all unbalanced segregation patterns, but it cannot distinguish between a normal and a balanced chromosome complement. It was initially developed by Conn and colleagues (1999). It has been widely applied in most PGD centres including those at Guy's and St Thomas', Brussels Free University, the Karolinska Hospital group in Stockholm, and the St. Barnabas group in the USA. The availability of commercial subtelomeric probes has increased the number of couples being treated in the past few years, compared to previously, when laboratory-prepared probes had to be used, making the development of FISH protocols more time consuming (Conn *et al.*, 1998). However, subtelomeric probes have lower efficiency of hybridisation, especially if they are compared with repetitive probes (Simopoulou *et al.*, 2003). Thus, we prefer to use two centromeric and one locus-specific probe for the PGD of reciprocal translocations, if the chromosomes involved and the position of the breakpoints permit it.

The "flanking" probe strategy has been applied for the PGD of the two reciprocal translocations in this study. In case A, 46,XY,t(5;19)(p12;p12) it was not possible to use a centromeric probe, due to cross-hybridisation. Thus a commercial dual locus-specific probe was utilised for chromosome 5 and a YAC locus-specific probe was used to detect chromosome 19, as at that time a commercial subtelomere was not available for this chromosome. The FISH protocol took a long time to optimise, as several problems involving culturing and DNA extraction of the YAC probe had to be overcome. In the end the DNA for this probe was sufficient for the analysis of the biopsied blastomeres only.

In case B, 46XX,t(11;22)(q23.3;q11.2), a commercial dual locus-specific probe was used for chromosome 22, and a commercial centromeric probe was used for chromosome 11. The latter probe emitted an orange-yellow fluorescence that was produced by the combination of red and green centromeric probes. This couple underwent two cycles of PGD, with an embryo transfer following the second cycle. During this cycle, failure of hybridisation was observed for the green centromeric probe for chromosome 11. The latter was attributed to the batch of the probes, as is discussed in 3.2.2. After careful consideration involving the colour and size difference between the locus-specific and centromere probes, two embryos were identified as normal or balanced and were transferred, leading to a normal live birth. The same probe combination was applied by Van Assche and colleagues (1999) in a PGD case involving a male carrier of this translocation.

Our group (Simopoulou *et al.,* 2003) described the application of the "flanking" probe FISH strategy for the PGD of six different reciprocal translocations. Embryo transfers took place in all of them. A biochemical pregnancy and two clinical pregnancies were achieved for three different cases, the latter two leading to normal live births. A modification of the "flanking probe" approach involved the use of two probes proximal to the breakpoints and two distal (Munne *et al.,* 1998a). The application of four probes instead of three could eliminate the risk of misdiagnosis due to the failure of hybridisation of one of the probes, but has to be balanced against the reduced efficiency of hybridisation with the increasing number of probes.

The second FISH approach for the PGD of reciprocal translocations was developed by Munne and colleagues (1998a) and involved the use of probes that spanned on the

breakpoints of a translocation. More specifically, such probes would give two distinct signals when hybridised to normal chromosomes. Their hybridisation on the derivative chromosomes would appear as an association of the colours that these probes were labelled with (Munne *et al.*, 1998a). This approach had the advantage that it was able to distinguish between normal and balanced products. It was applied for the PGD of a reciprocal translocation, 46,XY,t(3;4)(p24;p15). Spanning probes were developed from YAC clones, labelled in green for chromosome 3 and red for chromosome 4, and were also combined with a centromeric probe for chromosome 3, labelled in aqua. The couple underwent two PGD cycles, and identification of normal, balanced and unbalanced embryos was achieved during FISH analysis of biopsied blastomeres in both. Results were confirmed by re-analysis of the spare embryos (Munne *et al.*, 1998a). The disadvantage of this FISH strategy was that it was entirely patient-specific, and thus time consuming and not cost-effective.

Willadsen and colleagues (1999) and Verlinsky and Evsikov (1999) described the injection of human blastomeres or second polar bodies in cow eggs or mouse zygotes, with the aim of obtaining metaphase chromosomes from them. Both approaches have been used in the diagnosis of reciprocal translocations, leading to the birth of chromosomally balanced children (Willadsen *et al.*, 1999; Evsikov *et al.*, 2000). There is however, a high risk of chromosome loss when spreading a single metaphase.
PGD of reciprocal translocations was also carried out on metaphase chromosomes obtained from first polar bodies, fixed six hours after oocyte retrieval and analysed with whole chromosome paints combined with telomere probes (Durban *et al.*, 1998; Munne *et al.*, 1998d Munne *et al.*, 1998e). Full chromosome analysis of first polar bodies with the aid of spectral imaging was described by Marquez and colleagues (1998) for the diagnosis of reciprocal translocations in female carriers. The requirement of a well spread metaphase plate with clear chromosomes, the high risk of chromosome loss when spreading a single metaphase, the artefactual precocious separation of chromatids due to PB degeneration in culture, and the signal morphology could all lead to misdiagnosis using this PGD approach. Another event that could lead to a possible misdiagnosis is the occurrence of an interstitial crossover followed by segregation of balanced and unbalanced sets of chromosomes during the

second meiotic division (Munne, 2002). All the above made this strategy difficult to use on a regular basis.

Another group of patients requiring PGD to achieve a healthy pregnancy is that of balanced carriers of Robertsonian translocations. Such translocations are formed by the fusion of two acrocentric chromosomes. Male carriers sometimes display low sperm counts (Wells and Delhanty, 2001). This was the case for both male carriers of Robertsonian translocations in this study. Compared with the PGD strategies employed for the detection of unbalanced products from reciprocal translocations, the FISH protocols applied during PGD for Robertsonian translocations are much simpler. Thus, such cases can be diagnosed with the use of a minimum of two locus-specific and/or telomeric probes, each one hybridising to the acrocentric chromosomes that are involved in this rearrangement. When chromosome 21 is involved, it is preferable to use three probes, i.e. two hybridising on 21 and the third on the long arm of the other chromosome. In this way, chromosome 21 that is more likely to produce a viable trisomy could be detected with certainty and misdiagnosis due to hybridisation failure and/or signal overlap could be avoided.
The dual labelling of chromosome 21 was applied for the PGD of case D in this study. The male partner was a balanced carrier of a Robertsonian translocation, his karyotype being 45,XY,der(13;21)(q10;q10). The probes used for this case included a locus-specific probe for the long arm of chromosome 13, a locus-specific probe for the long arm of chromosome 21, and telomeric probe for 21q. These probes were used in both PGD cycles, during which the telomere probe for 21 appeared to be much fainter, compared with the two locus-specific probes. It also frequently failed to hybridise. The latter was attributed to the quality of this probe, as it was supplied by a different company than the one that provided the locus-specific probes. This had as an effect the diagnosis to be carried out based on the signals visible for the locus-specific probe for chromosome 21 for some blastomeres. Dual-coloured FISH was used for the diagnosis of case E. The carrier of the Robertsonian translocation was again the male partner, whose karyotype was 45,XY,der(13;14)(q10;q10).

A comparable approach was used by Conn and colleagues (1998), who carried out five PGD cycles for two couples, whose karyotype was 45,XY,der(13;14)(q10;q10)

and 45,XX,der(13;21)(q10;q10). As this study took place when commercial locus-specific probes were not widely available for every chromosome, it used probes that were prepared from YAC clones, but the FISH strategy was exactly the same with the one used for this study.

Durban and co-workers (2001) described an alternative FISH approach for the detection of Robertsonian translocations in female carriers. In this study, PGD to aid four female Robertsonian translocation carriers to initiate a normal pregnancy, was carried out by biopsying the first polar body, and using a combination of locus-specific probes, subtelomere probes and whole chromosome paints. The group claimed a diagnosis success rate varying between 80-100% with the use of first polar bodies. However, this approach has many disadvantages, as described above.

Two phenotypically and karyotypically normal couples were referred to the UCL centre for PGD for possible gonadal mosaicism for trisomy 21. The couple in case F had two ectopic pregnancies, and a Down's syndrome pregnancy which were all terminated, leading to inability to conceive naturally. The couple in case G had given birth to two children with Down's syndrome and had a termination of a trisomy 21 pregnancy. Chronologically couple G was referred first, but they decided against treatment due to family reasons. The initial FISH protocol that would be used for couple G involved the investigation of chromosome 21 only, with the use of a band-specific and a telomeric probe both hybridising to the long arm of this chromosome. A different approach was applied during the PGD of couple F. Hence, only one locus-specific probe was applied for the detection of chromosome 21, and an extra locus-specific probe was added for identifying chromosome 13. This modification occurred, as the telomere probe for chromosome 21 was not very efficient, giving relatively faint signals, combined with a high rate of hybridisation failure. The extra probe for chromosome 13 acted as a positive control for the FISH procedure, and also enabled the investigation of an extra chromosome, that tends to cause abnormalities. The ages of the female partners from these couples at the time of PGD referral were 39 for couple F and 37 for couple G.

No gametes, either oocytes or sperm, were available from couple F, during two PGD cycles. Thus, it was not feasible to directly investigate the possible presence of gonadal mosaicism for trisomy 21 in any of the two partners. There was no evidence

of mosaicism for trisomy 21 for this couple, upon examination of lymphocyte interphase nuclei from both partners.

Two locus-specific probes hybridising to different positions on the long arm of chromosome 21 were used by Conn and colleagues (1999) during the PGD of a couple that had a normal child, a child affected with Down's syndrome, and two other trisomy 21 conceptions. Polymerase chain reaction (PCR) analysis of DNA polymorphisms had previously revealed that the origin of the extra chromosome 21 was maternal. Confirmation that the female partner was a gonadal mosaic for trisomy 21 came from analysis of four unfertilised oocytes, three of which showed hyperhaploidy for chromosome 21, with one having a normal haploid complement (Conn *et al.,* 1999).

Embryo transfers took place for all five patients during nine PGD cycles. Data for couples A and D are also analysed in a larger report that has recently been published by our group (Simopoulou *et al.,* 2003), describing the outcome of 11 PGD cycles for 8 patients carrying six reciprocal translocations, one Robertsonian translocation and one intrachromosomal between arm insertion. Combination of this data with the outcome of the rest of the PGD cases outlined in this thesis leads to seventeen PGD cycles for the diagnosis of chromosome abnormalities, carried out over six years. Embryo transfers took place during all these cycles resulting to five pregnancies, one biochemical and four clinical, and the birth of four healthy infants. Table 6.1 demonstrates the above. In addition, one couple underwent a further cycle of treatment, leading to an ongoing twin pregnancy. Thus, in total, five of the eleven couples treated had a positive outcome.

Table 6.1: Summary of the outcome of 17 cycles of PGD for 11 couples carrying a chromosome abnormality (Cases H-M from Simopoulou *et al.*, 2003)

Patients and karyotypes	Cycles	Oocytes retrieved	Oocytes fertilised	Embryos biopsied	Normal/ balanced	Abnormal*	Embryos transferred	Outcome
A: 46,XY,t(5;19)(p12;p12)	1	20	15	13	1(8.3%)	12(91.7%)	1	No pregnancy
B:46,XX,t(11;22)(q23.3;q11.2)	2	26	14	11	2(18.2%)	11(84.6%)	2	Normal live birth
D: 45,XY,der(13;21)(q10;10)	2	22	17	15	6(40%)	9(60%)	6	No pregnancy
E: 45,XY,der(13;14)(q10;q10)	2	25	21	17	6(35.3%)	10(58.8%)	5	No pregnancy^{+}
F: 46,XX, 46,XY, possible gonadal mosaicism for trisomy 21	2	43	37	33	10(30.3%)	27(72.3%)	7	No pregnancy
H: 46,XX,t(5;11)(q34;q25)	3	34	29	22	8(36.4%)	18(69.2%)	7	Normal live birth
I: 46,XX,t(1;2)(q42.1;p23)	1	16	11	11	2(20%)	8(80%)	2	No pregnancy
J: 46,XX,t(16;17)(p13.3;p11.1)	1	8	8	7	4(50%)	4(50%)	3	Biochemical pregnancy
K: 46,XX,INS(7)(p22 q32 q31.1)	1	16	16	11	7(70%)	3(30%)	2	Normal live birth
L: 46,XX,t(8;12)(q11.2;q12)	1	21	17	13	2(15%)	11(85%)	1	Normal live birth
M: 46,XY,t(1;18)(p32;q23)	1	16	14	12	1(8.3%)	11(91.7%)	1 frozen/thawed	Balanced embryo frozen as blastocyst
Total	17	247	199	165	49(29.7%)	124(75.2%)	37	

*The percentage of normal/abnormal embryos is based on the embryos that have been analysed (including biopsied embryos but also embryos not suitable for biopsy spread and FISH-ed on day 4 or 5) and provided results for embryo classification. Embryos were considered abnormal when they consisted of an unbalanced chromosome constitution either due to abnormal meiotic segregation or due to post-zygotic errors. $^{+}$Twin pregnancy now ongoing.

6.2 Analysis of preimplantation embryos from nine cycles for the PGD of chromosome abnormalities

This part of the study involved the investigation of embryos from carriers of either structural or numerical chromosome abnormalities with application of FISH.

PGD was carried out for two different reciprocal translocations. In case A, the male partner was the carrier of the rearrangement, which involved chromosomes 5 and 19. This couple had experienced years of infertility, attributed to the severe oligospermia of the male partner. In case B, the female partner was the carrier of the translocation, that had taken place between chromosomes 11 and 22. Unlike other such rearrangements that are usually unique in the population, the t(11q;22q) translocation is frequently observed in humans. Four pregnancies were established for this couple, in the year preceding PGD treatment, but had all spontaneously aborted.

One PGD cycle was carried out for case A. All generated embryos were normally developing and of very good morphology. FISH investigation of all biopsied blastomeres and non-transferred embryos revealed that the majority of them were chaotic having literally almost every cell with a different chromosome complement. This observation affirms that good embryonic morphology cannot predict chromosome constitution. The generation of such highly abnormal embryos was attributed to extensive mitotic non-disjunction. The latter in combination with chromosome breakage, which was observed for one embryo from another reciprocal translocation patient (46,XXt(5;11)(q35;q25)), as described in the larger report published by our group (Simopoulou *et al.,* 2003) are two of the main factors leading to post-zygotic mosaicism. Chromosome breakage was evident in the analysis of blastomeres from three embryos that were examined with the application of CGH in two studies carried out by our group and a group in Australia (Wells and Delhanty, 2000; Voullaire *et al.,* 2000). Wells and Delhanty (2000) observed reciprocal losses and gains of parts of chromosomes 1, 2, and 7 in pairs of blastomeres from two different embryos. Breakpoints mapped to fragile chromosomal sites that are prone to breakage, which could take place due to depletion of nutrients from the culture

medium. Both groups postulated that such chromosome rearrangements could be the cause of significant imbalance, which in turn affects embryo viability (Wells and Delhanty, 2000; Voullaire *et al.*, 2000). The two different sets of embryos investigated in both studies had fertilised normally and were of good morphology, similar to the ones generated by the couple in case A.

One embryo was identified as normal or balanced and was transferred, but no clinical pregnancy ensued. The unavailability of the YAC probe, along with the high frequency of chaotic embryos made it impossible to determine the most common segregations for this translocation in the non-transferred embryos. Sperm analysis revealed that the alternate segregation was the most frequently occurring (54.3%), followed by adjacent-1 (18.4%), adjacent-2 (10.8%), and 3:1 disjunction (16.5%). Escudero and co-workers (2003) in their investigation of sperm from eleven reciprocal translocation carriers made a similar observation. They postulated that translocations with breakpoints near the centromeres of chromosomes, as was the case with the patient in this study, tend to form close configurations in meiosis I, leading to mainly 2:2 segregations (Escudero *et al.*, 2003).

Two cycles were carried out for the couple in case B. Fifteen embryos were generated during both cycles and their morphology was generally poor. No embryo transfer took place in the first IVF attempt due to the unexpected low fertilisation rate and the chaotic nature of the three embryos that were analysed. ICSI was used for the second PGD cycle, two embryos were identified as normal or balanced, and were transferred leading to the birth of a normal male baby. Re-analysis of all embryos from both cycles involved the investigation of chromosomes 11 and 22, but also of 15, 16, and 18 for the spare embryos of the first cycle, and 18, X, and Y for those of the second cycle. Five embryos were classified as chaotic, while the rest as mosaic aneuploid, mosaic balanced aneuploid, and mosaic balanced chaotic. One embryo was characterised as uniformly abnormal, but information was obtained only from the biopsied blastomeres, as the rest of the embryo was lost after the FISH procedure. The segregation patterns of the chromosomes participating in the translocation were established for five embryos and were: 2:2 alternate with a frequency of 40%, 3:1 interchange with a frequency of 40% as well, and 2:2 adjacent-1 with a frequency of 20%. Mitotic non-disjunction was also observed, sometimes in combination with one of the standard segregations. Cytogenetic investigation of liveborn unbalanced

offspring of both male and female carriers of the t(11q;22q) demonstrated that the majority are the products of tertiary trisomy for der(22) due to 3:1 non-disjunction (Iselius *et al.*, 1983). This was not observed in this study, as the 3:1 disjunction resulted in oocytes monosomic for either chromosome 11 or chromosome 22. Armstrong and colleagues (2000) analysed sperm from a male carrier of this translocation, and identified all types of segregation at metaphase II nuclei. They suggested that it was very likely that embryos carrying the unbalanced 2:2 translocation spontaneously miscarry early on during the pregnancy, while the ones trisomic for der(22) could survive at least until they are born (Armstrong *et al.*, 2000).

PGD was carried out for two cases of Robertsonian translocations, der(13;21) and der(13;14). In these cases, the carriers of the rearrangements were the male partners, who were both infertile. Each of the two couples had two cycles of PGD, during which twelve embryos were identified as balanced, and eleven of those were transferred. Analysis of the non-transferred embryos involved the investigation of the chromosomes forming the translocations, for the diagnosis to be confirmed. Their chromosome constitution was further examined scoring for chromosomes 4, 15, 18, X and Y, when embryo morphology permitted it. As previously, all the spare embryos were highly abnormal, with three being mosaic diploid, two being mosaic aneuploid and thirteen being entirely chaotic. Embryo morphology and development were again inferior. Both these could have been attributing factors for the inability of these two couples to establish a clinical pregnancy. Similar observations were made by Conn and colleagues (1998) in their investigation of 45 preimplantation embryos generated by two carriers of different Robertsonian translocations, 45,XY,der(13;14)(q10;q10) and 45,XX,der(13;21)(q10;q10) during five PGD cycles. Six embryos (13%) were identified as balanced for both couples, and were transferred, but no clinical pregnancy was established. All the remaining embryos were highly abnormal, 36% being aneuploid or aneuploid mosaic, and 51% chaotic. The high frequency of abnormal embryos generated by carriers of Robertsonian translocations noted in this study and that of Conn *et al.* (1998) suggests that there could be two distinct causative factors leading to the reduced fertility observed for such patients: the aneuploid segregation of the parental Robertsonian translocation, combined with a post-zygotic

factor that has as an effect the unregulated distribution of chromosomes in early cleavage stages in a significantly high number of embryos.

Determination of the segregations of the chromosomes involved in the translocation occurred directly by sperm analysis in case D, and indirectly according to the chromosome constitution of the embryos in case E. The most common segregation pattern in both cases was the alternate with a frequency of 85.7% in case D and 91.6% in case E. Adjacent-1 followed with a frequency of 14.3% in case D and 8.3% in case E. Post-zygotic errors including mitotic non-disjunction and chromosome loss attributed to the formation of the highly abnormal non-transferred embryos. Gametic analysis of male carriers of Robertsonian translocations has shown that a very large number of spermatozoa are either normal or balanced. Ogawa and colleagues (2000) and Escudero and colleagues (2000) in their studies of male Robertsonian translocation carrier sperm detected normal/balanced cells with frequencies ranging from 75-87% in each of the patients they examined. Comparable rates were seen in this study.

Couple F was referred to our centre for PGD because of the suspicion that one of the two partners was a gonadal mosaic for trisomy 21. Two PGD cycles were carried out and ten normal embryos were identified, seven of which were transferred. This patient did not manage to achieve a pregnancy in any of these cycles. Examination of chromosomes 13 and 21 occurred for the non-transferred embryos from both cycles, whereas the ones generated during the second attempt were also investigated for chromosomes 15, 18, and 22. Out of these, one was identified as normal for all the chromosomes. Biopsy of one cell had taken place for this embryo, but no result was obtained during diagnosis. Hence it was excluded from transfer. The remaining were as follows: seven uniformly abnormal or inconclusive, seven mosaic diploid, five mosaic aneuploid, and eight chaotic. These abnormalities were in their majority products of post-zygotic errors, namely mitotic non-disjunction, chromosome loss and/or gain. Meiotic errors were observed for two different embryos. Chromosome 21 was affected in one of them, whilst both chromosomes 13 and 21 were affected in the other, as discussed in 3.4.3. The chromosome abnormalities observed in the non-transferred embryos generated in the two PGD cycles were of different types. More specifically, in the first PGD cycle almost all spare embryos consisted of chaotic

chromosome constitutions, whereas during the second PGD cycle the observed abnormalities were the result of meiotic errors and mitotic non-disjunction. The inability of this couple to achieve a clinical pregnancy, even though they produced a relatively high number of balanced embryos, could be attributed to the advanced maternal age (39 during both treatment cycles) that could have led to both the meiotic chromosome abnormalities but also to the high incidence of mosaicism observed during both cycles. It has been generally accepted that mosaicism observed during the cleavage stage of embryo development and caused by post-zygotic errors does not increase with advancing maternal age (Munne and Cohen 1998). Recently the group at St Barnabas evaluated 1235 cleavage stage embryos derived from routine IVF patients by applying FISH for the detection of chromosomes 13, 18, 21, X, and Y (Munne *et al.,* 2002b). They determined that aneuploid mosaicism due to mitotic non-disjunction tends to be more frequent in embryos coming from women of advanced age. In an attempt to explain this phenomenon, they suggested that older oocytes could contain damaged stored mRNA and other components that are critical during the first cleavage divisions, up until the activation of the embryonic genome. Hence, depending on the degree of this damage, these components could lead to either meiotic or post-zygotic errors, seen as mitotic aneuploid mosaicism in the embryo (Munne *et al.,* 2002). This could have been the case for this patient, as the number of mosaic aneuploid embryos generated in both cycles was almost equal to the chaotic ones.

A total of 94 embryos were chromosomally investigated throughout this part of the study, and only twenty-five of them were classified as balanced. The remaining sixty-nine were either uniformly abnormal, mosaic diploid, or mosaic aneuploid. The majority however, of all generated embryos were classified as chaotic. Other studies have shown that embryos from some carriers of reciprocal and Robertsonian translocations are in the majority mosaic and chaotic. More specifically, Iwarsson and co-workers (2000) examined 64 biopsied and normally developing preimplantation embryos derived from four Robertsonian and three reciprocal translocation carriers. Out of these 64 embryos 17 were balanced, whereas 47 (73%) were mosaic for the chromosomes involved in the translocation (Iwarsson *et al.,* 2000). Similar observations were made by our group (Simopoulou *et al.* 2003) in the study of embryos coming from eight patients, all being carriers of a chromosome

rearrangement, as already described. Post-zygotic errors leading to mosaicism and chaos were detected in 75% of the non-transferred embryos investigated (Simopoulou *et al.,* 2003). Analysis of embryos from patients with structural chromosome abnormalities has also taken place with the application of CGH (Malmgrem *et al.,* 2002). This study involved the examination of 94 blastomeres from 28 embryos that were generated by 13 couples. Mosaicism affected the chromosomes involved in the rearrangement, but also other chromosomes, and all embryos investigated were either mosaic or chaotic. The group noticed that some patients were more prone to produce chaotic embryos, compared to others (Malmgrem *et al.,* 2002). The latter was also shown by Delhanty and colleagues (1997), and is confirmed by the data obtained in this study.

It has also been suggested that translocations may behave in a certain way during meiosis, predisposing only to a small proportion of embryos with a normal or balanced karyotype, and thus only a few or even no embryos available for transfer (Conn *et al.,* 1999). Additionally, several groups have carried out research in the possible interchromosomal effect and its association with structural rearrangements. The latter is defined as the effect a chromosomal rearrangement could have on the meiotic behaviour of chromosomes that are not involved (Pellestor *et al.,* 2001). FISH has been applied for the analysis of sperm of two male reciprocal translocation carriers, t(1;13) and t(3;19) (Oliver-Bonet *et al.,* 2002). Chromosomes 6, 18, 21, X and Y were scored in both cases and a possible interchromosomal effect was seen only for the second translocation carrier whose sperm demonstrated high rates of disomy 21 (Oliver-Bonet *et al.,* 2002). Different results were obtained from a larger study of 172 embryos from 28 carriers of both reciprocal and Robertsonian translocations, and being examined for chromosomes 13, 16, 18, 21, 22 and some for X, Y and 1 (Gianaroli *et al.,* 2002a). The group observed a higher incidence of aneuploidy for other chromosomes than those involved in the rearrangement in the case of the Robertsonian translocation carriers, but not for the ones belonging to the reciprocal group (Gianaroli *et al.,* 2002a). Comparable findings were seen for the embryos of the two Robertsonian translocation carriers in this study, but the sample size was too small to draw definite conclusions. Moreover, infertile males in general show increased rates of sperm aneuploidy so this fact may give a spurious interchromosomal effect.

The high rate of mosaic and chaotic embryos detected in this and other similar studies, is the outcome of many different factors. More specifically, during the first embryonic cleavage divisions, cell cycle checkpoints that normally regulate mitosis are either absent or function with reduced efficiency (Delhanty and Handyside, 1995). This factor most likely affects the normal chromosome segregation in the embryos (Delhanty and Handyside, 1995). In addition, the different drug regimes given to women in order for their stimulation to be achieved, along with the embryo culture conditions could influence chromosome division at this early stage of development (Munne *et al.,* 1997). Another factor affecting embryo survival could be the 5-10 minute incubation of the embryos in $Ca^{2+}Mg^{2+}$ free medium, which disrupts the gap cell junctions of the embryo, to allow the blastomeres to be acquired easier during the biopsy. In our centre we have increased the size of the medium droplet and the biopsy occurs as soon as the embryo is placed in it. Hence, the 10-minute incubation in the $Ca^{2+}Mg^{2+}$ free medium is avoided, and the repair of the cellular gap junctions that enable embryo compaction to proceed may be faster.

6.3 Conclusions

The main objective of this part of the study was firstly to develop robust and reliable FISH protocols for their implementation in the PGD of seven carriers of three different types of chromosomal abnormality, and secondly to examine the non-transferred embryos generated from these couples, to determine the various segregation modes and the frequencies of meiotic and mitotic errors. PGD strategies were devised for all seven patients, and were applied clinically in five cases. Even though the number of embryos investigated was relatively small, the results obtained are significant, as they contribute extra information on the preferential segregation patterns for both reciprocal and Robertsonian translocations and the chromosome constitution of embryos generated by such patients. Moreover, examination of embryos from patient F revealed that the repeated implantation failure was attributed to the high rate of post-zygotic errors observed in these embryos, rather than to the possible gonadal mosaicism. This patient could be another example of the advanced maternal age effect on mitotic aneuploid mosaicism, which was also demonstrated by

Munne and colleagues (2002), as discussed in the previous section. Effectively this patient is expected to go through a third PGD cycle, but this time her embryos will be investigated for six autosomes, 13, 15, 16, 18, 21, and 22. These chromosomes are examined in our centre during aneuploidy screening cases for which only one cell is biopsied per embryo. In the case of patient F, however, two cells will be biopsied, as previously, as her main problem is the generation of highly mosaic embryos. Investigation of six chromosomes instead of the two that were targeted in the previous cycles should lead to the identification of the most suitable embryo(s) to transfer.
The value of PGD to aid carriers of structural chromosomal abnormalities to achieve a normal pregnancy is demonstrated by the birth of a normal male baby in the case of the carrier of t(11q;22q), that had suffered four early spontaneous miscarriages prior to treatment, but also in the larger study by our group (Simopoulou *et al.,* 2003) in which clinical pregnancies followed three of the eight PGD cycles, two being established after the first treatment.

FISH as a diagnostic tool for the detection of chromosome abnormalities in embryos is a very efficient technique. It has, however certain limitations. Technical problems include the decrease in efficiency with the addition of extra probes, signal overlaps that could lead to misdiagnosis and loss of nuclei during the procedure, the latter being frequently experienced during this study. All these must always be assessed during the development of the proposed PGD strategy. FISH is mostly hampered by embryo mosaicism that could lead to misdiagnosing an abnormal embryo as normal, especially in cases when only one blastomere is being biopsied. The single cell biopsy is being followed by several PGD groups, including the Guy's and St Thomas' centre. This group advocates that biopsy of two cells significantly reduces the embryo mass, decreasing in this way the potential for implantation (Pickering *et al.,* 2003). There is more than one example both in this and the larger study carried out by our group (Simopoulou *et al.,* 2003), during which one of the nuclei was lost and a result was obtained from the other. In addition, re-analysis of the non-transferred embryos generated from this group of patients revealed that chromosomally balanced and abnormal cells could co-exist in the same embryo. The latter confirms our view that in cases of patients who are carriers of chromosomal abnormality diagnosis should be carried out on two blastomeres, rather than one.

The data obtained from this study demonstrates and confirms the observation that the generation of chaotic embryos is both patient-specific and a prognostic factor for the establishment of a clinical pregnancy (Delhanty *et al.,* 1997). The only couple that managed to achieve a pregnancy during the study was in case B. The female partner was able to start but not maintain pregnancies in the past. This combined with the fact that out of the five couples investigated, this couple had produced the second lowest number of chaotic embryos, meant that there was a higher chance for them to achieve a pregnancy with the right embryo. The latter happened after their second PGD attempt.

Counselling of prospective patients for the PGD of chromosome abnormalities should emphasise the fact that multiple attempts could be necessary for a pregnancy to be established. It should also stress the fact of embryo mosaicism and the possibility of not identifying an embryo suitable for transfer from the first attempt. Even if no such embryo is identified should such couples go ahead, the data obtained from the FISH examination should be discussed to provide such patients with the ability of making an informed choice as to how to proceed after treatment.

FISH has an extra limitation in that it is unable to target all 23 chromosomes. Recently the use of multiplex fluorescent PCR and DNA fingerprinting has been applied for the detection of chromosomes 13, 18, 21, X and Y, as an alternative to FISH for the detection of aneuploidy in single embryonic blastomeres (Findlay *et al.,* 2002; Katz *et al.,* 2002). Single-cell PCR has generally been hampered by ADO, which tends to affect 10% of amplifications (D. Wells, personal communication). The use of multiple markers per chromosome, however, increases single-cell multiplex PCR accuracy, and could be used to identify a larger number of chromosomes in a single amplification, compared to the number being diagnosed by FISH (A. Handyside, personal communication). CGH is at the moment the only technique with the ability to screen the entire genome of a single cell, and during the past two years has been applied clinically to screen embryonic blastomeres (Wilton *et al.,* 2001) and first polar bodies (Wells *et al.,* 2002). The disadvantages of this technique in its clinical application include the fact that it is vary laborious and requires good knowledge of both molecular and cytogenetic methods (Munne and Wells, 2002). Another drawback is

the time required for a result to be obtained. Current applications to blastomeres involve the freezing of the embryos (Wilton *et al.,* 2001). Modifications of the protocol leading to reduction in hybridisation time would possibly enable its wider application for the detection of both structural and numerical chromosome abnormalities.

In conclusion, PGD can be a very useful tool for couples that have experienced either years of infertility, multiple spontaneous abortions, or continuous implantation failures as a result of a chromosomal abnormality. As PGD is still relatively new and complicated, because it involves the combination of reproductive medicine and science, good communication between all the participants in the same centre and worldwide is essential (Vandervost *et al.,* 2000). The latter is accomplished by the ESHRE PGD Consortium. Reproductive medicine and science are currently evolving very rapidly. This means that PGD will hopefully become more accurate and more effective to aid childless couples to become parents.

6.4 FISH analysis of human metaphase II oocytes and corresponding first PBs

Out of all types of chromosome anomaly, constitutional aneuploidy is considered to be the most significant clinically, occurring in at least 5% of recognised pregnancies. Genetic investigations on human gametes and embryos have demonstrated that errors during female meiosis I are the main cause of numerical abnormalities. During the second part of this study, FISH was employed in three consecutive rounds for the analysis of unfertilised meiosis II oocytes and their corresponding first PBs when these were available. The probes used were specific for chromosomes of differing sizes representing all groups apart from F. The aim was to gain information concerning the segregation patterns in these cells that came from women undergoing assisted conception, in an attempt to answer questions related to mechanisms leading to maternal aneuploidy.

A total of 265 oocytes, some consisting of PBs as well, were considered suitable for this FISH investigation. These came from 168 patients with an average maternal age of 32.5 years. The collaborating centres were two: the Assisted Conception Unit at the University College London Hospitals Trust, and the Assisted Conception Unit at Tayside University Hospitals NHS Trust, in Dundee, Scotland.

Ten chromosomes were examined including 1, 4, 12, 13, 16, 17, 18, 21, 22, and X. Both centromeric and locus-specific probes were used for their investigation. Strict scoring criteria were adopted for the identification of anomalies in these cells. More specifically, we were scoring as abnormal, oocytes and/or PBs that showed extra signals, representing the gain of a whole chromosome or chromatid. Absence of signals was not scored, as the latter could not be distinguished from chromosome loss during the spreading process or hybridisation failure. An oocyte and/or PB was characterised as having undergone premature division of chromatids, when two or more chromatid diameters were separating the two signals. In these cases the cell was not considered to be abnormal per se, but to have an increased potential to cause an aneuploid conception due to random segregation at anaphase II, following fertilisation. These scoring criteria were employed

by Mahmood and colleagues (2000) and Cupisti and colleagues (2003) in earlier investigations by our group.

6.4.1 Processing of oocytes and PBs

Two different techniques were employed in order for the fixation of the eggs (oocyte + PB) on slides to be achieved. The majority of cells were fixed using the method suggested by Tarkowski (1966) with modifications, as described by Mahmood *et al.* (2000). Some eggs were fixed according to a different protocol based on the gradual fixation method suggested by Kamiguchi and colleagues (1993). As mentioned in the Results section, this study was hampered by the high material loss rate. This was the main reason of the application of the two spreading methods. Effectively, applying these two methods made no difference to the frequency of cell loss, chromosome numbers and morphology, and the ability of oocytes to remain on slides during the three consecutive rounds of FISH. Moreover, the gradual fixation method led to chromosome preparations that were surrounded by cytoplasm, making the scoring of signals very difficult.

The Tarkowski spreading protocol has been applied in several similar studies including those by Cupisti *et al.* (2003), Durban *et al.* (1998), Munne *et al.* (1995), and Pellestor *et al.* (1993) with or without modifications. Durban and colleagues (1998) in their study of first PBs reported on a very low rate of chromosome loss, due to the use of a fixative that consisted of equal amounts of ethanol and acetic acid, leading to its slower evaporation. The gradual fixation method was applied by Pellestor and colleagues (2003) in their investigation of unfertilised oocytes with the use of R-banding. The application of this method enabled them to score for both extra and missing chromosomal material in these cells (Pellestor *et al.,* 2003). A completely different protocol was employed by Martini and co-workers (2000). In this study the oocytes were spread on slides with the use of Tween/HCL in a way comparable with the one used for blastomere/embryo spreading. The latter resulted in the formation of "fragments" that had lost their chromosome shape and could easily be removed together with the cytoplasm. Corresponding PBs were not examined, as they could not be distinguished from the oocytes.

In the current study identification of cells initially took place by staining them with DAPI and locating them under fluorescence. This step was replaced by staining the oocytes and PBs with Giemsa, as it was thought that the DAPI and the prolonged exposure to fluorescent light affected their DNA leading to poorer chromosome morphology and frequent loss of material after the first round of FISH. The staining of the cells was abandoned altogether towards the end of this study, as it was observed that the Giemsa was also affecting chromosome quality and leading to an even higher rate of chromosome loss. Different treatments were also applied in order to reduce this, including ageing the cells at room temperature for 2-4 weeks, leaving them overnight in an incubator at 37°C or at 65°C, as suggested by Sandalinas *et al.* (2002), or a combination of both, storing them at either 4°C or –20°C prior to the first, second or third round of FISH, and spreading them on charged slides. From the eggs that were lost others were relatively new, i.e. spread during the year of examination, and others were very old and had been stored at 4°C or –20°C for years. Thus, there was not a standard pattern that could explain the reason as to why these cells were being lost with such high frequency.
The results obtained from the FISH investigation of unfertilised oocytes and their corresponding first PBs will be discussed in the following section.

6.4.2 Aneuploidy detection in human metaphase II oocytes and first PBs

FISH analysis was attempted for 265 meiosis II oocytes, some of them also consisting of their corresponding first PBs. Of these, 51 oocytes and 11 PBs yielded results after the first round of FISH, 27 oocytes and 8 PBs after the second round of FISH, whereas 7 oocytes and 2 PBs were examined in the final FISH round. The remaining cells were all lost, leading to a decrease in the sample size for this part of the study. Information was obtained for 31 patients, whose average age was 32.5 years with the youngest being 22 and the oldest 44. It has been demonstrated by population data that in an unselected group of women of average age of about 33 years it would be expected that approximately 8% of their oocytes would consist of either extra or missing chromosome material, and the groups mostly affected would be D, E, and G and also chromosome X due to their small size and the fewer chiasmata formed between the two homologues (Zenzes and Casper,

1992). Added to chromosomes belonging to these groups we also chose to examine larger chromosomes, belonging to groups A, and C, to check whether they were prone to undergo non-disjunction during the first female meiosis.

Extra chromosomes were scored for four oocytes and one PB all from different patients. The youngest patient in this group was 31 and the oldest 35, leading to an average age of 32.2 years. The latter is below the age at which an increase in aneuploid conceptions becomes marked. This implies that some of the mechanisms leading to maternal aneuploidy could be age-independent, especially in the infertile population. Two patients had oocytes containing an extra copy of 17, two had oocytes hyperhaploid for chromosome 13 and one patient had one PB with an extra chromatid 22. Surprisingly no abnormalities were scored for chromosome 21. The latter was attributed to the small size of the sample, and the frequent absence of this chromosome from the oocytes and PBs investigated. The estimated hyperhaploidy rate was 9.8%.
No abnormalities were scored for the larger chromosomes 1, 12, and X. The latter was not unexpected for 1 and 12, as many similar studies, including these by our group, have demonstrated the preferential involvement of the smaller chromosomes and chromosome X in oocyte aneuploidy (Cupisti *et al.*, 2003; Pellestor *et al,* 2003; Sandalinas *et al.*, 2002; Mahmood *et al.*, 2000; Dailey *et al.*, 1996).

The results from this study were partially included in the report by Cupisti and colleagues (2003). In that report a total of 236 eggs from 124 patients were successfully examined for chromosomes 1, 9, 12, 13, 16, 18, 21, and X. The abnormality rate (hyperploidy only) in this study was 4%, and smaller chromosomes were preferentially affected to a statistically significant extent (see Appendix C). Both extra whole chromosome and single chromatid anomalies were identified, among the fourteen hyperhaploidies that were scored. Table 6.2 summarises the frequency of anomalies found in eggs per chromosome investigated. From this study it was also postulated that aneuploidy could be affected by age independent mechanisms as the average age of patients with abnormalities was almost identical to that of the normal group (Cupisti *et al.*, 2003).

Table 6.2: Frequency of anomalies (hyperploidy) found in oocytes or 1st polar bodies per chromosome. (from Cupisti *et al.*, 2003).

Chromosome	Eggs[a] scored	Whole chromosome anomalies	Single chromatid anomalies	% of anomalies
1	83	0	0	0
9	84	0	0	0
12	62	0	0	0
13	95	3	0	3.2
16	97	1	1	2.1
18	95	1	1	2.1
21	96	2	4	7.0
X	105	0	1	1.1

[a]An egg consists of an oocyte and a first polar body, but may each may be present alone without the other

Anahory and colleagues (2003) investigated 104 unfertilised oocytes and 56 first PBs, by applying a combination of centromeric or locus-specific probes and whole chromosome paints for chromosomes 9, 13, 16, 18, 21, and X, in sequential FISH rounds. The aneuploidy rate (hyperploidy plus hypohaploidy) was higher than the one found in the study of Cupisti and colleagues (2003), and was estimated to be 11.5%. Abnormalities affected chromosomes 9, 16, 18, 21, and X, and ten oocytes were identified as having either extra whole chromosomes or single chromatids. The authors claimed that this FISH approach was advantageous, as it enabled the detection of whole chromosomes, rather than the visualisation of two dots, seen with the application of single probes (Anahory *et al.*, 2003). The spreading method used for this study was based on the Tarkowski protocol. However, the authors did not take into account the artifactual chromosome loss and were scoring both for extra but also for absent chromosomes.

Ninety-three first PBs were examined for chromosomes 13, 16, 18, 21, and 22 by Munne and co-workers (2000c). This study was carried out in the context of PGD for aneuploidy screening, so the patients were in the older age group. Similarly to the findings of our study, and the two mentioned above, abnormalities affecting all these chromosomes were detected, with the aneuploidy rate being higher compared to the studies above, at 33.3%. As with the Anahory investigation, the authors of this report were scoring for extra and missing chromosomes, not taking into consideration the possibility of artifactual loss during the spreading process.

An even higher aneuploidy rate of 47.5% was reported in the study of first PBs and 54 corresponding meiosis II oocytes, carried out by Pujol *et al.* (2003a). The aim of this investigation was the validation of PB analysis for prediction of aneuploidy in the oocyte. The chromosomes investigated belonged mostly to groups D, E, and G, including 13, 15, 16, 17, 18, 21, 22 and X. The only representatives of groups A-C that were examined were chromosomes 1 and X. Comparison of the observations made for the PBs and their oocytes led to the conclusion that 28.5% of the results obtained were artifactual. They did not identify a difference in the frequency with which the various chromosomes were involved in aneuploidy (Pujol *et al.*, 2003a). The latter contradicts the findings presented in all the other studies, including the one described in this project that all show the preferential involvement of the small chromosomes in maternal aneuploidy. This and the very high aneuploidy rate could be explained by the fact that almost all the oocyte/PB pairs they examined were obtained at the germinal vesicle stage of oogenesis and were left to mature *in vitro*. This, however, is not discussed in the report.

Germinal or gonadal mosaicism has been determined as one of the mechanisms leading to female aneuploidy (Cozzi *et al.*, 1999; Mahmood *et al.*, 2000; Delhanty, 2001; Cupisti *et al.*, 2003). This mechanism was evident in this study as well, and was observed for two out of the five patients whose oocytes or PBs were considered to be abnormal. In the first case germinal mosaicism for trisomy 13 was evident, by the scoring of an extra copy of this chromosome in one oocyte, whilst the corresponding PB had a normal complement. In the second case the first PB consisted of an extra chromatid for chromosome 22, with the corresponding oocyte having the normal single copy of this chromosome. In both

cases the cells consisted of about 23 chromosomes, so it was highly unlikely for all of them to have lost the extra copy of the chromosome in question. Such an event could take place in an otherwise karyotypically normal individual. In cases such as these it is possible for the trisomic germ cell line to undergo secondary non-disjunction (Cozzi *et al.*, 1999). There have been two reports by our group on evidence for gonadal mosaicism obtained by the analysis of oocytes by FISH. In one case a couple requested PGD for recurrent trisomy 21 conceptions, and FISH analysis of their preimplantation embryos along with unfertilised oocytes and their PBs revealed that the female partner was a gonadal mosaic for this trisomy (Cozzi *et al.*, 1999). In another report two patients were identified to be gonadal or germinal mosaics for trisomies 13 and 21. Both cases were unsuspected and were detected during an oocyte and PB study, very similar to the one described here (Mahmood *et al.*, 2000). Gonadal mosaicism is thought to be associated with confined placental mosaicism (CPM), as the primordial germ cells and the chorionic stroma originate from the same progenitor cells (Buehr, 1997). Stavropoulos and colleagues (1998) demonstrated the above by identifying a case of a conceptus that had CPM for trisomy 16 and was also a gonadal mosaic for this abnormality. Since CPM affects at least 1% of pregnancies, investigated by chorionic villus sampling (CVS), it is likely that gonadal mosaicism is more frequent than is assumed. Evidence for germinal mosaicism was also identified in the Pujol *et al.*, (2003a) study. Hence, three PBs from the same patient were detected to have extra whole chromosomes, whereas the corresponding oocytes were characterised as normal, haploid. Chromosomes 13, 15, 16, 17, and 22 were affected. The authors attributed these abnormalities to unique, multiple or successive segregation errors that could have taken place during the mitotic divisions of the precursor oogonia, rather than in the presence of a trisomic germ cell line (Pujol *et al.*, 2003a).

Balanced predivision of chromatids was noted for nearly all the examined chromosomes, with chromosome 21 being observed to be more frequently involved. This phenomenon was initially described by Angell (1991) and involves the premature separation of chromatids prior to anaphase I. The chromatids are then are at risk of segregating randomly during meiosis II and possibly leading to the formation of a trisomic

conception. For this reason, the observation of this balanced predivision was not considered as an abnormality, as these chromatids still had the potential to segregate normally, leading in this way to an oocyte that had the expected chromosome complement. Several studies have proposed that this balanced predivision of chromosomes to their sister chromatids was an artifact attributed to prolonged culture conditions of the cells and not a true event that could potentially result to female aneuploidy (Munne *et al.*, 1995, Dailey *et al.*, 1996). This hypothesis was abandoned after the findings of a further study by the same group on 47 fresh oocytes that were produced from 13 fertile donors (Sandalinas *et al.*, 2002). These oocytes were analysed by SKY, and 12 were identified to contain chromosomes that had undergone balanced predivision to their sister chromatids. It was concluded that the premature separation of oocyte chromosomes is one of the factors involved in female aneuploidy. This is confirmed by the fact that half of all anomalies detected by FISH analysis of oocytes affect single chromatids. The group also identified a direct association of advancing maternal age and increased frequency of chromatid predivision (Sandalinas *et al.*, 2002).
The situation is different for the PBs, that undergo rapid degeneration once in culture. In this case the balanced premature separation of chromosomes could be the result of this rapid degeneration, rather than being a true event. The latter was observed by Clyde and colleagues (2001) in their study of one oocyte and its corresponding first PB by M-FISH. More specifically, once cytokinesis in the PB was complete, degeneration of the cell began. This was observed after having cultured the PB for approximately 6 hours. Balanced predivision was also noted for two other PBs from the same patient, that were fixed on slides in less than six hours (Clyde *et al.*, 2001).
The observations made in the current study concerning predivision are most likely a combination of both artifactual and true events, especially in the case of oocytes. In general, the latter were left in culture 24-48 hours before being fixed. In cases of inferior chromosome morphology, balanced predivision was attributed to cellular degeneration. If chromosome quality was good, then this event was considered more likely to be true.

An observation that was made in the SKY study by Sandalinas *et al.* (2002) was that the balanced predivision affected chromosomes belonging to groups E-G more frequently,

compared to the larger ones. This was also noted in this study, with chromosome 21 having separated into its sister chromatids in six oocytes. Chromosomes 12, 13, 17, and 22 were scored as prematurely separated in two oocytes each, whereas the larger chromosome 4 underwent premature division in two oocytes and one PB, the latter most likely being due to the cell's degeneration. Such observations in combination with the fact that smaller chromosomes are more prone to non-disjunction during the first female meiotic division are all attributed to the number of chiasmata that are formed during meiotic recombination. The smaller the chromosome, the fewer the number of crossing-over events. Chiasmata are crucial for the correct segregation of homologues during meiosis (Tease *et al.,* 2002). Several studies have been carried out in an attempt to elucidate the patterns of female recombination that could potentially lead to malsegregation of chromosomes (Robinson *et al.,* 1993; Sherman *et al.,* 1994; Fisher *et al.,* 1995; Hassold *et al.,* 1995; Lamb *et al.,* 1996; Nicolaidis and Petersen, 1998; Brown *et al.,* 2000). All these came to the common conclusion that variations in recombination patterns such as failure of crossing-over, reduction in crossing over frequency combined with formation of distal chiasmata, or an increase in crossing-over frequency combined with the formation of proximal chiasmata, all act as risk factors contributing to non-disjunction. Tease and colleagues (2002) used an immunocytological approach to investigate the chromosome recombination patterns in human fetal oocytes. They found that only one distal crossover is formed between the two homologues for chromosome 21. The latter could explain the observation of the frequent balanced predivision for this chromosome, made in this study.

The effect of maternal age in oocyte aneuploidy was not investigated in this study due to the small sample size. The negative influence of advanced maternal age on female meiosis has been demonstrated, however, by various other studies on unfertilised oocytes and first PBs. The first was that of Dailey *et al.* (1996). The group examined 383 oocytes from 107 patients with an average maternal age of 36.2 years, for chromosomes X, 13, 18, and 21. They reported an increase in non-disjunction of bivalent chromosomes from 1.5% in women 25-34 years of age to 24.2% in women of 40 years or older. Hence the association of advanced maternal age with chromosome malsegragtion was obvious (Dailey *et al.,* 1996). A patient with an extra whole chromosome 18 and an extra

chromatid for 21 in the same oocyte was identified in the study of Mahmood and colleagues (2000). This patient was 38 years old and the oldest in the group. The presence of two different anomalies in a single cell clearly demonstrates the adverse effects of maternal ageing in the chromosome complement of oocytes (Mahmood *et al.*, 2000). Pellestor and colleagues (2003) carried out a much larger study of 3,042 unfertilised oocytes from women aged between 19-46. The cells were examined with R-banding. They identified that maternal age is directly correlated with both whole chromosome non-disjunction, but also with abnormal single chromatid events and concluded that multiple factors are related in this maternal age effect, both environmental and intrinsic (Pellestor *et al.*, 2003).

Throughout this study no extra copies were scored for the larger chromosomes 1, 4, 12, and X. Absence of these chromosomes was attributed to technical loss. Data from cleavage stage embryos analysed both with FISH and CGH have shown monosomies for such chromosomes (Harper *et al.*, 1995; Voullaire *et al.*, 2000; Wells and Delhanty, 2000). Events such as these could be the result of a different mechanism, namely anaphase lag, which seems to affect larger chromosomes (Harper *et al.*, 1995; Voullaire *et al.*, 2000; Wells and Delhanty, 2000).

The estimation of the true aneuploidy rate in studies that are examining female gametes either karyotypically or via FISH methods occurs by doubling the hyperhaploidy rate to account for the hypohaploids that result from the true chromosome loss. Zenzes and Casper (1992) reported on the hyperhaploid rates from 11 karyotyping studies of 1120 oocytes. The average rate from the combination of these studies was 6.6%. More recently Pellestor and colleagues (2002) carried out a larger karyotyping study on 1397 oocytes and identified a hyperhaploidy rate of 4.1%. Moreover, the average rates from FISH studies range between 4-11%, scoring, however, only for extra chromosomes. There have been reports of much higher aneuploidy rates, namely 45.2 % (Verlinsky *et al.*, 2001) and 44% (Martini *et al.*, 2000). In the first case the group applied FISH on the 1st and 2nd PBs with aim of predicting the chromosome complement of the corresponding oocytes in the context of PGD for aneuploidy. The authors were scoring for both extra

and missing chromosome material, without accounting for the artifactual loss of chromosomes due to processing. In addition, they aimed to identify and transfer chromosomally balanced embryos, so as for the success rate of IVF in older women to be improved. Hence, any oocyte that was suspected of an abnormality was excluded (Verlinsky *et al.,* 1999; 2001). In the second case (Martini *et al.,* 2000) the high aneuploidy rate is most likely due to the way the oocytes were processed. They were treated with pronase to remove corresponding PBs and were spread in a way that grossly affected their morphology. Removal of the PB had as an effect the inability to investigate the corresponding cell and confirm possible abnormalities.

Hyperhaploidy can be reliably demonstrated with the application of FISH and other related techniques, such as SKY, and this is evident from the results obtained in this and other similar studies. The estimation of hypohaploidy, however, requires a molecular cytogenetic technique that would ensure no artifactual loss of genetic material. Comparative Genomic Hybridisation is a DNA based method that enables the screening of an entire genome being achieved in a single hybridisation step. CGH has been developed by our group for the analysis of blastomeres coming from cleavage stage embryos (Wells and Delhanty, 2000) and also of first PBs during a clinical PGD case (Wells *et al.,* 2002). This method was employed in this study in an attempt to examine all 23 chromosomes in meiosis II oocytes and corresponding first PBs to investigate maternal aneuploidy and its causal factors further.

6.5 CGH analysis of human metaphase II oocytes and corresponding first PBs

During the last stage of this study, CGH was employed to screen all 23 chromosomes of unfertilised oocytes and their corresponding first PBs. This molecular cytogenetic technique would enable the examination of chromosomes that were not targeted during the previous FISH study, and would possibly provide data on hypohaploidy, as well.

Investigation was attempted for 85 cells (40 oocytes, and 45 PBs), and 37 of these were pairs. All the patients that donated their cells for this part of the study were undergoing infertility treatment at the Assisted Conception Unit at Tayside University Hospitals, NHS Trust, Dundee, Scotland. Maternal ages ranged between 22 and 39 years (mean 31.9). These couples were mostly being treated due to male factor infertility. Problems with the female partner were evident in three cases, including one patient with polycystic ovary syndrome (PCOS), one with anovulation, and one with idiopathic infertility.

Prior to its application for the examination of oocytes and PBs, CGH was evaluated on genomic, and trisomic fibroblast DNA, and on normal and abnormal single cells. This evaluation, along with the technical problems experienced during the development of an accurate and reliable protocol for the analysis of minute DNA amounts will be discussed below.

6.5.1 Technical aspects of CGH

The CGH protocol used for the analysis of all oocytes and PBs was as described by Wells and colleagues (2002) with certain modifications. Three sequential stages were involved. These included the initial DNA amplification with the DOP-PCR, the enzymatic incorporation of the fluorescent labels, followed by hybridisation onto normal male metaphases. Evaluation of these stages took place by carrying out seven positive control experiments:

1. Normal female genomic DNA against normal male genomic DNA

2. Fibroblast DNA trisomic for 13 against normal male genomic DNA
3. Fibroblast DNA trisomic for 21 against normal female genomic DNA
4. Fibroblast DNA trisomic for 22 against normal male genomic DNA
5. DNA from a clump of three normal female buccal cells against DNA from a clump of three normal male buccal cells
6. DNA from a clump of three normal female buccal cells against DNA from a clump of three normal female buccal cells
7. DNA from a single fibroblast cell trisomic for 18 against normal male genomic DNA

Genomic and fibroblast DNAs were of relatively low concentrations and these along with that from the clumps (3-5 cells) and single cells were all amplified with the use of a whole genome amplification (WGA) reaction, namely DOP-PCR. The primer employed in this reaction is capable of annealing to a huge number of sites scattered throughout the genome, leading to efficient amplification of more than 85% of loci (Wells *et al.*, 1999). Its application for these samples led to the generation of DNA fragments between 300-1500bp in size, and concentrations of approximately 800-1000ng. These were more than sufficient for the further processing of all samples. Evaluation of the remainder of the CGH protocol, in the context of nick translation efficiency and assessment of the accuracy and sensitivity of the computer software employed for the experiment analysis and interpretation for DNA samples with the same or different concentration was also feasible. Karyotypes were accurately assessed in every case, with all trisomies correctly identified. These seven experiments were also used as reference when comparable results were obtained from the CGH analysis of the oocytes and PBs.

Wells and colleagues (1999) were the first to describe the application of CGH to single fibroblasts, buccal cells, and amniocytes. They tested four different WGA approaches, including DOP-PCR, tagged PCR (T-PCR), primer extension preamplification (PEP), and *Alu*-PCR for the amplification of these cells. The DOP-PCR was the method that provided the most efficient coverage of the genome, and generated the highest DNA quantity that was sufficient for the CGH analysis of these cells (Wells *et al.*, 1999). This approach was different from the one applied in this study, as the fluorescent labels for

both test and reference DNAs were incorporated during amplification, instead of by nick translation. The authors of that report were the first to demonstrate the detection of aneuploidy with CGH analysis of cells amplified by DOP-PCR (Wells *et al.*, 1999).

A similar study was carried out by Voullaire and colleagues (1999). They also applied the DOP-PCR for the amplification of single fibroblasts that were trisomic for 13, 18, or 21. Genomic DNA that was also amplified by the DOP-PCR was used as reference. The fragment sizes of the amplified products were comparable to the ones generated in this investigation, i.e. their size varied between 300-2000bp. They also applied the nick translation procedure for the labelling of the PCR products. Analysis and interpretation of the obtained results verified the ability of the CGH to detect trisomies of even small chromosomes, such as 21 at the single cell level with the resolution being of the order of 40Mb (Voullaire *et al.*, 1999). The latter was seen in this study as well, as fibroblast samples trisomic for the smaller chromosomes 18, 21 and 22 were analysed and these trisomies were identified with the same resolution.

An alternative WGA protocol was described by Klein and colleagues (1999) called "Ligation-Mediated" PCR. During this WGA method, the single cell DNA was initially digested after cell lysis, with the use of the *Mse*I restriction endonuclease. The digestion lasted approximately three hours. Subsequently, two primers were used that were left to anneal and amplify the digested single cell DNA overnight. The authors claimed that this approach enabled the faithful amplification of the genome. This was its advantage over the DOP-PCR, which preferentially over-amplified certain parts of the genome (Wells *et al.*, 1999). However, "Ligation-Mediated" PCR required a longer period of time for the amplification to be complete, whereas the amplified products were generated within 6 hours with the application of the DOP-PCR. The utilisation of CGH at the single cell level was evaluated not only for research purposes but also with the aim of applying this technique clinically for the analysis of gametes and blastomeres from embryos. Such applications necessitated the design of a protocol that would yield results within the time frame of an embryo transfer, i.e. by day 5 the latest. Hence, the WGA approach described by Klein and colleagues (1999) was not appropriate, as it was both time consuming and had the additional restriction endonuclease digestion step, increasing in this way both the

complexity of the WGA protocol, and the possibility of contamination to enter the reaction.

Three groups of differently prepared oocytes and PBs were investigated with CGH throughout this part of the study, as is described in section 5.1. Results were obtained for all three groups, with the CGH being most successful for the cells belonging to the third category (single cells suspend in approximately 2μl of sterile PBS and overlaid with oil), and least for the cells belonging to the first category (single cells suspended in 100μl of sterile PBS). The latter were stored in the –80°C for approximately four months prior to their analysis. It was suspected that the combination of the high amount of PBS in which the cells were suspended and their prolonged storage in the –80°C could have adversely affected the DNA amplification. The high PBS volume necessitated centrifugation and removal of excess fluid prior to the DOP-PCR. This could have increased the risk of cell loss. In addition, the long storage of the cells could have resulted in DNA degradation, leading to poor amplification and fragment sizes that were too small for CGH, especially after nick translation, which introduces further DNA strand breaks. Many cells were known to have been lost or lysed during their processing, and consequently the low amplification success rate was not unexpected (26 cells demonstrated the expected smears from a total of 85 for which CGH was attempted). The amplified products from all cells were analysed by agarose gel electrophoresis, and smears were visible for three cells out of those that had not yielded results with CGH. In these three cases failure was attributed to inferior DNA quality and possible degradation, due to prolonged storage time. Absence of the characteristic smears for the rest of the failed cells suggested their loss during processing. All the above observations led to the conclusion that CGH would be most successful on cells that were suspended in a small amount of PBS, overlaid with oil and being stored in the –80°C for a relatively short period of time, i.e. two months or less.

Difficulties arose in the interpretation of the captured images as well. More specifically, the majority of the oocytes used in this study had been exposed to sperm either via IVF or ICSI. So the presence of sperm DNA was possible for the IVF oocytes and almost a

certainty for the ICSI ones. Hence, we considered that the CGH analysis was probably carried out on both the maternal and the paternal genomes. In the cases of the PBs, the presence of sperm was very unlikely, as these cells were already extruded prior to fertilisation, and thus represented solely the maternal genome. It was postulated that for the purposes of analysis the DNA from haploid oocytes and PBs would behave as diploid female DNA, and thus when a whole chromosome was missing this would correspond to a nullisomy in the diploid state, whereas absence of a single chromatid would correspond to a monosomy. Another problem encountered during interpretation was the relative insensitivity of the CGH software to detect abnormalities caused by single chromatids in DNA from single haploid cells, especially those involving their possible absence from the complement.

Despite the technical problems encountered during the CGH analysis of such cells, data was collected for 11 oocytes and 15 first PBs. Among these, several were characterised as abnormal, and chromosomes that are not typically targeted with FISH were seen to show anomalies. This demonstrated the value of CGH in its ability to screen entire chromosome complements.

6.5.2 Chromosome abnormalities in oocytes and PBs investigated with CGH

The second and main aim of this project involved the extended investigation into the mechanisms causing female aneuploidy by examining unfertilised oocytes and their corresponding first PBs. CGH was selected to conclude this investigation, due to its capability to examine all 23 chromosomes and identify most gains and losses of DNA. A normal haploid chromosome complement was detected for 8 oocytes and 10 PBs, four of which were corresponding, whereas CGH failure was observed for 5 PBs and 6 oocytes. Eggs (oocytes and PBs) were donated from fourteen patients, with a mean maternal age of 31.9 (22-39) years. This was slightly lower compared to the average maternal age of the patients that participated to the FISH part of the study.

The reference DNA, with which the test DNA samples were compared, was initially from a normal male (46,XY), but this changed, as the study proceeded to DNA from a normal

female (46,XX). This modification occurred for three reasons: firstly, the use of female DNA would act as an internal control in cases that the examined cells were contaminated with male DNA from the handler, secondly it would enable the detection of chromosome X hyperhaploidy in the test sample, and thirdly it would possibly aid in the distinction between loss of whole chromosomes and single chromatids. The argument for this was that neither the reference DNA nor the test DNA, especially in the cases of PBs, contained Y chromosome material. Hence, if the CGH analysis detected a chromosome for which DNA was missing in the test, comparison between the fluorescence intensity of the green fluorochrome on the chromosome in question and the Y chromosome, would most likely identify whether this loss was attributed to a whole chromosome or a single chromatid. More specifically, if there was no green visible and the chromosome in question was very faint, similar to the bottom part the Y chromosome then loss of a whole chromosome would have taken place (nullisomy in the diploid state). If however, there were some green fluorescence visible for that chromosome, then the loss would be attributed to a single chromatid (monosomy in the diploid state).

In practice, it was observed that the CGH software used was more sensitive in detecting the presence of extra chromosomal material rather than its absence. This was also noted by other groups that were using this version of software to carry out their CGH analysis (Malmgrem *et al.*, 2002; D. Wells, personal communication).

During analysis and interpretation of the captured images in some cases, the presence of hybridisation artefacts was observed for the heterochromatic regions, and both the short and long arm telomeres of certain chromosomes, including 1, 9, 16, and Y and the satellite regions of the acrocentric chromosomes. These were caused due to the extreme suppression of these regions by the Cot-1 DNA and any low level fluorescence at these sites was attributable to background. Consequently, these regions were not considered during interpretation. In addition, in cases where abnormal results were obtained for chromosomes 19 and 22, they were interpreted with caution, due to the fact that these chromosomes are also known to be prone to labelling artefacts. Other CGH studies have reported on the observation of artefacts on these chromosome regions, such as Lomax and colleagues (2000), Tabet and colleagues (2001), and Wilton and colleagues (2001).

Chromosome abnormalities in the form of both gains and losses were detected for seven cells, three oocytes and four PBs. Of these one oocyte and two PBs were carrying abnormalities affecting more than one chromosome. Gains were identified for chromosomes 4, 5, 8, 9, 12, 13, and X and losses for chromosomes X, 21 and 22. These cells were donated from five patients, whose ages ranged between 22-39, with an average maternal age of 25 years.

The most interesting case was that of patient 4412, who was the youngest of the group (22 years of age) and the one with the most abnormal cells. Four oocytes and their corresponding PBs were examined. Of these, one oocyte and two PBs consisted of the normal haploid chromosome complement, whilst three cells (two oocytes and one PB) were characterised as abnormal. The anomalies scored involved the presence of extra DNA material for chromosome 13 and absence of DNA material for chromosome 22 in the first oocyte and the corresponding PB characterised as normal, and the absence of DNA material for chromosome X and possibly the presence of extra DNA material for chromosome 21 in the second oocyte, with its corresponding PB consisting of the reciprocal extra DNA for chromosome X and missing DNA for chromosome 21. The fact that the reciprocal loss and gain of DNA material was not detected in one oocyte-PB pair could be explained, due to the relative inability of the CGH to fully detect anomalies, or as a consequence of aneuploidy originating in the sperm.

This patient was undergoing fertility treatment due to PCOS. The observation of these highly abnormal cells in combination with the very young age of this patient led to the hypothesis that one of the causal factors for these aneuploid cells were the polycystic ovaries. Two other reports described patients with PCOS and aneuploid gametes. More specifically, Sengoku and colleagues (1997) examined 74 oocytes from PCOS patients in their investigation of an association of the poor fertilisation rate of oocytes coming from such patients and their chromosome complement. Aneuploidy was detected in ten oocytes, and four appeared to be diploid (Sengoku *et al.*, 1997). More recently, Clyde and colleagues (2001) analysed one oocyte and its corresponding PB, from a 33-year old PCOS patient, with the application of M-FISH. They identified three extra chromatids for chromosomes 15, 19, and 22 in the oocyte, with their reciprocal loss from the PB. These

abnormalities were the result of premature division of chromosomes into their sister chromatids, as discussed in the previous section.

The observations made in these studies, along with the data presented in this thesis suggest that PCOS patients could be at a relatively higher risk of producing aneuploid oocytes, but further examination of gametes generated from such patients is necessary to confirm this hypothesis. Production of highly aneuploid oocytes from polycystic ovaries could be attributed to the abnormal folliculogenesis that is observed in these patients. The follicles of PCOS patients arrest at a diameter of 5-8 mm, which is much earlier than a mature follicle is expected to ovulate (Webber *et al.*, 2003). The latter is the result of the increased concentrations of the tonic luteinizing hormone (LH) that are observed at this stage leading to the generation of oocytes of inferior quality, with poor fertilisation potential (Stanger *et al.*, 1985; Howels *et al.*, 1986; Regan *et al.*, 1990).

The fact that the CGH software used for analysis was possibly incapable of detecting abnormalities caused by the loss of single chromatids was also evident during the analysis of oocyte 1134.3 and its corresponding PB. In this case, the oocyte was at the germinal vesicle stage at the time of egg collection, was not exposed to sperm and was left to mature *in vitro*. Its analysis resulted in the identification of extra chromosome material for 13. The reciprocal loss was not detected in the corresponding PB. The latter could either be attributed to the presence of an extra chromatid for 13 in the oocyte combined with the incapability of the software to recognise the missing chromatid in the PB, or to the fact that this patient was a germinal mosaic for trisomy 13, with the extra chromosome segregating in the oocyte.

Gains of larger chromosomes, namely 4, 5, 8, 9, 12, and X were detected in three PBs. The PB no. 1254.6 was characterised as highly abnormal, as it presented with gains of three chromosomes. Chromosomes 4, 12 and X were also investigated during the FISH part of this study, both in PBs and oocytes, but no anomalies were scored for them. As mentioned above, PBs were considered to be free of sperm, representing solely the maternal genome. Thus, these gains were regarded as being true observations, and were not attributed to artefacts of the hybridisation process. Molecular cytogenetic studies of

the female gametes, including the one described here have demonstrated the preferential involvement of chromosomes smaller in size than 13 in oocyte aneuploidy. Extra copies of chromosomes belonging to larger groups were also scored, but were not as frequent.

CGH was clinically applied for the analysis of 12 PBs from one patient, in the context of PGD for aneuploidy screening (Wells *et al.*, 2002). During this investigation, results were obtained for 10 cells and only one of them was identified as consisting of the normal haploid chromosome complement. Both gains and losses of chromosomes were detected in the 9 aneuploid PBs, with smaller chromosomes 14, 16, 20, 21, and 22 being more frequently involved. Among the abnormal PBs two were described to be carrying anomalies of larger autosomes, one consisting of an extra copy of chromosome 2, and the other missing chromosome 5. Further investigation occurred by FISH analysis of blastomeres from the resulting embryos with probes targeting the chromosomes that came up as abnormal with CGH. Almost all abnormalities observed in the PBs were confirmed by FISH in the embryos, one of them being classified as trisomic for chromosome 5, due to a meiotic error. The oocyte corresponding to the PB with the extra chromosome 2 had failed to fertilise, and this result was not confirmed by FISH (Wells *et al.*, 2002).

Extra or missing copies of larger autosomes were scored in the examination of 47 fresh unfertilised oocytes, carried out by Sandalinas and colleagues (2002). These cells were assessed with the application of SKY, which enabled the detection of all 23 chromosomes and the scoring of both extra and missing copies. Meiotic errors were more frequently seen to be affecting the smaller chromosomes 14, 16, 17, 18, 19, 20, 21, and 22. However, five cells consisted of extra or missing whole chromosomes and/or chromatids, including 6, 8, 9, and 12 (Sandalinas *et al.*, 2002).

Pellestor and colleagues (2002) applied the R-banding method for the analysis of 1397 unfertilised meiosis II oocytes. Among these, five cells were carrying extra copies of 3, 6, 9, 11, and 12, either in the form of whole chromosomes or single chromatids. Similar to the highly abnormal PBs observed in this study, Pellestor's investigation detected cells with multiple anomalies, with up to 10 chromosomes being affected. Since this was a

karyotyping study exact assignment for a chromosome was not always feasible (Pellestor *et al.,* 2002), and the identification of chromosome loss was not reliable.

Meiotic errors affecting larger autosomes were also observed during the CGH analysis of preimplantation embryos. Wells and Delhanty (2000) used this method in the examination of 64 blastomeres from 12 cleavage-stage embryos. High levels of mosaicism were detected, caused mainly due to post-zygotic errors. In addition, one third of the examined embryos were classified as aneuploid due to meiotic errors, with chromosomes 1, 21 and X being affected (Wells and Delhanty, 2000). A similar CGH investigation was carried out on 126 blastomeres from embryos derived from patients with repeated implantation failure (Voullaire *et al.,* 2002). Errors of meiotic origin led to the presence of extra copies of chromosomes 11, 13, 16, and 21 and absence of chromosomes 8, 9, 10, 14, 15, 16, 21 and X, observed in a total of 12 embryos. From these, eight were generated from patients of advanced maternal age (Voullaire *et al.,* 2002).

From all the above it can be concluded, that larger autosomes can malsegregate during meiosis, but this phenomenon is not as common as the one involving the non-disjunction of the smaller chromosomes.

Theoretically, the application of CGH for the extended investigation of unfertilised meiosis II oocytes and their corresponding first PBs gives a more comprehensive rate of aneuploidy, as both the presence and the absence of chromosomes can be scored. The aneuploidy rate derived from the data obtained during this part of the study was estimated to be approximately 27%. This is relatively high, but results from a small sample size such as this, are easily skewed by the inclusion of unusual data from individual samples. For example, if patient 4412 were excluded from the group, on the basis that the abnormalities scored in her eggs were attributable to the PCOS, then the aneuploidy rate becomes 15.4%. The latter is still higher compared to the 9.8% hyperhaploidy rate that was the result of the FISH analysis of oocytes and PBs. However, this is expected as the application of FISH allowed the examination of 10 chromosomes only and was also hampered by the inability to score for missing chromosomes due to the artifactual chromosome loss. Voullaire and colleagues (2002) in their CGH investigation of

preimplantation embryos found that the incidence of meiotic aneuploidy in that group was about 10%. A similar rate was calculated in the R-banding study of Pellestor *et al.* (2002), whereas in the FISH study of Cupisti *et al.* (2003) the hyperhaploidy rate was estimated to be 4%. Doubling to include hypothetical corresponding hypohaploidy would give a rate of 8% not significantly different from the 10% experienced by others. Considering the young average age of the patients participating in this study, our data on the aneuploidy rate do not seem exceedingly high.

In summary, the CGH part of this project should be viewed as a pilot study. Much has been learned of the difficulties involved when this technique is employed for the analysis of minute amounts of DNA. The main problem that needs to be overcome so as for a method such as CGH to find a possible wider clinical application for the analysis of cells such as PBs is the current inability to distinguish between chromosome and chromatid abnormalities and also the difficulty in consistently identifying reciprocal gains and losses in oocyte and PB pairs. An alternative approach would be to combine FISH and CGH. Hence, and since PBs tend to be more fragile and degenerate rapidly, they could be analysed with CGH, whilst the corresponding oocytes could be placed on slides and analysed via FISH. In this way it should be possible to target specifically abnormalities detected with the CGH and examine whether they were genuine. A similar study was carried out recently by Gutierrez-Mateo and colleagues (2004) who also used CGH for the analysis of oocytes and their corresponding 1st PBs. Thirty unfertilised oocytes and their PBs, along with seven single 1st PBs were examined in this way. The aneuploidy rate was of the range of 48%, which is much higher compared to the one estimated from the results of this study. The latter could be attributed to the fact that all the examined cells in the Guiterrez-Mateo study were at the MI stage at the time of retrieval and were left to mature *in vitro*, whereas the majority of the oocytes investigated in the study described in this thesis were at the MII stage when collected. Even so, the results obtained in this project clearly demonstrate the potential of CGH to detect a wider range of anomalies, compared to similar FISH studies.

6.6 Conclusions

Karyotyping and FISH studies of preimplantation embryos and unfertilised meiosis II oocytes have demonstrated that errors taking place during the first meiotic division in females are the main cause of constitutional numerical chromosome abnormalities. This has been backed up by earlier molecular studies investigating various chromosome polymorphisms of fetal material from spontaneous abortions (Hassold *et al.,* 1984). The main mechanisms proposed are two: the first involves the malsegregation of whole univalent chromosomes (Griffin, 1996), and the second the predivision of sister chromatids prior to meiotic anaphase I (Angell *et al.,* 1991; 1994). Smaller autosome aneuploidies have been particularly associated with advancing maternal age, while absent or aberrant genetic recombination has previously been identified as a generally predisposing factor (Dailey *et al.* 1996). The research objective of the second part of this project was the extensive investigation into the mechanisms leading to female aneuploidy of specific chromosomes in IVF patients, the majority of whom were below the age of 35 years. This was attempted by employing two different methods, FISH and CGH for the analysis of meiosis II oocytes and their corresponding PBs when these were available.

Both methods were hampered by several technical difficulties, which led to the collection of data for only a small fraction of cells for which analysis was attempted. FISH as a technique was much simpler and results were obtained within 24 hours. However, out of the 265 eggs that was the starting number for this part of the study, results on almost all 10 chromosomes investigated were obtained from only 7 oocytes and 2 PBs. Partial results were obtained from 51 oocytes, while the remaining cells were all lost during the sequential FISH rounds. In addition, both the spreading methods used clearly led to the loss of individual chromosomes. Hence, during the FISH part of the study, only abnormalities due to extra copies of chromosomes and/or single chromatids were scored.

CGH is a DNA-based method, and its advantage over FISH is that it enables the screening of all 23 chromosomes, providing in this way information on chromosomes that are not targeted by FISH, and also giving an idea about the hypohaploidy rate, as the

cells are placed intact in tubes. In practice, however, the development of a CGH protocol that would yield reliable results when applied on cells such as oocytes and PBs took longer than expected, the actual technique was very labor intensive, and required knowledge of both molecular and cytogenetic methods. In addition, the software used for the interpretation of the captured images was sometimes (depending on the quality of the hybridisation and the analysis of the captured images) not sensitive enough to pick up absence of chromosomal material, especially in cases of single chromatids. This in combination with the suboptimal quality of the examined cells were most likely the reasons why reciprocal losses and gains of DNA were detected for only one oocyte-PB pair, out of the seventeen investigated.

One of the main problems with CGH, especially in its potential to be clinically applied, is the required hybridisation time. Wilton and colleagues (2001) were the first group to apply CGH for the clinical diagnosis of aneuploidy, and the protocol they used had a hybridisation time of 72 hours, which was longer than the timeframe of an embryo transfer. Hence all embryos were cryopreserved. This cryopreservation could be very damaging to the biopsied embryos. This was observed in a subsequent CGH study by the same group (Wilton *et al.,* 2003), in which only 54% of the embryos survived with half of their cells undamaged (Munne and Wells, 2003). The hybridisation time used in this study was also for 72 hours, but a similar CGH protocol has been shown to yield results after 25-30 hours (Wells *et al.,* 2002). The latter was not investigated here due to lack of time.

Even though the sizes of the final sample for both the FISH and the CGH parts of this study were smaller than expected, some conclusions could be drawn from the obtained results. Hence, combination of both the FISH and CGH observations made from the analysis of unfertilised meiosis II oocytes and their corresponding PBs revealed several mechanisms leading to maternal aneuploidy. In particular, the classical model of non-disjunction of whole chromosomes leading to them being present as extra copies, or absent from the oocytes was observed and was the most common in both studies. Moreover, the precocious separation of chromatids prior to anaphase I with their subsequent random segregation was also evident from the FISH results. Evidence for the

presence of gonadal or germinal mosaicism for a trisomic cell line was seen in three different patients out of a total of 49 assessed, two participating in the FISH study, and possibly one in the CGH investigation. The latter could mean that this phenomenon tends to be more frequent than expected, at least in this infertile population. The preferential involvement of the smaller autosomes was also observed, especially from data obtained by FISH analysis. However, involvement of larger autosomes was also evident from the CGH results. Several cells, either oocytes or PBs were also identified to be carrying multiple abnormalities.

This was the first study during which the combination of FISH and CGH was applied for the extensive investigation of different groups of female gametes. Its significance lies firstly to the fact that it enabled the identification of the involvement of chromosomes ranging in size in oocyte aneuploidy. Added to this, it also allowed the comprehensive analysis of six cells (oocytes and PBs) coming from a very young patient with PCOS, and the identification of three highly abnormal ones, versus three cells with a normal chromosome complement. The most important finding was that the average age of the patients with abnormalities was much lower than 35. This implies that some of the mechanisms of aneuploidy described above could be acting in an age-independent manner in this group of younger IVF patients and may be influenced by their infertile aetiology. Further CGH examination of oocytes and their corresponding 1st PBs is however required to confirm the observations made in this work. Such information could be crucial in the counseling of infertile couples, and the treatment options that should be offered to them in order for the successful beginning of a clinical pregnancy to be achieved. Infertile patients found to be at high risk of producing aneuploid gametes may benefit from Preimplantation Genetic Screening (PGS) for chromosome abnormalities. Such methods have been reported to improve implantation rates and lower the incidence of spontaneous abortions and trisomic pregnancies in certain subgroups of IVF patients (advanced maternal age, 3 or more failed IVF attempts, repeated spontaneous pregnancy loss) (Gianaroli *et al.*, 1997a,b; Munne *et al.* 2002a).

6.7 Future work

During the final part of this study, CGH was evaluated and modified in order to enable the investigation of chromosomes in oocytes and their corresponding first PBs. This protocol has been shown to work reliably on the DNA from these cells, but also on blastomere DNA from preimplantation embryos. The next step is to evaluate the modified CGH protocol and its potential in clinical application for PGD. Selection of embryos after CGH analysis has been shown to lead to improved IVF success rates, as well as a reduction in the incidence of spontaneous abortions and aneuploid syndromes in high risk patients (Wilton *et al.,* 2003). The preferred approach would be to carry out CGH on the first PB, which would be removed prior to fertilisation. In this way, cryopreservation of embryos should be avoided.

Prior to its clinical application, this approach will be validated by the separation of the oocyte and its corresponding PB. PBs will be placed in tubes and will be analysed by CGH, whereas the corresponding oocytes will be spread on slides and will be analysed by FISH. In these cases, probes for chromosomes investigated during the current study will be combined with others shown by the CGH analysis of the PBs to be abnormal. In this way, data will be collected both about the significance of the effects of the different mechanisms of aneuploidy established in this study, the accuracy of CGH versus FISH, and the frequency of aneuploidy for each of the examined chromosomes. It is hoped that the use of oocytes matured *in vitro* (IVM) will improve the success rate of both types of analysis, as it will be possible to obtain oocytes and 1st PBs without them running the risk of degenerating due to extended time in culture, and avoiding in this way the adverse effects on chromosome morphology and DNA quality. The downside of using IVM oocytes, is that they may not be representative of mature eggs, as far as the frequency of chromosome abnormalities is concerned.

Modifications of the current CGH protocol, involving both the WGA method and the hybridisation time will be attempted in order to further improve its accuracy. An

alternative WGA approach to the DOP-PCR procedure is a method termed multiple displacement amplification (MDA). This method uses a φ29 DNA polymerase and random exonuclease-resistant primers, whilst DNA amplification takes place at 30°C (Dean *et al.*, 2002). It has been shown that MDA provides a highly uniform representation of the genome, with the amplification bias being less than 3-fold among eight chromosomal loci, compared to the 4-6-fold observed with the application of the DOP-PCR (Dean *et al.*, 2002). As far as the reduction time in hybridisation is concerned, the current protocol will be evaluated to examine whether it could yield analysable results after 25-30 hours, as was shown by Wells and colleagues (2002). The use of DNA microarrays to act as hybridisation templates, instead of the target metaphase chromosomes that have been used so far could further reduce the period of hybridisation, and improve the analysis of the obtained results, increasing the simplicity of interpretation by avoiding the need to karyotype metaphase chromosomes.

Once the above validation is complete and the CGH protocol is considered reliable and robust, it will be possible for it to be applied clinically, in cases of patients that have a previous history of trisomic conceptions, repeated miscarriages or repeated IVF failure. The clinical application will involve the analysis of first PBs with CGH, followed initially by selective FISH analysis of blastomeres from embryos derived from the corresponding oocytes. The latter procedure is already being carried out at the UCL Centre for PGD. Theoretically, the combination of the two molecular cytogenetic methods will enable the selection of embryos that have a normal chromosome complement, increasing the probability of a clinical pregnancy and the birth of a healthy baby. A similar approach is currently being carried out by Verlinsky and colleagues at the Reproductive Genetics Institute, in Chicago USA. Preliminary results have not shown a decline in embryo viability, despite the additional manipulation (Y.Verlinsky, ISPD Conference, 2004). Further data on the effect of a double biopsy (PB plus blastomere) on embryonic survival were provided in a recent study carried out by Magli and colleagues (2004). They evaluated implantation rates among three groups of embryos (1st- PB biopsy only, 2nd - PB and blastomere biopsy, 3rd – blastomere biopsy only) generated from patients that were undergoing PGS. Chromosomes X, Y, 13, 15, 16, 18, 21, and 22 were

examined by two sequential FISH rounds. The authors of this report concluded that the implantation rates did not vary significantly among the three embryo groups, being 15% for the first, 26% for the second, and 25% for the third, demonstrating in this way that a combined PB-blastomere biopsy does not seem to adversely affect embryo viability (Magli *et al.* 2004).

Chapter 7

Bibliography

7.1 Publications Arising from Thesis

7.1.1 Articles

1. Cupisti S., Conn C.M., Fragouli E., Whalley K., Mills J.A., Faed M.J.W., Delhanty J.D.A. (2003). Sequential FISH analysis of oocytes and polar bodies reveals aneuploidy mechanisms. Prenat. Diagn. 23: 663-668

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7.1.2 Abstracts

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Appendices

A: Appendix to Materials and Methods

1A Cell Culture Media

Media for bacteria and yeast culture were prepared with de-ionised double-distilled water, they were then autoclaved at 15lbs psi 121°C for 30 minutes and stored at room temperature (15-25°C), under sterile conditions.

1A.1 Iscove's /10% FCS/ GPS/ PHA- for peripheral lymphocyte culture.

Sterile Iscoves modified Dulbeccos medium; 10% v/v heat-inactivated fetal calf serum; 10µl/ml phytohaemagglutinin (PHA); 20µl/ml GPS. (GPS: 0.2M L-Glutamine, 300mg/ml Penicillin, 500mg/ml Streptomycin monosulphate stored at -20°C).

1A.2 Roswell Park Memorial Institute (RPMI) 1640 medium/ 15% FCS/ GPS/ $NaHCO_3$/PHA- for peripheral lymphocyte culture.

Sterile RPMI medium; 15% v/v heat inactivated fetal calf serum; 30µl/ml $NaHCO_3$; 20µl/ml phytohaemagglutinin (PHA); 10µl/ml GPS. (GPS: 0.2M L-Glutamine, 300mg/ml Penicillin, 500mg/ml Streptomycin monosulphate stored at -20°C).

1A.3 Versene solution- for fibroblast FISH preparation, DNA extraction and single cell isolation.

0.02% (w/v) EDTA in 1lt of Hank's medium

1A.4 SD medium- for yeast culture.

7g/l Bacto yeast nitrogen base (without amino acids); 20g/l glucose; 55mg/l adenine and tyrosine. After autoclaving, 1% filter-sterilised casamino acid solution added.

1A.5 2xTY medium- for bacterial culture

16g/l Bacto tryptone; 10g/l Bacto yeast extract; 5g/l NaCl; 1g/l glucose

1A.6 Medium for agar plates/stabs

1% w/v Bacto-agar added to the culture medium (SD or 2xTY) and autoclaved. For agar plates/stabs this was heated until liquefaction, cooled before addition of required antibiotic supplement and poured into sterile petri dishes or bijou's straight away. The latter were left to dry under aseptic conditions.

1A.7 Glycerol stocks

Three types were prepared, each containing different amounts of glycerol and yeast culture.

1. 250µl of yeast overnight culture; 650µl 100% sterile glycerol
2. 500µl of yeast overnight culture; 250µl 100% sterile glycerol
3. 850µl of yeast overnight culture; 150µl 100% sterile glycerol

All were prepared in 1ml cryo-tubes. They were stored in the -20°C and then in the -80°C.

2A Solutions and buffers

De-ionised double distilled was used for the preparation of all the solutions and buffers. They were all stored at room temperature, unless stated otherwise.

2A.1 General solutions

2A.1.1 PBS: 10mM phosphate buffer; 2.7mM KCl; 137mM NaCl; pH 7.4

2A.1.2 20xSSC: 0.15M NaCl; 15mM Sodium citrate; pH 7

2A.1.3 10x TBE: 90mM Tris-HCl pH 8; 90mM Boric acid; 2mM EDTA

2A.1.4 TE: 10mM Tris-HCl, pH 8; 0.1mM EDTA

2A.1.5 10xTNE: 1M Tris; 1.5M NaCl; 0.1mM EDTA

2A.1.6 Fixation solution (fix): 3:1 Dried Methanol: Acetic Acid

2A.1.7 70% Acetic acid: 7ml Acetic acid, 3ml double distilled H_2O

2A.2 Plasmid/Cosmid DNA maxiprep solutions

2A.2.1 Cell Resuspension Solution: 0.5M Glucose; 0.25M Tris-HCl, pH 8; 0.1M EDTA

2A.2.2 Cell Lysis Solution: 0.2M NaOH; 1% SDS.

2A.2.3 Neutralisation Solution: 5M potassium acetate; 40% v/v glacial acetic acid

2A.3 *Alu*PCR reagents (Stored at -20°C)

2A.3.1 10xPCR buffer (HT biotechnology Ltd): 0.1M Tris-HCl, pH 9; 0.5M KCl: 15mM $MgCl_2$; 1% Triton X-100; 0.1% w/v gelatin.

2A.3.2 dNTP mix: 2mM each dATP, dCTP, dGTP, dTTP

2A.3.3 *Alu* oligonucleotide primers:

ALU 1F: GGA TTA CAG GCG TGA GCC A

ALU 1R: GCC ACT GCA CTC CAG CCT G (Pharmacia) (Liu *et al.*, 1993)

2A.4 DOP PCR reagents (Stored at -20°C)

2A.4.1 10xPCR buffer (HT biotechnology Ltd): 0.1M Tris-HCl, pH 9; 0.5M KCl: 15mM $MgCl_2$; 1% Triton X-100; 0.1% w/v gelatin.

2A.4.2 dNTP mix:

2A.4.3 DOP oligonucleotide primer: CCGACTCGAGNNNNNNATGTGG (Oswel) (Telenius *et al.*, 1992)

2A.5 Nick translation reagents (Stored at -20°C)

2A.5.1 dNTP mix: 0.2mM each dATP, dCTP, dGTP; 0.1mM dTTP; 0.1mM label-dUTP.

2A.5.2 10x nick translation buffer: 0.5M Tris-HCl, pH 7.5; 0.1M $MgSO_4$; 1M DTT.

2A.5.3 Nick translation enzyme: DNA polymerase I; DNase 1; 50% glycerol; 50mM Tris-HCl, pH 7.2; 0.1mM DTT; 0.5 mg/ml nuclease-free BSA

2A.6 FISH solutions

2A.6.1 Pepsin buffer: 0.01N HCl; 0.1mg/ml pepsin.

2A.6.2 Paraformaldehyde buffer: 1xPBS; 1% paraformaldehyde.

2A.6.3 LSI buffer for locus specific probes (Vysis):

2A.6.4 CEP buffer for repetitive sequence probes (Vysis):

2A.6.5 Hybridisation buffer for locus specific probes (lab-prepared)/COSMIX buffer: 50% deionised formamide; 10%w/v dextran sulphate; 2xSSC pH 8. Stored at -20°C

2A.6.6 Hybridisation buffer for repetitive sequence probed (lab-prepared): 60% deionised formamide; 10%w/v dextran sulphate; 2xSSC pH 8. Stored at -20°C

2A.6.7 SSCT: 4xSSC; 0.05% Tween 20

2A.6.8 Antifade medium: 1.25μg/ml 4'6-diamidino-2-phenylindole (DAPI) in Vectarshield mounting medium (Vector), Stored at 4°C, protected from light.

2A.7 CGH solutions

2A.7.1 Pronase for oocyte and PB separation: 100mg Pronase (Sigma), mixed with 33ml PBS without Ca^{2+}, Mg^{2+}. Aliquots of 1ml prepared and stored at –20°C

2A.7.2 Hybridisation buffer for CGH probes (lab-prepared)/COSMIX buffer: 50% deionised formamide; 10%w/v dextran sulphate; 2xSSC pH 8. Stored at -20°C

2A.7.3 70% Formamide: 10ml double-distilled de-ionised H_2O, 5ml 20x SSC, 35ml formamide

2A.7.4 SSC/Triton: 50ml 4x SSC, 50µl Triton (Sigma)

2A.8 Spreading solutions

2A.8.1 1% Tween 20: 1ml Tween 20 in 99ml of double distilled deionised H_2O

2A.8.2 1N HCl: 1ml of concentrated HCl in 11ml of double distilled deionised H_2O

2A.8.3 Spreading solution for blastomeres and embryos: 1ml of 1% Tween, 0.1ml 1N HCl, 8.9ml of double distilled deionised H_2O.

2A.8.4 Fixative I for oocyte spreading: 5:1:4 Methanol: Acetic acid: H_2O

2A.8.5 Fixative II for oocyte spreading: as 2A.7.6

2A.8.6 Fixative solution I for oocyte spreading: Methanol- 10ml, Acetic acid- 2ml, H_2O- 8ml

2A.8.7 Fixative III for oocyte spreading: Methanol- 15ml, Acetic acid- 15ml, H_2O- 5ml

2A.9 Solutions used for DNA extraction from blood

2A.9.1 TKM1 (low salt buffer): 10mM Tris-HCl pH 7.6, 10mM KCl, 10mM $MgCl_2$, 2mM EDTA

2A.9.2 TKM2 (high salt buffer): 10mM Tris-HCl pH 7.6, 10mM KCl, 10mM $MgCl_2$, 0.4 M NaCl, 2mM EDTA

2A.10 Lysis buffer for fibroblast DNA extraction

1.21gr Tris, 0.19gr EDTA, 0.2gr SDS, 1.17gr NaCl in 100ml of double-distilled de-ionised H_2O. Solution autoclaved. 10mg/ 100ml of Proteinase K added after autoclaving.

B: Appendix to Chapter 3. Cytogenetic analysis of embryonic blastomeres using the ISCN Nomenclature

Table 3.1 (ISCN): Case A, 46,XY,t(5;19)(p12;p12); FISH analysis of embryos from one PGD cycle using the dual LSI EGR1 locus-specific probe for chromosome 5 (D5S721 5q31-SO, D5S23 5p15.2-SG) and one locus-specific probe for chromosome 19 (YAC19, 19q13.2-orange) for the biopsied cells. Non-transferred embryos were FISHed with the LSI EGR1 for chromosome 5 only.

Embryo	FISH Analysis Result [No. of Blastomeres]
1 Biopsied Cell Remainder	nuc ish 5q31(D5S72x1), 19q13.2 (YAC19 x1) [1] nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x3) [1] nuc ish 5q31 (D5S721x2), 5p15.2 (D5S231x2) [1] nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x2) [1] nuc ish 5q31 (D5S721x1), 5p15.2 (D5S231x 3) [1]
2 Biopsied Cell Remainder	nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x2), 19q13.2 (YAC19 x1) [2] nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x2) [1] nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x3) [1] nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x1) [1] nuc ish 5q31 (D5S721x3), 5p15.2 (D5S23x2) [1] nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x2) [1]
5 Remainder	nuc ish 5q31 (D5S721 x2), 5p15.2 (D5S23x4) [3] nuc ish 5q31 (D5S721x4), 5p15.2 (D5S23x4) [1] nuc ish 5q31 (D5S721 x3), 5p15.2 (D5S23x4) [1] nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x3) [1]
7 Biopsied Cell Remainder	nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x2), 19q13.2 (YAC19x2) [2] Transferred
8 Biopsied Cell Remainder	nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x3) [1] nuc ish 5p15.2 (D5S23x3) [1] 1 nucleus with no signals nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x3) [2] nuc ish 5q31 (D5S721x3), 5p15.2 (D5S23x3) [2]
9 Biopsied Cell Remainder	nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x1), 19q13.2 (YAC19x1) [1] Lost
10 Biopsied Cell	nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x2), 19q13.2 (YAC19x1) [1]

10 Remainder	nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x2), 19q13.2 (YAC19x1) [1] nuc ish 5q31 (D5S721x3), 5p15.2 (D5S23x2) [2] **nuc ish 5q31 (D5S721x3), 5p15.2 (D5S23x4) [1]** nuc ish 5q31 (D5S721x3), 5p15.2 (D5S23x3) [1]
11 **Biopsied Cell** Remainder	**Lost** nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x2) [4] nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x1) [2]
13 Biopsied Cell **Remainder**	nuc ish 5q31 (D5S721x4), 5p15.2 (D5S23x1) [2] **nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x1) [1]** **nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x2) [1]** nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x1) [1]
14 Biopsied Cell Remainder	nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x2) [1] nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x1)[1] nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x2) [4] nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x1) [1] nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x1) [1]
15 Biopsied Cell Remainder	Lost nuc ish 5q31 (D5S721x1), 5p15.2 (D5S23x3) [4]
17 **Biopsied Cell** Remainder	**Lost** nuc ish 5q31 (D5S721x3), 5p15.2 (D5S231x1) [1] nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x1) [5]
18 Biopsied Cell Remainder	nuc ish 5q31 (D5S721x3), 5p15.2 (D5S23x3) [1] nuc ish 5q31 (D5S721x2), 5p15.2 (D5S23x3) [1] nuc ish 5q31 (D5S721x3), 5p15.2 (D5S23x2) [1] nuc ish 5q31 (D5S721x3), 5p15.2 (D5S23x3) [2]

Table 3.2 (ISCN): Case B, 46XX,t(11;22)(q23.3;q11.2); FISH analysis of embryos from two PGD cycles using a centromeric probe for chromosome 11 (CEP11, D11Z1 orange),and the dual LSI VCFS locus-specific probe for chromosome 22 (D22S75 22q11-SO, ARSA 22q13-SG). Non-transferred embryos from the 1st PGD cycle were re-probed with the centromere probes for chromosomes 15 (D15Z3, SO), 16 (D16Z2 SG), and 18 (D18Z1 SA). Non-transferred embryos from the 2nd PGD cycle were re-probed with the centromere probes for chromosomes 18 (as 1st cycle), X (DXZ1 SG), and Y (DYZ3 SO).

Embryo	FISH Analysis Result [No. of Blastomeres]
1/1st cycle	
Biopsied	Not biopsied
Cell	nuc ish 22q11 (D22S75x2), 22q13 (ARSAx2), 15q11.2 (D15Z3x2), 16q11.1 (D16Z2x2) [1]
Remainder	nuc ish 22q11 (D22S75x2), 22q13 (ARSAx2), 15q11.2 (D15Z3x2), 18cen (D18Z1x2) [1]
2/1st cycle	
Biopsied	Not biopsied
Cell	nuc ish 22q13 (ARSAx1) [1]
Remainder	nuc ish 11cen (D11Z1x1) [1]
10/1st cycle	
Biopsied	nuc ish 11cen (D11Z1x1), 22q11 (D22S75x2), 22q13 (ARSAx2) [1]
Cell	nuc ish 11cen (D11Z1x1), 22q11 (D22S75x4), 22q13 (ARSAx3),
Remainder	**16q11.1 (D16Z2x1) [1]**
	nuc ish 11cen (D11Z1x1), 22q11 (D22S75x2), 22q13 (ARSAx3), 16q11.1 (D16Z2x1) [1]
11/1st cycle	
Biopsied	nuc ish 22q11 (D22S75x4), 22q13 (ARSAx1) [1]
Cell	nuc ish 22q11 (D22S75x2) [1]
	nuc ish 22q11 (D22S75x2), 22q13 (ARSAx1) [1]
	nuc ish 22q11 (D22S75x3), 22q13 (ARSAx1), 16q11.1 (D16Z2x2),
Remainder	18cen (D18Z1x1) [1]
	nuc ish 22q11 (D22S75x3), 22q13 (ARSAx1), 15q11.2 (D15Z3x2),16q11.1 (D16Z2x2), 18cen (D18Z1x2) [1]
	nuc ish 22q11 (D22S75x2), 22q13 (ARSAx1), 15q11.2 (D15Z3x1),16q11.1 (D16Z2x1) [1]
	nuc ish 11cen (D11Z1x1), 22q13 (ARSAx1), 15q11.2 (D15Z3x1),16q11.1 (D16Z2x1), 18cen (D18Z1x2) [1]
	nuc ish 11cen (D11Z1x2), 22q11 (D22S75x2), 15q11.2 (D15Z3x1) [1]
12/1st cycle	
Biopsied	nuc ish 22q11 (D22S75x3/4) [1]
Cell	nuc ish 22q11 (D22S75x1), 22q13 (ARSAx5)
	nuc ish 22q11 (D22S75x2), 22q13 (ARSAx2), 15q11.2
Remainder	(D15Z3x2),16q11.1 (D16Z2x3), 18cen (D18Z1x2) [1]
	nuc ish 22q11 (D22S75x2), 22q13 (ARSAx2), 15q11.2 (D15Z3x2),16q11.1 (D16Z2x1), 18cen (D18Z1x2) [1]

1/2nd cycle Biopsied Cell Remainder	No result nuc ish 11cen (D11Z1x1), 22q11 (D22S75x2), 22q13 (ARSAx2), 18cen (D18Z1x2), Xcen (DXZ1x3)[1] nuc ish 11cen (D11Z1x1), 22q11 (D22S75x2), 22q13 (ARSAx2), 18cen (D18Z1x2), Xcen (DXZ1x3), Ycen (DYZ3x2)[1] nuc ish 11cen (D11Z1x1), 22q11 (D22S75x3), 22q13 (ARSAx3), 18cen (D18Z1x2), Xcen (DXZ1x1), Ycen (DYZ3x2) [1]
2/2nd cycle Biopsied Cell Remainder	nuc ish 11cen (D11Z1x2), 22q11 (D22S75x1), 22q13 (ARSAx1) [1] nuc ish 11cen (D11Z1x2), 22q11 (D22S75x3), 22q13 (ARSAx2) [1] nuc ish 11cen (D11Z1x2), 22q11 (D22S75x2), 22q13 (ARSAx2) [1]
3/2nd cycle Biopsied Cell Remainder	nuc ish 11cen (D11Z1x2), 22q11 (D22S75x2), 22q13 (ARSAx2) [1] Transferred
5/2nd cycle Biopsied Cell Remainder	nuc ish 22q11 (D22S75x1), 22q13 (ARSAx3) [1] nuc ish 11cen (D11Z1x2), 22q11 (D22S75x3), 22q13 (ARSAx2) [1] nuc ish 11cen (D11Z1x1), 22q11 (D22S75x1), 22q13 (ARSAx1) [1]
6/2nd cycle Biopsied Cell Remainder	nuc ish 11cen (D11Z1x2), 22q11 (D22S75x2), 22q13 (ARSAx2) [2] Transferred
7/2nd cycle Biopsied Cell Remainder	nuc ish 11cen (D11Z1x2), 22q11 (D22S75x2)[1] nuc ish 11cen (D11Z1x1), 22q11 (D22S75x2), 22q13 (ARSAx2) [1] nuc ish 11cen (D11Z1x2), 22q11 (D22S75x2), 22q13 (ARSAx2) [1]
8/2nd cycle Biopsied Cell Remainder	nuc ish 22q11 (D22S75x4), 22q13 (ARSAx3) [2] Lost
9/2nd cycle Biopsied Cell Remainder	No result nuc ish 22q11 (D22S75x1), 18cen (D18Z1x1), Xcen (DXZ1x1), Ycen (DYZ3x1) [1] nuc ish 22q11 (D22S75x2), 22q13 (ARSAx1), Xcen (DXZ1x1), Ycen (DYZ3x1) [1] nuc ish 22q11 (D22S75x3), 22q13 (ARSAx1), 18cen (D18Z1x1), Xcen (DXZ1x1), Ycen (DYZ3x1) [1] nuc ish 11cen (D11Z1x3), 22q11 (D22S75x3), 22q13 (ARSAx1), 18cen (D18Z1x3), Xcen (DXZ1x2), Ycen (DYZ3x3) [1]

Table 3.3 (ISCN): Case D, 45XY,t(13;21)(q10;q10); FISH analysis of embryos from two PGD cycles using the locus-specific probe LSI13 for chromosome 13 (RB1 SG), the locus-specific probe for chromosome 21(D21S529 SO), and the subtelomere probe for chromosome 21 (TEL21 orange). Non-transferred embryos from the 1st PGD cycle were re-probed with the centromere probes for chromosomes 18 (D18Z1), X (DXZ1 SG), and Y (DYZ3 SO).

Embryo	FISH Analysis Result [No. of Blastomeres]
2/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), 21qter (TEL21qx2) [1] Lost
3/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 21qter (TEL21qx2) [1] Transferred
4/1st cycle Biopsied Cell Remainder	nuc ish 21q13.2 (D21S529x3) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x3), 18cen (D18Z1x2), Xcen (DXZ1x1), Ycen (DYZ3x1) [1] nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x1) [1] **nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x3) [1]** nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x1), Ycen (DYZ3x1) [1]
5/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 21qter (TEL21qx2) [2] Transferred
7/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21qter (TEL21qx2) [1] Transferred
9/1st cycle Biopsied Cell Remainder	Lost Inconclusive
1/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 21qter (TEL21qx2) [2] Transferred
2/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), 21qter (TEL21qx1) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1) [1] nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x1) [1]
3/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1), 21qter (TEL21qx1) [1] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), 21qter (TEL21qx2) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 21qter (TEL21qx1) [1] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2) [1]

	nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x1) [1]
4/2nd cycle Biopsied Cell Remainder 4/2nd cycle Remainder	nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x1), 21qter (TEL21qx1) [1] nuc ish 13q14 (RB1x1) [2] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1) [1] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x1), 21qter (TEL21qx1) [1]
5/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 21qter (TEL21qx2) [1] Transferred
6/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 21qter (TEL21qx2) [1] Transferred
7/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x2), 21qter (TEL21qx1) [1] nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x2) [2] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), 21qter (TEL21qx1) [1] nuc ish 13q14 (RB1x6), 21q13.2 (D21S529x2) [1] nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x2), 21qter (TEL21qx2) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1), 21qter (TEL21qx3) [1]
8/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x3), 21qter (TEL21qx2) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1), 21qter (TEL21qx2) [1] nuc ish 13q14 (RB1x5), 21q13.2 (D21S529x3), 21qter (TEL21qx1) [2] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), 21qter (TEL21qx2) [1] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x3) [2]
9/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x3) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x3), 21qter (TEL21qx1) [1] nuc ish 21q13.2 (D21S529x1), 21qter (TEL21qx1) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] nuc ish 13q14 (RB1x2), 21qter (TEL21qx2) [1]

Table 3.4(ISCN): Case E, 45XY,t(13;14)(q10;q10); FISH analysis of embryos from two PGD cycles using the locus-specific probe LSI13 for chromosome 13 (RB1 SG), and the TelVysion 14q subtelomere probe for chromosome 14 (D14S308 SO). Non-transferred embryos from the 1st PGD cycle were re-probed with centromere probes for chromosomes 4 (D4Z1 SG), 15 (D15Z3 SO), and 18 (D18Z1 SA). Non-transferred embryos from the 2nd PGD cycle were re-probed with the centromere probes for chromosomes 18 (D18Z1 SA), X (DXZ1 SG), and Y (DYZ3 SO).

Embryo	FISH Analysis Result [No. of Blastomeres]
1/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 14qter (D14S308x1) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [3] nuc ish 13q14 (RB1x2) [4] **nuc ish 13q14 (RB1x2), 4cen (D4Z1x2) [1]** nuc ish 13q14 (RB1x3), 14qter (D14S308x2), 15q11.2 (D15Z3x1), 18cen (D18Z1x2) [1]
2/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [1] Transferred
3/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [2] Transferred
4/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [1] **nuc ish 13q14 (RB1x2), 14qter (D14S308x2), 4cen (D4Z1x2), 15q11.2** (D15Z3x2), 18cen (D18Z1x2) [3] nuc ish 13q14 (RB1x1), 14qter (D14S308x1), 4cen (D4Z1x1), 18cen (D18Z1x1) [1] nuc ish 13q14 (RB1x1), 14qter (D14S308x1), 4cen (D4Z1x1), 15q11.2 (D15Z3x2), 18cen (D18Z1x2) [1]
5/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [1] Transferred
6/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x2), 4cen (D4Z1x2), 15q11.2 (D15Z3x2), 18cen (D18Z1x2) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x1), 4cen (D4Z1x2), 15q11.2 (D15Z3x1), 18cen (D18Z1x3) [1]
7/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x1) [2] nuc ish 13q14 (RB1x1), 14qter (D14S308x1), 4cen (D4Z1x2), 15q11.2 **(D15Z3x2), 18cen (D18Z1x2) [2]** **nuc ish 13q14 (RB1x1), 14qter (D14S308x1) [1]**

7/1st cycle Remainder	nuc ish 13q14 (RB1x3), 14qter (D14S308x1) [1] nuc ish 13q14 (RB1x3), 14qter (D14S308x1), 4cen (D4Z1x1), 15q11.2 (D15Z3x1) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x2), 4cen (D4Z1x2), 15q11.2 (D15Z3x2), 18cen (D18Z1x2) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x1), 4cen (D4Z1x3), 15q11.2(D15Z3x3), 18cen (D18Z1x3) [1]
8/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x3), 14qter (D14S308x3) [2] nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [2] nuc ish 13q14 (RB1x2), 14qter (D14S308x2), 15q11.2 (D15Z3x1) [1] nuc ish 13q14 (RB1x1), 14qter (D14S308x1), 15q11.2 (D15Z3x1) [1] nuc ish 13q14 (RB1x1), 14qter (D14S308x1), 4cen (D4Z1x1), 15q11.2 (D15Z3x1) [1] nuc ish 4cen (D4Z1x2), 15q11.2 (D15Z3x1), 18cen (D18Z1x1) [2] nuc ish 13q14 (RB1x3), 14qter (D14S308x1), 4cen (D4Z1x3), 15q11.2 (D15Z3x2), 18cen (D18Z1x2) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x1), 4cen (D4Z1x1), 15q11.2 (D15Z3x1), 18cen (D18Z1x1) [1] nuc ish 13q14 (RB1x2), 4cen (D4Z1x2), 15q11.2 (D15Z3x1), 18cen (D18Z1x2) [1] nuc ish 13q14 (RB1x3), 14qter (D14S308x2), 4cen (D4Z1x2), 15q11.2 (D15Z3x2), 18cen (D18Z1x2) [1]
1/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x1), 14qter (D14S308x1) [1] nuc ish 13q14 (RB1x3), 14qter (D14S308x4), 18cen (D18Z1x2), Xcen (DXZ1x2), Ycen (DYZ3x2) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x2), 18cen (D18Z1x3), Xcen (DXZ1x3), Ycen (DYZ3x2) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x4), 18cen (D18Z1x1), Xcen (DXZ1x3), Ycen (DYZ3x2) [1]
2/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x1), 14qter (D14S308x2) [1] nuc ish 13q14 (RB1x1), 14qter (D14S308x2) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x1) [2] nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [1] nuc ish 13q14 (RB1x3), 14qter (D14S308x1) [1]
3/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [1] Transferred
5/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 14qter (D14S308x1) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x3) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [3] nuc ish 13q14 (RB1x2), 14qter (D14S308x1) [1] nuc ish 13q14 (RB1x1), 14qter (D14S308x2) [1]

6/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [2] Transferred
7/2nd cycle Biopsied Cell Remainder	Lost nuc ish 13q14 (RB1x1), 14qter (D14S308x1) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x2) [1]
8/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x1), 14qter (D14S308x1) [1] Lost
9/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x1), 14qter (D14S308x3) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x3), 18cen (D18Z1x2), Xcen (DXZ1x2), Ycen (DYZ3x1) [2] nuc ish 13q14 (RB1x1), 14qter (D14S308x3), 18cen (D18Z1x2), Xcen (DXZ1x2), Ycen (DYZ3x1) [1] nuc ish 13q14 (RB1x2), 14qter (D14S308x3), 18cen (D18Z1x2), Xcen (DXZ1x4), Ycen (DYZ3x2) [1]

Table 3.5(ISCN): Case F, possible gonadal mosaicism for trisomy 21; FISH analysis of embryos from two PGD cycles using the locus-specific probe LSI13 for chromosome 13 (RB1 SG), and the locus-specific probe for chromosome 21(D21S529 SO). Non-transferred embryos from the 2nd PGD cycle were re-probed with centromere probes for chromosomes 15 (D15Z1 SO), and 18 (D18Z1 SA) and the locus-specific probe for chromosome 22 (bcr SG).

Embryo	FISH Analysis Result [No. of Blastomeres]
1/1st cycle Biopsied Cell Remainder	Lost nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [5] nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x4) [7] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2) [2]
2/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] Frozen
3/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] Frozen
4/1st cycle Biopsied Cell Remainder	No signals visible Inconclusive

5/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [2] Transferred
6/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] Frozen
7/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [2] Frozen
8/1st cycle Biopsied Cell Remainder	Lost nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x2) [2] nuc ish 21q13.2 (D21S529x2) [2] nuc ish 21q13.2 (D21S529x1) [2] nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x4) [3] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x5) [1]
9/1st cycle Biopsied Cell Remainder	Inconclusive Frozen
10/1st cycle Biopsied Cell Remainder	Inconclusive, background Inconclusive
11/1st cycle Biopsied Cell Remainder	Not biopsied nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x4) [1]
12/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x3) [1] nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x4) [2]
13/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x1) [1] Inconclusive
14/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x3) [1] nuc ish 21q13.2 (D21S529x4) [3]
15/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] Frozen
16/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [2] Transferred
17/1st cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x3) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x4) [1] nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x4) [2] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1]

18/1st cycle Biopsied Cell Micronucleus Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] nuc ish 21q13.2 (D21S529x1) [1] **nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x3) [4]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [13] nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x4) [8] **nuc ish 13q14 (RB1x8), 21q13.2 (D21S529x4) [1]**
3PN/1st cycle Remainder	nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x3) [11] **nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x3) [1]** nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2) [1] **nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x3) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [2]
1/2nd cycle Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx2) [2] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x1), **18cen (D18Z1x1), 22q11.2 (bcrx1) [1]** nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x1), lost after 2nd round, **[1]** **nuc ish 15cen (D15Z1x1) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x1), 18cen (D18Z1x2), 22q11.2 (bcrx2) [1]
2/2nd cycle Biopsied Cell Remainder	Lost nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx2) [6]
3/2nd cycle Biopsied Cell Remainder	**nuc ish 21q13.2 (D21S529x2), covered with cytoplasm [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] **Transferred**
4/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx2) [1] Transferred
5/2nd cycle Biopsied Cell Remainder	**nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x2) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x2), 15cen (D15Z1x1), **18cen (D18Z1x1), 22q11.2 (bcrx1) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), **18cen (D18Z1x2), 22q11.2 (bcrx2) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x3), 22q11.2 (bcrx2) [1] **nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x1),), 15cen (D15Z1x2),** 18cen (D18Z1x3), 22q11.2 (bcrx2) [1]

6/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x3) [1] **nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 22q11.2 (bcrx3) [1]** nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx2) [1] **nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x3), lost after 2nd round [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), **18cen (D18Z1x2), 22q11.2 (bcrx2) [2]** nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), lost after 2nd round [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x1), 22q11.2 (bcrx2) [1] **nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1), lost after 2nd round [1]** **nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x3), 22q11.2 (bcrx2) [1]**
7/2nd cycle **Biopsied Cell** Remainder	**nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2)[1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1) [1] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), 15cen (D15Z1x1), **18cen (D18Z1x1), 22q11.2 (bcrx2) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx3) [1] **nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1), 15cen (D15Z1x2),** 18cen (D18Z1x2), 22q11.2 (bcrx2) [3] **nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), 15cen (D15Z1x2),** 18cen (D18Z1x2), 22q11.2 (bcrx2) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), lost after 2nd round [1]
9/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2) [1] nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x1), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx2) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), **18cen (D18Z1x2), 22q11.2 (bcrx2) [1]** nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x1), 15cen (D15Z1x1), 18cen (D18Z1x1), 22q11.2 (bcrx1) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x3), 18cen (D18Z1x2), 22q11.2 (bcrx2) [1] nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x2), 15cen (D15Z1x3), 18cen (D18Z1x2), 22q11.2 (bcrx3) [1] nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x5), 22q11.2 (bcrx2) [1]
10/2nd cycle **Biopsied Cell** Remainder	**No signals [1]** **Lost [1]** Fragmented nuclei, no signals visible

11/2nd cycle Biopsied Cell Remainder	**nuc ish 21q13.2 (D21S529x1) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x1), **18cen (D18Z1x2), 22q11.2 (bcrx2) [3]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx2) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx3) [2] **nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x2), 15cen (D15Z1x2),** 18cen (D18Z1x2), 22q11.2 (bcrx2) [1] nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), **18cen (D18Z1x3), 22q11.2 (bcrx1) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), lost after 2nd round [1] **nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2),** 18cen (D18Z1x2), 22q11.2 (bcrx2) [1]
12/2nd cycle **Biopsied Cell** **Remainder**	**nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x3) [1]** nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2) [1] **nuc ish 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx3) [1]** nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x1), 15cen (D15Z1x1), 18cen (D18Z1x2), 22q11.2 (bcrx3) [1] **nuc ish 15cen (D15Z1x3), 18cen (D18Z1x1) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1), 15cen (D15Z1x1), 18cen (D18Z1x2), 22q11.2 (bcrx3) [1] **nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x3), 15cen (D15Z1x2),** 18cen (D18Z1x2), 22q11.2 (bcrx2) [1] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), lost after 2nd round [1]
13/2nd cycle **Biopsied Cell** Remainder	**nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1]** No signals [1] Lost
14/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x1) [1] No signals [1] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x1), lost after 2nd round [3] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x1), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx3) [1] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x1), 15cen (D15Z1x2), **18cen (D18Z1x2), 22q11.2 (bcrx2) [1]**
15/2nd cycle Biopsied Cell Remainder	nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx2) [2] Transferred

16/2nd cycle Biopsied Cell Remainder	**nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1) [1] nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x2), 15cen (D15Z1x2), **18cen (D18Z1x2), 22q11.2 (bcrx3) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx2) [1] **nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x3), 15cen (D15Z1x3),** 18cen (D18Z1x2), 22q11.2 (bcrx1) [1] **nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x2), 15cen (D15Z1x3),** 18cen (D18Z1x1), 22q11.2 (bcrx1) [1] nuc ish 13q14 (RB1x3), 21q13.2 (D21S529x2), 15cen (D15Z1x1),), **18cen (D18Z1x4), 22q11.2 (bcrx1) [1]**
19/2nd cycle Remainder	nuc ish 13q14 (RB1x5), 21q13.2 (D21S529x3), 15cen (D15Z1x4), **18cen (D18Z1x1), 22q11.2 (bcrx1) [1]**
20/2nd cycle (0PN) Biopsied Cell Remainder	**nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1) [1]** nuc ish 13q14 (RB1x2), 21q13.2 (D21S529x1), 18cen (D18Z1x1), **22q11.2 (bcrx1) [1]**
21/2nd cycle (3PN) Biopsied Cell Remainder	Lost nuc ish 13q14 (RB1x1), 21q13.2 (D21S529x2), 15cen (D15Z1x2), 18cen (D18Z1x2), 22q11.2 (bcrx1) [1] nuc ish 13q14 (RB1x4), 21q13.2 (D21S529x3), 15cen (D15Z1x2), 18cen (D18Z1x3), 22q11.2 (bcrx3) [1]

C: Appendix to Chapter 4. Cytogenetic analysis of metaphase II oocytes and 1st PBs with the ISCN Nomenclature

Table 4.1 (ISCN): FISH analysis of human metaphase II oocytes using the satellite III probe for chromosome 1(1q12, SG), the centromeric probe for chromosome 12 (D12Z1, orange), and the centromeric probe for chromosome X (DXZ1, SO)

Oocyte/patient no.	FISH Analysis Result
5coll(t)	ish 12cen (D12Z1x1), Xcen (DXZ1x1)
6col2(b)	ish 12cen (D12Z1x1), Xcen (DXZ1x1)
3782.1	ish.1q12 (x1), 12cen (D12Z1x1), Xcen (DXZ1x1)
3783.2	ish 1q12 (x1), 12cen (D12Z1x1), Xcen (DXZ1x1)
3855.1	ish Xcen (DXZ1x1)
1009.4	ish 12cen (D12Z1x1), Xcen (DXZ1x1)
971.3	ish 1q12 (x1), 12cen (D12Z1x1), Xcen (DXZ1x1)
968.1	ish 1q12 (x1), Xcen (DXZ1x1)
968.2	ish 1q12 (x1), Xcen (DXZ1x1)
3416.11	ish Xcen (DXZ1x1sep), Xcen (DXZ1x1sep)
3416.12	ish Xcen (DXZ1x1)

Table 4.2 (ISCN): FISH analysis of corresponding 1st PBs using the satellite III probe for chromosome 1(1q12, SG), the centromeric probe for chromosome 12 (D12Z1, orange), and the centromeric probe for chromosome X (DXZ1, SO)

PB/patient no.	FISH Analysis Result
968.1 PB	ish 1q12 (x1), 12cen (D12Z1x1sep), 12cen (D12Z11x1sep), Xcen (DXZ1x1 doublet)
3416.11 PB	ish 12cen (D12Z1x1), Xcen (DXZ1x1)

Table 4.3 (ISCN): FISH analysis of human metaphase II oocytes using the centromeric probe for chromosome 4 (D4Z1, SG), and the centromeric probe for chromosome 17 (D17Z1, SO)

Oocyte/patient no.	FISH Analysis Result
3969.2	ish 17cen (D17Z1x1)
3969.3	No signals
3969.4	ish 4cen(D4Z1x1sep), 4cen(D4Z1x1sep), 17cen(D17Z1x1)
3967.3	No signals
3967.8	ish 17cen (D17Z1x1)
3967.9	ish 17cen (D17Z1x1sep), 17cen (D17Z1x1sep)
1084.4	ish 17cen (D17Z1x1)
1084.6	ish 17cen (D17Z1x1)
1084.8	ish 17cen (D17Z1x1)

Table 4.4 (ISCN): FISH analysis of corresponding 1st PBs using the centromeric probe for chromosome 4 (D4Z1, SG), and the centromeric probe for chromosome 17 (D17Z1, SO)

PB/patient no.	FISH Analysis Result
3969.4 PB	No signals
3967.3 PB	ish 17cen (D17Z1x1)
3967.9 PB	ish 17cen (D17Z1x1sep), 17cen (D17Z1x1sep)
1084.8 PB	ish 17cen (D17Z1x1)

Table 4.5 (ISCN): FISH analysis of human metaphase II oocytes using the centromeric probe for chromosome 4 (D4Z1, SG or SO), the centromeric probe for chromosome 12 (D12Z1, orange), and the centromeric probe for chromosome 17 (D17Z1, SO or SG)

Oocyte/patient no.	FISH Analysis Result
3892.1	ish 12cen (D12Z1x1), 17cen (D17Z1x1)
3892.2	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1)
3892.5	ish 12cen (D12Z1x1), 17cen (D17Z1x1)
1033.4	ish 12cen (D12Z1x1)
1118.5	ish 17cen (D17Z1x1)
1118.6	ish 17cen (D17Z1x1)
3847.1	ish 12cen (D12Z1x1)
1054.1	ish 4cen (D4Z1x1), 17cen (D17Z1x1)
3839.1	ish 12cen (D12Z1x1)
4077.1	ish 4cen (D4Z1x1), 17cen (D17Z1x1)
4091.2	ish 4cen (D4Z1x1)
1169.3	ish 4cen (D4Z1x1), 17cen (D17Z1x1)
4130.6	ish 4cen (D4Z1x1), 17cen (D17Z1x1)
1152.1	ish 4cen (D4Z1x1)
4176.5	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1)
4176.8	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1)
4181.1	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1)
4181.2	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1)
4181.3	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1)
4181.4	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1)
4184.2	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1)
1209.2	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x2)
1243.6	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1sep), 17cen (D17Z1x1sep) or 4cen (D4Z1x1sep), 12cen (D12Z1x1sep), 17cen (D17Z1x1sep), 17cen (D17Z1x1sep)
4257.1	ish 12cen (D12Z1x1)
4257.2	ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1)
4257.3	ish 4cen (D4Z1x1), 12cen (D12Z1x1)
4292.7	ish 12cen (D12Z1x1), 17cen (D17Z1x1)
4292.9	ish 4cen (D4Z1x1), 17cen (D17Z1x1)
4264.1	ish 4cen (D4Z1x1), 17cen (D17Z1x2)
4264.2	ish 12cen (D12Z1x1)
4264.3	ish 4cen (D4Z1x1), 12cen (D12Z1x1)
4264.4	ish 4cen (D4Z1x1sep), 4cen (D4Z1x1sep), 12cen (D12Z1x1), 17cen (D17Z1x1)

Table 4.6 (ISCN): FISH analysis of corresponding 1st PBs using the centromeric probe for chromosome 4 (D4Z1, SG or SO)), the centromeric probe for chromosome 12 (D12Z1, orange), and the centromeric probe for chromosome 17 (D17Z1, SO or SG)

PB/patient no.	FISH Analysis Result
3892.1 PB	ish 12cen (D12Z1x1), 17cen (D17Z1x1)
4181.3 PB	**ish 4cen (D4Z1x1sep), 4cen (D4Z1x1sep), 17cen (D17Z1x1)**
4181.4 PB	ish 4cen (D4Z1x1)
4184.2 PB	ish 12cen (D12Z1x1), 17cen (D17Z1x1sep), 17cen (D17Z1x1sep)
1243.6 PB	**ish 4cen (D4Z1x1), 12cen (D12Z1x1), 17cen (D17Z1x1sep), 17cen** (D17Z1x1sep) or *ish 4cen (D4Z1x1sep), 12cen (D12Z1x1sep), 17cen (D17Z1x1sep),* 17cen (D17Z1x1sep)

Table 4.7 (ISCN): FISH analysis of human metaphase II oocytes using the locus-specific probe LSI13 for chromosome 13 (RB1 SG), and the locus-specific probe LSI21 for chromosome 21 (D21S529 SO).

Oocyte/patient no.	FISH Analysis Result
5coll(t)	**ish 21q13.2 (D21S529x1)**
3782.1	ish 13q14 (RBx1)
1005.2	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
1084.2	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
1084.4	ish 13q14 (RBx2)
1084.6	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
3892.1	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
3967.3	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
3967.9	ish 13q14 (RBx1)
3969.3	ish 13q14(RBx4sep), 21q13.2 (D21S529x1)
3892.5	ish 21q13.2 (D21S529x1)
1033.4	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
3839.1	**ish 13q14 (RBx1)**
3887.6	ish 13q14 (RBx1)
1001.5	**ish 21q13.2 (D21S529x1)**
H-1	**ish 13q14 (RBx1)**
1147.1	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
4081.1	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
4176.5	**ish 21q13.2 (D21S529x1)**

4181.3	ish 13q14 (RBx1), 21q13.2 (D21S529x2sep)
4181.4	No signals
4184.2	ish 13q14 (RBx1), 21q13.2 (D21S529x2sep)
1243.6	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
4257.1	ish 21q13.2 (D21S529x2sep)
4257.2	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
4257.3	ish 13q14(RBx2sep), 21q13.2 (D21S529x1)
4292.7	ish 21q13.2 (D21S529x1)

Table 4.8 (ISCN): FISH analysis of corresponding 1st PBs using the locus-specific probe LSI13 for chromosome 13 (RB1 SG), and the locus-specific probe LSI21 for chromosome 21 (D21S529 SO).

PB/patient no.	FISH Analysis Result
1084.4 PB	No signals
1084.6 PB	ish 13q14 (RBx1 double signal), 21q13.2 (D21S529x1)
3892.1 PB	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
3967.3 PB	ish 21q13.2 (D21S529x2sep)
3969.3 PB	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
3892.5 PB	ish 13q14 (RBx1), 21q13.2 (D21S529x1)
4181.4 PB	ish 13q14 (RBx1), 21q13.2 (D21S529x2sep)
1243.6 PB	ish 13q14 (RBx1), 21q13.2 (D21S529x2sep)

Table 4.9 (ISCN): FISH analysis of human metaphase II oocytes using the locus-specific probe LSI22 for chromosome 22 (bcr SG).

Oocyte/patient no.	FISH Analysis Result
5coll(t)	ish 22q11.1 (bcrx1)
1084.4	22q11.1 (bcrx1 double signal)
3839.1	ish 22q11.1 (bcrx1)
1243.6	22q11.1 (bcrx1 double signal)
4292.7	ish 22q11.1 (bcrx1)
4176.5	ish 22q11.1 (bcrx1)
4181.4	ish 22q11.1 (bcrx1)

Table 4.10 (ISCN): FISH analysis of corresponding 1st PBs using the locus-specific probe LSI22 for chromosome 22 (bcr SG).

PB/patient no.	FISH Analysis Result
1243.6 PB	ish 22q11.1 (bcrx2 signals)
4181.4 PB	ish 22q11.1 (bcrx1), 22q11.1 (bcrx1sep)

6.4.2 Aneuploidy detection in human metaphase II oocytes and first PBs/ Statistical analysis (as carried out for data presented in Cupisti *et al.*, 2003, and in Table 6.2)

The chi-squared test of independence for frequency in a 2 by 2 contingency table was used. The 2 by 2 contingency table is:

	Small	Not small	Total
Anomalies present	13	1	**14**
No anomalies	370	333	**703**
Total	**383**	**334**	**717**

The null hypothesis is that the rates of anomalies in small and in non-small chromosomes are the same. The value of the chi-squared test statistic (with 1 degree of freedom) is 8.93, and the *p*-value is 0.003.

As the *p*-value is quite small, there is strong evidence that the chromosome anomaly rates in the two groups differ from each other.

The odds of an anomaly in the smaller chromosomes is 11.7 times the odds of an anomaly in the larger chromosomes. It's possible to attach a 95% confidence interval (CI) to the ratio to give some idea of how precisely is being estimated. The 95% CI for this ratio of odds from the data presented in Cupisti *et al.*,2003 and partly in this thesis, goes from of 1.5 to of 90.0.

This interval does not contain 1 (the ratio obtained when the anomaly odds are the same in the two groups), but it does include a wide range of "plausible" values for the ratio of odds. This means the estimation of the magnitude of the difference in anomaly rates between the two groups cannot take place very precisely from this data set.