Investigation of aneuploidy in preimplantation embryos

by

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‘I, Thalia Mamas, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.’

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To my father, mother and sister

ABSTRACT

Aneuploidy is common in human preimplantation embryos. This thesis examines aneuploidy detection using an array platform, aneuploidy in embryos from fertile couples and recombination in gametes through the detection of cross-over events in embryos.

The first aim of this project was the optimisation of array comparative genomic hybridisation (aCGH) to examine all chromosomes in single blastomeres and trophectoderm samples from embryos, prior to clinical implementation. Accurate detection of errors was possible on single cells from epithelial cell lines of known chromosomal status. The same cell lines were used to mimic mosaic trophectoderm samples to examine the effect of mosaicism on aCGH. Aneuploidy could be confidently detected when more than 50% of the cells in the sample were abnormal.

Aneuploidy studies have been mainly performed on embryos, from couples undergoing preimplantation genetic screening (PGS), which are expected to be highly abnormal. The second aim was to determine the aneuploidy level in embryos from couples undergoing preimplantation genetic diagnosis (PGD) for monogenic disorders. Fluorescence *in situ* hybridisation (FISH) was used to examine five chromosomes in 86 embryos from 19 couples and all chromosomes were examined in 53 embryos from six couples by aCGH. Diploid mosaic embryos were the most predominant group when FISH analysis was carried out, whereas the majority of embryos were euploid after aCGH. Post-zygotic rather than meiotic errors were more common in embryos from PGD cycles when compared to embryos from PGS cycles.

Aneuploidy is known to associate with aberrant recombination. The third aim was to examine meiotic recombination. Polymorphic markers on five chromosomes were used to detect cross-over events in 77 embryos from 10 couples. Female recombination was higher than male. Increasing age had a negative effect on recombination. No significant effect of recombination on morphology and aneuploidy was observed, however euploid embryos had more recombination than aneuploid.

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Abbreviations

1C-4C DNA	1 chromatid – 4 chromatids DNA
aCGH	Array comparative genomic hybridisation
ADO	Allele drop out
AE	Axial elements
AMA	Advanced maternal age
ART	Assisted reproductive technologies
BAC	Bacterial artificial chromosome
bp	Basepair
BROVCA1	Breast-ovarian cancer-1
^{O}C	Degrees Celsius
Ca	Calcium
CEP	Chromosome enumeration probe
CG	Chromosome gain
CGH	Comparative genomic hybridisation
Ch	Channel
CL	Chromosome loss
cm	Centimeters
cM	Centimorgans
CNP	Copy number polymorphisms
Co-denaturation	Simultaneous denaturation
CPM	Confined placental mosaicism
CRGH	Centre for Genetic and Reproductive Health
CVS	Chorionic villi sample
Cy	Cyanine
DAPI	4',6-diamidino-2-phenylindole
dH_2O	Deionised water
DM1	Myotonic dystrophy type 1
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DO	Dragonfly orange
DOP-PCR	Degenerate oligonucleotide primed PCR
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGA	Embryonic genome activation
ESHRE	European Society of Human Reproduction and Embryology

FA	Female
FAM	6-Carboxyfluorescein
FAP	Familial adenomatous polyposis
FISH	Fluoresence *in situ* hybridisation
FPCR	Fluorescence polymerase chain reaction
hCG	Human chorionic gonadotrophin
HCl	Hydrochloric acid
HFEA	Human Fertilisation and Embryology Authority
HiFi	High Fidelity
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IR	Implantation rate
IVF	*In vitro* fertilisation
KCl	Potassium chloride
kb	Kilobase
L	Litres
LBR	Live birth rate
LD	Linkage disequilibrium
LDU	Linkage disequilibrium unit
LE	Lateral elements
LFR	Long fragment read
LSI	Locus specific identifier probe
MA	Male age
Mb	Megabase
m/c	Miscarriage
mCGH	Metaphase comparative genome hybridisation
MDA	Multiple displacement amplification
MHC	Major histocompatibility complex
mg	Milligrams
μg	Micrograms
Mg	Magnesium
$MgCl_2$	Magnesium chloride
MI	First meiotic division
MII	Second meiotic division
Min	Minutes
μl	Microliters

ml	Millilitres
μm	Micrometres
mm	Millimetres
mM	Micromolar
MND	Mitotic non disjunction
MSA	Mean spot amplitude
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NF1	Neurofibromatosis type 1
nm	Nanometer
NOA	Non obstructive azoospermia
NRES	National Research Ethics Service
OA	Obstructive azoospermia
ORT	Ovarian reserve test
PB	Polar body
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Primer extension preamplification
pg	Picogram
PGD	Preimplantation genetic diagnosis
PGH	Preimplantation genetic haplotyping
PGS	Preimplantation genetic screening
PMT	Photomultiplier tube
PN	Pronuclei
POP	Performance optimised polymer
PR	Pregnancy rate
PSSC	Premature separation of sister chromatids
PVA	Polyvinyl alcohol
RCT	Randomised controlled trial
Rec	Recombination
REC	Research ethics committee
RIF	Repeated IVF failure
RM	Recurrent miscarriage
RNA	Ribonucleic acid
rpm	Revolutions per minute

RT	Room temperature
SA	Spectrum aqua
SAC	Spindle assembly checkpoint
SBR	Signal to background ratio
SC	Synaptonemal complex
SCC	Sister chromatid cohesion
SD	Standard deviation
Sep-denaturation	Separate denaturation
SG	Spectrum green
SNP	Single nucleotide polymorphism
SO	Spectrum orange
SSC	Saline sodium citrate
STR	Short tandem repeat
SYCE	Synaptonemal complex central element
SYCP	Synaptonemal complex-specific protein
TBE	Tris/Borate/EDTA
TE buffer	Tris/EDTA buffer
TE	Trophectoderm
TEX12	Testis-expressed sequence 12
TFM	True fetal mosaicism
TIFF	Tagged image file format
TOP	Termination of pregnancy
Tris	Tris(hydroxymethyl)aminomethane
UPD	Uniparental disomy
UCL	University College London
UV	Ultraviolet
V	Volts
WGA	Whole genome amplification
w/v	Mass to volume
YY	Yakima Yellow

1 Introduction

1.1 Aneuploidy

Aneuploidy is defined as the deviation from a multiple of the haploid number of chromosomes in a cell. In humans, studies have shown that aneuploidy is the primary cause of pregnancy loss and birth defects as well as failure to establish a pregnancy following assisted reproduction technologies (ART, Nagaoka *et al*, 2012). Aneuploidy may even be the reason of poor fertility in humans when compared to other species (Delhanty, 2001). It has been estimated that, almost 5% of human conceptions are aneuploid. It is known that among all recognised pregnancies around 15% to 20% result in spontaneous abortions (Hassold, 1986). Analysis of spontaneous abortions during the first trimester of pregnancy has revealed that up to 70% carry a chromosomal anomaly (Fritz *et* al, 2001). Aneuploid pregnancies that survive to term will lead to the birth of children with developmental defects and mental retardation. Many studies have been performed presenting the varying level of aneuploidy at different developmental stages. A recent study has reported that 43.8/10000 births carry a chromosomal anomaly (Wellesley *et al*, 2012). These are all values from established pregnancies during which analysis is possible. Early loss during the first weeks of gestation of aneuploid pregnancies may go undetected and therefore the true level of aneuploidy may be higher. Indeed, this can be proven by studies performed in preimplantation embryos and gametes.

1.1.1 Cell division – Mitosis and Meiosis

Mitosis is the process of the division of all somatic cells (reviewed by Mitchison and Salmon, 2001). The number of chromosomes in the daughter cells, the products of mitosis, is the same as in the progenitor cell. Mitosis follows interphase and DNA replication. The steps of mitosis include prophase, when chromosomes condense and the microtubules of the mitotic spindle are formed from tubulin. The next stage is metaphase when the chromosomes attach to the spindle and align at the centre of the cell, called the equatorial plate, followed by anaphase when sister centromeres part and sister chromatids of each chromosome move to the opposite poles of the cells. Sister chromatid separation is possible through cleavage of chromatid cohesion by separase (Hauf *et* al, 2001). Aurora B kinase is responsible for the association of separase with the centromeres at the onset of anaphase (Yuan *et al*, 2009). Securin ensures the correct timing of separase function, as it inhibits separase until anaphase (Nasmyth *et* al, 2000). Mitosis is completed by telophase when the two daughter cells are formed by cytokinesis.

Through the process of meiosis haploid gametes are generated, carrying only one copy of all the chromosomes. Meiosis consists of two cell divisions between which the DNA is not replicated, meiosis I (MI) and meiosis II (MII). As in mitosis, meiosis starts after a cell cycle, during which DNA is replicated. This generates the primary gametocytes that contain a pair of all chromosomes each comprised of two sister chromatids [4C DNA (four chromatid DNA), figure 1.2] (Handel and Schimenti 2010).

The stage of prophase in meiosis I, which is the longest, is comprised of four sub stages. In summary, during leptotene and zygotene chromosome pairing occurs. In pachytene, the chromosomes synapse and finally desynapse at the diplotene stage. Looking at prophase I in detail, the chromosomes condense and the meiotic spindle is formed. The bivalents, pairs of homologous chromosomes, one paternal and one maternal in origin, line up during synapsis. This is possible with the formation of the synaptonemal complex (SC) along each chromosome. Meiosis-specific proteins of the cohesin group compose the SC. During leptotene, the synaptonemal complex-specific proteins 2 (SYCP2) and 3 (SYCP3) are responsible for forming the axial elements (AE) of the SC. During zygotene, they align to create the SC's lateral elements (LE) that are separated by the central zone. Important proteins responsible for the formation of the central zone are SYCP1 and the synaptonemal complex central element proteins 1 (SYCE1), 2 (SYCE2) and 3 (SYCE3), as well as the testis-expressed sequence 12 (TEX12) protein. Synapsis is completed by the pachytene stage (reviewed by Fraune *et al*, 2012) (figure 1.1). The SC disassembles at the end of prophase I.

Figure 1.1: Stages of prophase I

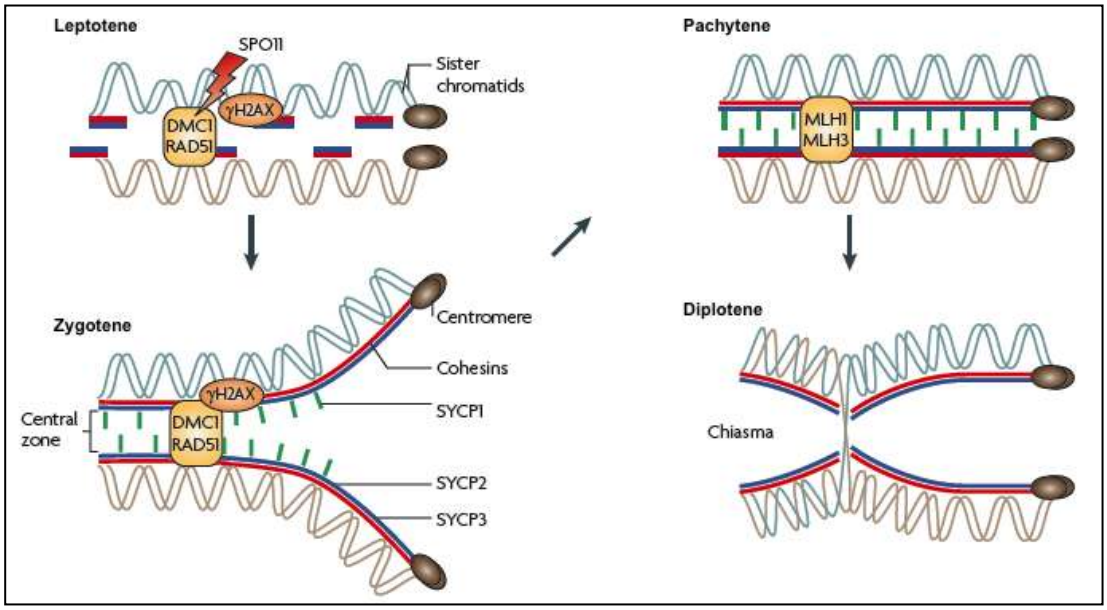


Figure 1.1: *Presentation of the formation of the synaptonemal complex to complete chromosome synapsis, as well as the initiation of double strand breaks and recombination at the leptotene through to the pachytene stage. Prophase I is completed at the diplotene stage with the disassembly of the synaptonemal complex and the formation of chiasmata. (Adapted by permission from Macmillan Publishers Ltd: Nat Rev Genet. Handel and Schimenti, 2010)*

Another important event occurring during prophase I, is recombination between homologous chromosomes. Meiotic recombination is responsible for variation between individuals, as well as the correct segregation of chromosomes in the daughter cells (Smith and Nicolas, 1998). It is initiated by double-strand breaks of the DNA by a meiosis-specific topoisomerase-like protein, SPO11 (Keeney *et al*, 1997). The double strand breaks trigger the homologous recombination repair machinery; γH2AX is formed after phosphorylation of the histone H2AX, which in turn induces the binding of recombination proteins, including DMC1 and RAD51, at the recombination nodules along the AEs of the synaptonemal complex. These proteins aid in the strand invasion between chromatids (Masson and West, 2001). At the pachytene stage, mismatch repair proteins MLH1 and MLH3 localise at the recombination nodules. Recombination is completed with the formation of cross-overs (Handel and Schimenti 2010) (figure 1.1). The visible result of recombination between homologous chromosomes is a chiasma, which involves two of the four chromatids of one bivalent (figure 1.1). One of the important functions of a chiasma is to stop separation of the homologous chromosomes prior to the beginning of anaphase I (Carpenter, 1994). There is a correlation between the length of the SC and the number of recombination events and gene density, which is irrespective of the physical length of the chromosome. For example, a longer SC has been

observed in the gene rich, high in recombination frequency chromosome 19 than in chromosome 18 that is similar in physical length and gene-poor (Sun *et al,* 2004). Moreover, the length of the SC is around two times longer in females than in males, resulting in increased of recombination in females, as discussed later in section 1.1.1.1 (Tease and Hultén, 2004).

The next step of MI is metaphase I, when homologous chromosomes align at the equatorial plate attached by the spindle at the opposite poles. The attachment to the microtubules of the spindle happens via the sister kinetochores, which are located in the centromeres. Tension is created that pulls away the maternal from the paternal chromosomes but they do not disjoin due to the chiasmata and sister chromatid cohesion (SCC) (Petronczki *et al*, 2003). SCC is maintained at the centromeres until anaphase II, in order to prevent separation of the sister chromatids. This maintenance is achieved by the function of cohesin proteins, like SMC1, SMC3 and STAG3 in mammalian cells (Prieto *et al*, 2001). A protein essential for the association of kinetochores with microtubules is SCC1 (Hoque and Ishikawa, 2002).

Attachment of all kinetochores to the microtubules is ensured prior to the onset of anaphase I, through the spindle assembly checkpoint (SAC). If any of the kinetochores are not attached, the SAC is activated and the process arrests at metaphase I (Sun and Kim, 2012). The SAC is also present during mitosis of all somatic cells with the same checkpoint function (Musacchio and Salmon, 2007). In anaphase I, SAC is silenced, provided that all kinetochores are properly attached to the microtubules of the spindle and the bivalents are separated. The centromeres of each chromosome do not part and finally recombined homologues move to the opposite poles at telophase I ending the first cycle of meiosis with two newly formed cells containing half the number of chromosomes (2C DNA, figure 1.2).

Meiosis II is a process similar to mitosis during which sister chromatids segregate, instead of whole chromosomes that segregate during meiosis I. Chromosomes again condense in prophase II, move to the equatorial plate in metaphase II, the centromeres separate in anaphase II, the centromere cohesion is lost and single chromatids move to the opposite poles. Similarly to meiosis I, the SAC regulates the transition, from metaphase to anaphase, to ensure that all kinetochores are attached to the spindle (Sun and Kim, 2012). Meiosis II ends in telophase II, when four haploid cells are formed (1C DNA, figure 1.2).

Despite the fact that the general features of meiosis are consistent between males and females, there are important differences in the timing needed for completion between the sexes (Hunt and Hassold, 2002). In females, meiosis starts during fetal life and arrests at prophase I before birth. At puberty meiosis I resumes in some oocytes, resulting in the production of a secondary oocyte and the first polar body. Meiotic arrest occurs again at the stage of metaphase II and is only resumed upon fertilisation when meiosis II is completed resulting in the formation of a mature oocyte and the second polar body. Meiosis in males is initiated at puberty and continues with no arrests throughout their lives. Meiosis I results in the production of two secondary spermatocytes and meiosis II in four haploid spermatids as shown in figure 1.2 (Handel and Schimenti, 2010).

Figure 1.2: Gametogenesis

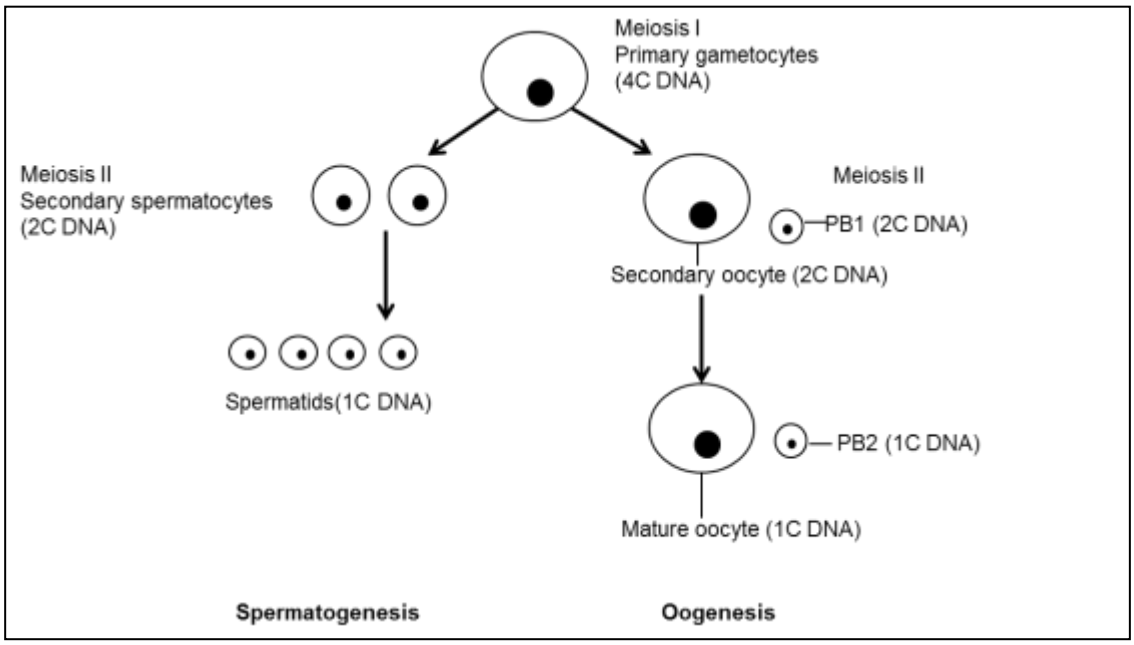


Figure 1.2: *The two meiotic divisions in spermatogenesis and oogenesis. The starting materials in both sexes are cells containing two pairs of all chromosomes with two chromatids each (4C DNA), following DNA replication. After MI in females, the secondary oocyte and the first polar body (PB) are formed and in males two secondary spermatocytes, all containing one pair of each chromosome with two chromatids each (2C DNA). After MII the mature oocyte and the second PB are formed in females upon fertilisation and in males four spermatids are formed that will give rise to spermatozoa. The products of MII in both sexes are all haploid cells containing one chromatid (1C DNA).*

1.1.1.1 Recombination. Differences between individuals and chromosomes.

Diversity between individuals is generated through meiotic recombination, when a new combination of alleles is formed. Several methods can be used to quantify recombination. This can be done directly, by examining chiasmata at the pachytene stage of gametogenesis. Immunofluorescence is utilised to detect the DNA mismatch repair protein MLH1 that has been found to localise at sites of crossing over during meiosis in mice (Baker *et al*, 1996) and humans (Barlow and Hultén *et al*, 1998). Although it is a reliable method in measuring cross-over events in sperm, in oocytes, detecting cross-overs through MLH1 is not as accurate. MLH1 immunofluorescence in prophase oocytes from fetal ovaries has revealed fewer cross-over events than that predicted from linkage analysis (Cheng *et al*, 2009). Another difficulty faced in studying recombination directly in the gametes is the scarcity of the samples, especially the oocyte.

Indirect approaches are more applicable, for example linkage analysis in families, utilising polymorphic markers across the genome and examining them across generations (Lamb *et al*, 2005). Genotyping is performed with the aid of genetic maps that show the order of the markers across the chromosomes, as well as the distances between each locus. These are genetic distances indicating the chance of recombination occurring between two loci and are measured in centiMorgans (cM, Lynn *et al*, 2004). The ability to analyse polymorphic markers across the genome has led to the creation of the International HapMap project. The purpose of this project is the identification of common sequence variants across different populations with the ultimate goal being the discovery of variants that are linked to common diseases and the development of new diagnostic and therapeutic tools (International HapMap Consortium, 2003). Mapping of over 3.1 million single nucleotide polymorphisms (SNPs) in four populations was described in Phase II of the HapMap. The populations were geographically diverse of European, African, Chinese and Japanese ancestry (International HapMap Consortium, 2007). Recently, HapMap 3 was published incorporating seven additional populations as well as the analysis of copy number polymorphisms (CNPs) (International HapMap 3 Consortium, 2010).

The non-random association of alleles at different loci in a haplotype is termed linkage disequilibrium (LD) and maps based on LD have been created (Slatkin*,* 2008). The metric LD maps are based on linkage disequilibrium units (LDU) that can describe the underlying structure of LD (Maniatis *et al,* 2002). These maps can be

visualized by plotting marker location in LDU against the marker distances in kilobases (kb). The LDU maps can provide information about current and historic recombination at a very fine resolution. Plotting these LDU maps reveals the non-linear relationship between physical distance and the underlying LD together with the "Block-Step" structure of a region. "Blocks" of LD represent areas of low haplotype diversity and therefore low recombination, whereas "Steps" define LD breakdown mainly caused by recombination since cross-over profiles have been shown to agree with LD patterns (Webb *et al,* 2008).

Recombination is not uniform across the genome. Some sites show increased recombination (hot spots) and other sites show lower or no recombination (silent spots) (Yu *et al*, 2001). Recombination hotspots occur on average every 200 kilobases or less in the human genome (McVean *et al,* 2004). The mammalian protein PRDM9 has been found to control the level of recombination in hotspot areas (Cheung *et al*, 2010). In humans, PRDM9 recognises a specific 13-mer that is in high abundance in recombination hotspots (Baudat *et al*, 2010). Recombination frequency is enhanced in telomeric and subtelomeric regions (Dib *et al*, 1996). There are differences between the chromosomes as well. Shorter chromosomes exhibit higher recombination rate. For example the recombination rate of the short chromosomes 21 and 22 is double than that observed on the long 1 and 2 chromosomes (Kong *et al*, 2002). Among all the autosomes, the highest recombination has been observed in chromosome 19 (Dib *et al*, 1996).

Recombination varies between sexes and individuals (Cheung *et al*, 2007). Recombination is higher in females than in males (Hassold *et al*, 2000). The recombination in the telomeric regions of the chromosomes is higher in males, whereas females show higher rates around the centromere. Moreover, variation in the recombination of the 22 autosomes between individuals has been observed within females (Broman *et al*, 1998; Kong *et al* 2002). This has also been observed in males, as analysis of MLH1 loci for the detection of recombination events in sperm from males with normal sperm parameters, has shown that there is variation between individuals in the distribution of cross-over events for all chromosomes (Sun *et al,* 2006b). Specifically for chromosome 19, recombination is quite uniform across the chromosome for females, whereas in males, as seen in other studies, it is greater around the telomeric regions (Mohrenweiser *et al*, 1998). Recombination hotspots are not the same between sexes as well. After comparing female and male maps it has been observed that 15% of recombination hotspots are specific to one

sex (Kong *et al*, 2010). An effect of advanced age has been observed in female recombination, in that it is increased, whereas no effect has been observed in male recombination (Kong *et al* 2004).

1.1.2 Incidence of aneuploidy

1.1.2.1 Cytogenetic and molecular cytogenetic methods to study aneuploidy

A variety of methods have been applied to gametes and embryos to study their chromosomal status. Initial studies were performed by traditional karyotyping (Jamieson et al, 1994). The main difficulty in this technique was the lack of metaphase spreads to perform the analysis. Fluorescence *in situ* hybridisation (FISH) was then utilised on interphase nuclei, easily accessible in gametes and embryonic cells. However, only a few chromosomes could be analysed. The most recent and comprehensive technique is comparative genomic hybridisation (CGH), with which all chromosomes can be analysed. The outcome of studies that have utilised either FISH or CGH may not be associated due to differences, advantages and limitations in these two techniques. These are presented in sections 1.2.3.1 and 1.2.3.2 of the Introduction.

1.1.2.2 Incidence of aneuploidy in the gametes

Chromosomal aneuploidy is more common in the oocytes than the sperm. Aneuploidy in the gametes and the embryos is studied post ART. Findings, however, in these couples may not reflect the general population.

Around 4% of sperm is aneuploid with the sex chromosomes having the highest frequency of aneuploidy (Templado *et al*, 2011). Contradictory to females, increasing male age does not contribute to an increase in aneuploidy frequency (Luetjens *et al*, 2002).

Aneuploidy analysis of the female gamete can either be performed by direct assessment of the chromosomal status of the oocyte or indirectly, through analysis of the polar bodies. Through a variety of studies, it is estimated that around 15-20% of human oocytes carry a chromosomal abnormality (Pellestor *et al*, 2006). Aneuploidy in the oocyte and the polar bodies is common even in females of young reproductive age. Two studies on oocyte and polar body complexes using comprehensive chromosome analysis by CGH from two groups of young women,

average age 22 and 32.5 years in each group, revealed an aneuploidy rate of 3% and 22% respectively (Fragouli *et al*, 2006; 2009). On the other hand, analysis of both polar bodies from 117 zygotes from a group of patients with repeated implantation failure and an average age of 39.1 years, showed an aneuploidy level as high as 65.5% (Fragouli *et al*, 2010). Even higher aneuploidy levels (72%) have been reported in polar bodies from women of advanced maternal age (average age = 40 years, Geraedts *et al*, 2011). Smaller chromosomes have been found to be more commonly involved in aneuploidy during oogenesis (Cupisti *et al*, 2003).

1.1.2.3 Incidence of aneuploidy in preimplantation embryos

Preimplantation embryo development starts after fertilisation (Day 0) of an oocyte by a sperm, with the formation of two pronuclei (2PN), one paternal, one maternal in origin. This is followed by a series of cleavage divisions that last three days resulting in embryos comprising of six to ten cells. During that period, embryonic genome activation (EGA) also occurs (Braude *et al*, 1988). Compaction occurs after EGA as the cells form tight junctions between them and the morula is formed, on day four. During the morula stage, the cells within the embryo start differentiating into two distinct cell lineages. By day five and six and after many cell divisions the embryo reaches the blastocyst stage. The features of a blastocyst are a fluid-filled blastocyst cavity and the inner cell mass (ICM) that is surrounded by the trophectoderm (TE). The ICM and the TE are two differentiated cell lineages. ICM will give rise to the embryo, whereas the placenta will be formed from the TE (Huppertz and Herrler, 2005). Implantation occurs on around day seven, post fertilisation (Niakan *et al*, 2012). Figure 1.3 presents the embryo preimplantation development.

Figure 1.3: Preimplantation embryo development

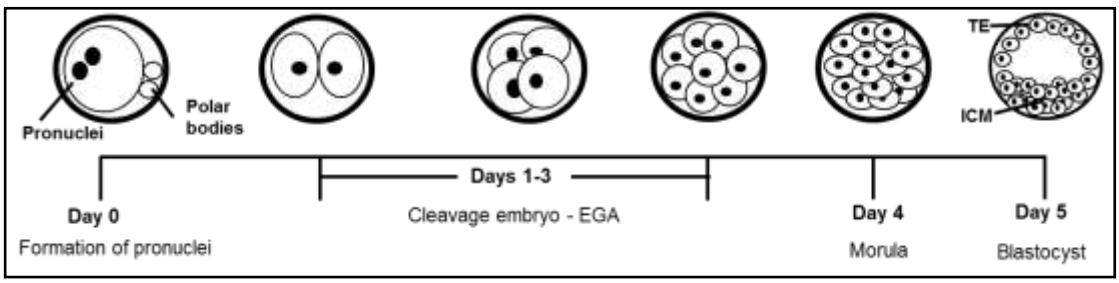


Figure 1.3: *Normal fertilisation occurs with the formation of the two pronuclei at the start of the preimplantation embryo development. A series of cell divisions follows, during which embryonic genome activation takes place. This is called the cleavage stage and lasts three days. On day four of development the cells compact and form the morula and on day five, after differentiation of the cells in two cell lineages, the inner cell mass and the trophectoderm, the blastocyst is formed.*

The majority of data on the level of aneuploidy in embryos comes from the results of preimplantation genetic diagnosis and screening cycles, as well as follow-up analysis of these cycles. Most of the studies use FISH to analyse chromosomes. However, complete disaggregation of embryos and analysis of all the chromosomes in every single cell reveals in detail the extensive aneuploidies that can be present in an embryo, such as mosaic and chaotic cell lines and chromosome breakage, apart from uniform aneuploidies originating in meiosis (Wells and Delhanty, 2000).

Abnormalities in the chromosome number of preimplantation embryos are very common and the majority can only be detected at that stage of development as up to 90% of the aneuploidies seen in the cleavage stage embryo do not survive the first trimester of pregnancy (Munné *et al*, 2004). Embryonic aneuploidy may be a result of meiotic error occurring during the production of the gametes but also mitotic error during the first cellular embryonic divisions, leading to mosaicism. A high rate of chromosomal abnormalities, mitotic and meiotic in origin, exists in both normally and abnormally developing embryos (Munné *et al*, 1993). Analysis of all cells from cleavage stage embryos has revealed that only 25% carry no aneuploid cells (Mantzouratou and Delhanty, 2011). Generally, monosomy is more frequent than trisomy in embryos. There are also differences in the rate of aneuploidy between chromosomes in cleavage stage embryos, with 22, 16, 21 and 15 being most commonly involved, whereas abnormalities affecting chromosomes 14, X, Y and 6 are the least common. These results were obtained after FISH analysis for 14 chromosomes in over 2000 embryos (Munné *et al*, 2004).

Uniform aneuploidies in the embryo can persist to the last stage of preimplantation development, the blastocyst. More than 50% of blastocysts are aneuploid (Fragouli and Wells, 2011). Trisomies, as well as monosomies (such as monosomy for chromosomes X and 21) that are known to exist in the first trimester of pregnancy, have been detected in blastocysts (Sandalinas *et al*, 2001). Apart from aneuploidy of whole chromosomes, either meiotic or mitotic in origin, structural chromosomal abnormalities are present in the preimplantation embryo. These segmental changes have been found to occur in 7-23% of embryos following analysis by CGH (Voet et al, 2011).

1.1.2.3.1 Preimplantation embryo mosaicism

Mosaicism is very common in embryos of the preimplantation stage. Mosaic embryos carry cells of more than one chromosomal complement and may be morphologically normal (Harper *et al*, 1995). Another, more extreme, chromosomal situation has been described in embryos, when each cell carries a different chromosomal complement. These are called chaotic embryos (Delhanty *et al*, 1997). It is estimated that over 50% of embryos generated through *in vitro* fertilisation (IVF) are mosaic (Delhanty, 2005).

Mosaicism arises through errors in the first mitotic divisions of the embryo post fertilisation (Kalousek, 2000). However, there have been suggestions that the fourth mitotic division in an embryo is the one generating most aneuploid blastomeres (Gonzalez-Merino *et al*, 2003). Mosaicism is irrespective of maternal age, as it has been found to exist in high levels in embryos from young women (Baart *et al*, 2006). In contrast to errors in meiosis that are mostly maternal in origin, mosaicism may be a result of paternal contribution. This was made apparent in cases of severe male infertility and especially those of non-obstructive azoospermia (NOA), in which the rate of chromosomal abnormalities due to mosaicism increased, when compared to cases of males with normal sperm parameters (Magli *et al*, 2009).

Mechanisms that lead to mosaicism, include post-zygotic chromosome loss, when cells carry monosomies, which results after anaphase lag, during which whole chromosomes show a delayed movement to the spindle pole, chromosome gain, represented with cells carrying trisomies, caused by chromosome duplication and mitotic non-disjunction, when cells carry reciprocal monosomies and trisomies of the same chromosome (Coonen *et al*, 2004). In some studies, chromosome loss has been found to be the most common mechanism of post-zygotic errors leading to mosaicism (Daphnis *et al*, 2005; Daphnis *et al*, 2008), whereas a different study has shown that mosaicism may arise by chromosome gains and losses at a similar rate (Fragouli *et al*, 2011a).

Malsegregation of the chromosomes, which will lead to mosaicism, may occur due to the deregulation of mitosis and malfunction of the centromeres during the first post-zygotic divisions (Kalousek, 2000). It has been observed that separation of the sister chromatids is not essential for the exit from mitosis or DNA replication in the next cell cycle. Therefore, defects in the cleavage of sister chromatids cohesion by

separase will not lead to a block of the cell cycle (Hauf *et al*, 2001). Moreover, increased levels of securin, the protein that controls separase function, may lead to malsegregation of chromosomes (Nasmyth *et al*, 2000). In early studies of mosaicism in embryos, it was suggested that abnormalities in cell cycle checkpoints were a source of error (Delhanty and Handyside, 1995). Analysis of gene expression in cleavage stage embryos revealed overexpression of cell cycle drivers resulting in the rapid division of cells and increase in the gene copy number but also to susceptibility of chromosome abnormalities (Kiessling *et al*, 2010).

1.1.2.3.2 Mosaicism during different stages of preimplantation embryo development and pregnancy

Mosaicism can persist at high levels through all stages of preimplantation development. Its clinical significance is not yet clear, however there is a hypothesis that diploid mosaic embryos with a high proportion of diploid cells have the ability to lead to a normal fetus (Fragouli *et al*, 2011a). A large number of studies have been performed to determine the presence and levels of mosaicism at all developmental stages with some presenting contradictory results. Mosaicism has been reported in up to 88% of embryos of all developmental stages, along with the hypothesis that it is a normal condition of *in-vitro* generated embryos (Gonzalez-Merino *et al*, 2003). One study showed that the frequency of mosaicism increased as the embryos developed. Of the 33 blastocysts analysed, 90.9% were found to be mosaic, carrying mostly diploid and polyploid cells. On the other hand, mosaicism in arrested embryos was represented by more chaotic abnormalities (Bielanska *et al*, 2002). A different study led to similar findings, where 95% of the blastocysts contained 70% or more diploid cells, whereas 65% of arrested mosaic embryos carried the same proportion of diploid cells (Ruangvutilert *et al*, 2000a). Conversely, lower levels of mosaicism in blastocysts when compared to arrested embryos, have also been reported (Evsikov and Verlinsky, 1998; Fragouli *et al*, 2008). Comparison of the aneuploidies occurring at the cleavage stage, in blastocysts and in first trimester pregnancies, showed that haploid, monosomic and some trisomic abnormalities seen in early preimplantation stages are lost in the blastocyst. On the other hand, abnormalities seen in early pregnancies are already established in the blastocyst (Clouston, 2002). In mosaic blastocysts the degree of aneuploidy is similar between the two cell lineages, the trophectoderm and the inner cell mass (Derhaag *et al*, 2003).

The decrease of the levels of mosaicism from the cleavage through to the blastocyst stage may be due to the ability of the embryos to compensate for these errors. An example of this is the extrusion of a micronucleus carrying the extra chromosome in trisomic cells (Li *et al*, 2005). Decrease of the level of mosaicism has also been observed even after the blastocyst stage on cultured day 14 embryos (Munné *et al*, 2005). Duplication of a monosomic chromosome is another form of error correction, which, however will result in uniparental disomy (UPD). Bi-parental inheritance will be lost, as the chromosomal pair will derive from one parent (Engel, 2006). This is detrimental if the chromosome that has undergone UPD contains imprinted genes. For example, Beckwith-Wiedermann syndrome can be caused by the duplication of the paternal chromosome 11, whereas Prader-Willi syndrome by the duplication of maternal chromosome 15 (Butler, 2009). Another possibility of correction during late stages of preimplantation development may arise through mitotic arrest of abnormal cells, through control of the cell cycle that is activated with EGA (Los et al, 2004).

Mosaicism has also been detected in chorionic villi samples (CVS) from early pregnancies at around 2%. Mosaicism in pregnancies may be localised in the placenta and is called confined placental mosaicism (CPM). CPM for trisomies of chromosomes 2, 3, 7, 8, 9, 16 and 22 have been found to occur in between nine and 91 pregnancies per 100000 (Wolstenholme, 1996). Mosaicisim has also been detected in the whole of the fetus and is called true fetal mosaicism (TFM, Grati *et al*, 2006). Mitotic non-disjunction followed by anaphase lag during the first post-zygotic divisions may also be the reason for detectable mosaicism in patients with mosaic trisomy 13, a viable condition with varying phenotypes (Jinawath *et al*, 2011).

1.1.3 Origin of aneuploidy

The parental origin of aneuploidies, as well as the stage at which they occur, vary between chromosomes. Most importantly, aneuploidy in the oocytes is more common than in sperm. In women, there is clear evidence on the positive correlation of increasing chromosomal errors in oocytes with increasing age (Hassold *et al*, 2007). Comprehensive chromosomal analysis of both polar bodies from zygotes has revealed that the effect of advancing female age is more profound in the occurrence of MII errors (Fragouli *et al*, 2011b).

Trisomy of chromosome 16, which leads to spontaneous abortion, can only be maternally derived from errors occurring in meiosis I (Hassold *et al*, 1995). Similarly,

95% of trisomy 21 cases, leading to Down's syndrome, are caused by errors in maternal meiosis (Antonarakis and the Down Syndrome Collaborative Group, 1991). Origin of aneuploidy among the acrocentric chromosomes 13, 14, 15, 21 and 22 is similar with errors arising in paternal meiosis not exceeding 17% of the cases. The majority of maternal errors occur in MI, whereas nondisjunction in paternally derived cases occur mostly in MII (Zaragoza *et al*, 1994). More than 96% of trisomy 22 cases are maternally derived with 90% of them resulting from errors during MI (Hall *et al*, 2007a). Similarly, the majority of Patau syndrome cases, trisomy 13, are maternally derived with 67% of the errors occurring during MI and the rest during MII (Hall *et al*, 2007b). The most frequent stage of error that leads to trisomy 18, or Edwards syndrome, is in maternal meiosis MII (Bugge *et al*, 1998).

Abnormalities of the sex chromosomes occur with a prevalence of 1.88 per 10000 births, with the most common being the 47,XXY condition, or Klinefelter's syndrome (Boyd *et al*, 2011). Maternal and paternal, mainly MI errors equally contribute to the generation of Klinefelter's syndrome (MacDonald *et al*, 1994). Autosomal monosomies are lethal for the early embryo. Therefore, information on the origin can only be obtained for the 45,X condition, Turner's syndrome (Hassold and Hunt, 2001). Molecular analysis of individuals with Turner's syndrome revealed that the majority of cases arise from errors during spermatogenesis (Jacobs *et al*, 1997).

1.1.4 Causes of aneuploidy

Aneuploidy in the gametes may arise through a variety of errors. Meiotic non-disjunction is the term used for the mal-segregation of chromosomes or chromatids during meiosis. Mechanisms that lead to meiotic non-disjunction of whole chromosomes, are "true" non-disjunction, when a bivalent fails to resolve chiasmata and both homologues segregate together, as well as non-disjunction resulting from the premature resolution or absence of chiasmata that may cause independent segregation of the homologues (Hassold and Hunt, 2001). These are illustrated in figure 1.4 Malsegregation of single chromatids during meiosis I may lead to the production of aneuploid gametes (Angel, 1991). Studies on a cohort of 100 oocytes showed that this may occur through the premature separation of the sister chromatids (PSSC) during anaphase of meiosis I (Angell *et al*, 1994). Recent CGH analysis on polar bodies revealed that single chromatid errors were more common than whole chromosome errors caused by non-disjunction in oocytes (Gabriel et al, 2011). Generation of aneuploid gametes following PSSC depends on the

segregation of the extra chromatid in anaphase II, post fertilisation. In 50% of cases the extra chromatid will pass to the second polar body and therefore the oocyte will be euploid (Angell *et al*, 1993).

Figure 1.4: Chromosome and chromatid errors in meiosis I and meiosis II

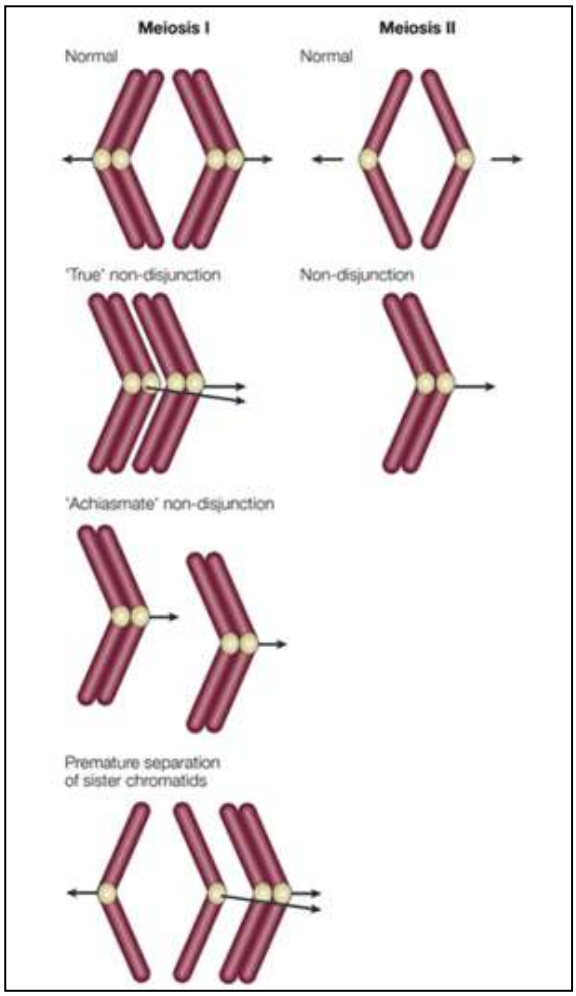


Figure 1.4: *During meiosis I, "true" and "achiasmate" non-disjunction of whole chromosomes, as well as premature separation of sister chromatids may occur. In meiosis II, aneuploidy may arise through non-disjunction of the sister chromatids. (Adapted by permission from Macmillan Publishers Ltd: Nat Rev Genet. Hassold and Hunt, 2001)*

One of the causes of non-disjunction is aberrant recombination during both meiotic divisions. A lot of information on the effect of recombination in the segregation of chromosomes comes from studies on trisomy 21, as it is the most common viable trisomy of the autosomes (Hassold *et al*, 1996). Early studies have shown that recombination is reduced along chromosome 21, causing non-disjunction during meiosis, thus resulting in the birth of children with Down's syndrome (Warren *et al*, 1987). The location of multiple recombination events on chromosome 21, and more specifically, at the location of 21q is important in the correct segregation of the

chromosome. A study on families with an infant carrying trisomy 21 using 15000 SNPs on 21q showed that the average distance of recombination events in maternal MI errors was reduced when compared to normal controls (Oliver *et al*, 2012). Recent data have shown that the cases of chromosome malsegregation due to changes in recombination, leading to maternal MII errors for chromosome 21, increased with maternal age (Oliver *et al*, 2008). Since 1968, it has been observed that the number and location of chiasmata were reduced as maternal age increased (Henderson and Edwards, 1968). Reduced recombination is also observed among non-disjoined chromosomes 21 in cases of Down's syndrome that are paternal MI in origin, but there is no difference in paternal MII errors (Savage *et al*, 1998). Recombination is also reduced in cases of non-disjunction of chromosome 16, especially around the centromere (Hassold *et al*, 1995), acrocentric chromosomes 13 and 22 (Hall *et al*, 2007a; 2007b) and in non-disjunction of the sex chromosomes in cases of 47,XXY and 47,XXX (MacDonald *et al*, 1994).

Meiotic abnormalities in sperm have been analysed with a variety of techniques (reviewed by Egozcue *et al*, 2005). Direct analysis of the synaptonemal complexes was performed using light and electron microscopy. Recent advances in immunofluorescence allow the analysis of synapsis and meiotic recombination. This is performed by analysing specific proteins of the synaptonemal complex (SYCP1 and SYCP3) along with the visualisation of a DNA repair and recombination protein, MLH1, located at the sites of chiasmata (Sun *et al*, 2005). This can be done in conjunction with multiplex FISH on sperm in order to assess recombination and aneuploidy simultaneously (Ma *et al*, 2006). Immunofluorescence methods have revealed complete absence of chiasmata in the sex chromosomes and chromosomes 21 and 22 in MI spermatocytes (Uroz *et al*, 2011). PSSC has also been detected as a mechanism for non-disjunction (Uroz *et al*, 2008) as well as defects in chromosome synapsis (Sun *et al*, 2007) in sperm.

Aneuploidy occurs at a significantly higher rate in sperm from infertile men than fertile (Sun *et al*, 2008) affecting the sex chromosomes in a higher frequency than the autosomes (Martin *et al*, 1996). A reduction in the recombination frequency has been observed in sperm from men with non-obstructive azoospermia (NOA) and men with sperm maturation arrest, than those with normal sperm parameters (Gonsalves *et al*, 2004). Evidence shows that decrease in recombination among the sex chromosomes in infertile men increase the occurrence of sperm with XY disomy (Ferguson *et al*, 2007). However, errors in meiosis occur regularly even in the sperm

of fertile men with the sex chromosomes, as well as chromosomes 21 and 22 being more susceptible to non-disjunction (Sun *et al*, 2006a).

Despite the fact that meiotic errors are common during spermatogenesis, the aneuploidy level observed in sperm is much lower than that observed in the oocyte. This may be due to the presence of a checkpoint during spermatogenesis that does not allow the progression of aneuploid sperm (Uroz and Templado, 2012). Indeed, aneuploid oocytes lack the chromosome-mediated checkpoint control that is needed for meiotic arrest or delay of the metaphase to anaphase transition in the case of unaligned chromosomes along the meiotic spindle (LeMaire-Adkins *et al*, 1997). The increased risk of aneuploidy in older women may be attributed to the fact that during the extended stage of prophase I, cohesion within the bivalents is weakened, leading to PSSC (Wolstenholme and Angel, 2000). Moreover, aging oocytes do not have the ability to resolve poor recombination and the SAC's efficiency is deteriorating resulting in an increase of chromosome malsegregation (Wang *et al*, 2011).

Another cause of aneuploidy that will lead to uniform abnormalities in the embryo is germline or gonadal mosaicism. In this situation, the errors do not occur during the meiotic divisions of gametogenesis, but have already been established in the germ cells prior to the onset of meiosis. Errors occurring in the premeiotic divisions will result in germline mosaicism. Gonadal mosaicism is the term used to describe mosaic cells that are present in the embryonic gonad (Delhanty, 2005). Analysis of oocyte and polar body complexes has revealed the presence of extra chromosomal material in both the oocyte and the first polar body, suggesting a trisomic germ cell line in chromosomally normal women (Cozzi *et al*, 1999, Mahmood *et al*, 2000). Evidence for gonadal mosaicism comes from direct cytogenetic analysis of ovarian fetal tissue. In a study on female fetuses, it was revealed that all eight analysed had a proportion of trisomic 21 ovarian cells at an average rate of 0.54%, with the abnormality occurring in meiotic and pre-meiotic cells (Hultén *et al*, 2008). This finding can be an explanation of recurrent aneuploid conception in young women. On the other hand, analysis of fetal testicular cells from male fetuses revealed that none of them were trisomic for chromosome 21, providing an extra explanation of the higher rate of cases of Down's syndrome that are maternal in origin (Hultén *et al,* 2010). The above finding is in line with the hypothesis that more stringent cell cycle control checkpoints exist during spermatogenesis than oogenesis.

1.2 Preimplantation genetic diagnosis and screening

Preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) are performed after IVF and biopsy of oocytes or embryos. Genetic analysis of polar bodies or embryonic cells can reveal those embryos carrying a genetic or chromosomal abnormality and only those that are unaffected are chosen for transfer back to the uterus (Wells and Delhanty 2001). Surplus, normal, good quality embryos can be cryopreserved and transferred in a later cycle. Cryopreservation of biopsied embryos does not affect their implantation and developmental potential when compared to cryopreserved blastocysts that have not undergone biopsy (El-Toukhy *et al*, 2009). Recently a new method for cryopreservation has been developed, called vitrification, which, when compared to slow freezing, shows significantly higher embryo survival, pregnancy and implantation rates (Keskintepe *et al*, 2009).

1.2.1 Biopsy

Biopsy of the oocyte or the embryo is essential in PGD and PGS to retrieve the sample for genetic analysis. Three different types of biopsy are used, all with several advantages and drawbacks. These are: biopsy of the polar bodies from the oocytes, biopsy of single blastomeres from cleavage stage embryos and biopsy of TE samples from blastocysts.

1.2.1.1 Polar body biopsy

Biopsy of the first polar body from the oocyte and/or the second polar body from the zygote can be performed. Biopsy of both polar bodies can either be sequential (Kuliev and Rechitsky, 2011), or simultaneous (Geraedts *et al*, 2011). The polar bodies, as resulting cells of meiosis, are not needed for fertilisation and embryo development. Polar body biopsy was first introduced for the detection of alpha-1-antitrypsin deficiency in a couple in which the woman was a carrier for the disorder. Diagnosis and selection of unaffected embryos was possible without the removal of embryonic samples (Verlinsky *et al*, 1990). The removal of the polar body can be performed after the opening of the zona pellucida by a laser followed by aspiration (Montag *et al*, 2004). Biopsy of the first and/or the second polar body is also used for the detection of chromosomal aneuploidies, since the majority of errors arise in maternal meiosis. Biopsy of just the first polar body is beneficial as errors in whole

chromosomes in the oocyte will be reciprocal in the polar body (Verlinsky *et al*, 1996).

The main disadvantage of biopsy at that stage is that indirect analysis of the oocyte through the polar bodies will miss the detection of errors that are paternal or post-zygotic in origin (Delhanty, 2011). Moreover, compensation of chromosomal errors arising through PSSC in meiosis I, which are present in the first polar body, may occur in meiosis II. As mentioned in section 1.1.4, following the segregation of chromatids in anaphase II, there is a 50% chance that the zygote will be euploid, whereas the second polar body will carry an abnormality reciprocal to the first polar body. This was demonstrated with the birth of a normal child from an oocyte with reciprocal aneuploid polar bodies (Scott *et al*, 2012a).

1.2.1.2 Cleavage stage biopsy

The removal of blastomeres from the cleavage stage embryo does not impair its further preimplantation development *in vitro* (Hardy *et al*, 1990). Moreover, a large prospective comparative follow-up study on babies born after PGD with biopsy at this stage showed that it did not impose any extra risks in regards to complications in the health of the babies at birth (Desmyttere *et al*, 2012). At cleavage stage biopsy single blastomeres are removed from day three embryos that have reached the six- to eight-cell stage by aspiration through a small opening drilled at the zona pellucida, which surrounds the embryo, usually by a laser.

A two-cell biopsy reduces the chance of error during diagnosis and increases the number of unaffected embryos that can be transferred (Lewis *et al*, 2001). A small, comparative study on the effects of the removal of one, two or three cells, performed on 188 cycles concluded that the implantation and pregnancy rates after two-cell removal were acceptable (Van de Velde *et al*, 2000). However, a larger study showed that the live birth rate in cases where one cell was biopsied was significantly higher than in those where two cells were biopsied and similar to standard ICSI cases, where no biopsy was performed (De Vos *et al*, 2009). Similarly, a randomised controlled trial (RCT) concluded that biopsy of one cell was less invasive than two, but on the other hand the diagnosis rate was significantly increased in PGD cases that utilised PCR for the analysis. A small but not statistically significant decrease in the live birth rate was observed after biopsy of two cells (Goossens *et al*, 2008). According to the European Society of Human

Reproduction and Embryology (ESHRE) PGD Consortium guidelines, two cells should be removed from one embryo only if it contains six or more cells (Harton *et al*, 2011a).

1.2.1.3 Blastocyst biopsy

Biopsy of TE from the blastocyst occurs at day five or six. An opening in the zona pellucida of the embryo is made one or two days before the biopsy by a laser through which the TE herniates allowing biopsy of around six cells with the assistance of a laser. The hole is made on the opposite side of the inner cell mass, which will lead to the formation of the embryo proper (Veiga *et al*, 1997). The ability to perform the diagnosis on a small number of cells overcomes issues faced in single-cell analysis, especially when single-cell DNA is amplified for the detection of single gene disorders (McArthur *et al*, 2005). One of the drawbacks of blastocyst biopsy is that not all embryos manage to reach that stage *in vitro* and as a result fewer embryos are available for analysis. On the other hand, a high pregnancy rate per oocyte retrieval has been reported post blastocyst biopsy with diagnosis of single gene disorders and structural chromosomal abnormalities (McArthur *et al*, 2008). Comparison between cleavage stage and blastocyst biopsy has shown that the latter results in higher implantation rates (Kokkali *et al*, 2007).

1.2.2 Preimplantation genetic diagnosis

Preimplantation genetic diagnosis (PGD) is an option for couples that are at risk of transmitting a genetic disease to their offspring or are carriers of structural chromosomal rearrangements and want to avoid the termination of an affected pregnancy following prenatal diagnosis (Verlinsky *et al*, 2004). It was first developed and applied for the determination of the sex of the embryo in cases of X-linked disorders with the use of polymerase chain reaction (PCR) amplification of loci on the Y chromosome (Handyside *et al* 1989; 1990). Fluorescence *in situ* hybridisation (FISH) was then used to replace PCR for the sex determination in cases of X-linked diseases (Delhanty *et al*, 1993) and for the detection of structural chromosomal abnormalities in couples with a balanced translocation (Munné, 2001). Suitable and optimised protocols, based on the amplification of embryonic DNA, are used for the diagnosis of autosomal dominant and recessive, as well as X-linked single gene disorders with a high diagnosis rate among the embryos analysed (Fiorentino *et al*, 2006). According to the latest data collection of the ESHRE PGD Consortium, 1363

cycles with oocyte collection for monogenic disorders and 774 for structural chromosomal abnormalities were performed between January and December 2008 (Goossens *et al*, 2012).

1.2.2.1 Techniques used in PGD for the detection of monogenic disorders

PCR amplification of embryonic cells is the most common technique used in PGD for monogenic disorders. Specific loci of interest can be amplified in a single PCR reaction on the biopsied cells, however, the number of loci is limited and the time needed for extensive optimisation of suitable protocols may be long (SenGupta and Delhanty 2012). Amplification of the whole genome, in order to facilitate the analysis of multiple loci across the genome, is an alternative technique. PGD using WGA was performed for the diagnosis of Familial Adenomatous Polyposis Coli (Ao *et al,* 1998) and since then, with suitable optimisation, a variety of whole genome amplification (WGA) methods have been applied clinically in PGD.

The WGA methods that have been successful in amplifying the minute amount of DNA found in a single cell are primer-extension preamplification (PEP, Zhang *et al*, 1992), degenerate oligonucleotide-primed-PCR (DOP-PCR, Telenius *et al*, 1992) and multiple displacement amplification (MDA). MDA is an isothermal whole genome amplification technique that uses φ29 DNA polymerase and random primers to produce fragments of amplified product that are larger than 10 kb long, representing the genome uniformly (Dean *et al*, 2002). MDA is able to amplify the whole genome of a single cell producing satisfactory results in PCR based downstream reactions (Handyside *et al*, 2004, Hellani *et al*, 2004, Spits *et al*, 2006) and has been used in PGD for the detection of a variety of monogenic disorders including fragile X syndrome, β-thalassaemia, cystic fibrosis and Marfan syndrome (Burlet *et al*, 2006, Hellani *et al*, 2004, Lledó *et al*, 2006). WGA is also used in preimplantation genetic haplotyping (PGH), which is indirect detection of mutations through linkage, using a large number of polymorphic markers linked to the disease-causing gene (Renwick *et al*, 2010).

Analysis of minute amounts of DNA, such as those in single cells, poses several problems and limitations, which, if not addressed, may lead to misdiagnosis. Contamination, allele drop out (ADO) and amplification failure may occur during manipulation and analysis of the samples, however, measures can be taken in order to minimise their effect. The first procedures performed during a PGD case, the

biopsy, tubing of the sample and the first amplification are more prone to contamination. Sources of contamination include the maternal cumulus cells surrounding the oocyte, sperm and external DNA (Wilton *et al*, 2009). Maternal and paternal contamination can be avoided by complete removal of the cumulus cells and the use of ICSI for fertilisation respectively. The use of gloves and protective clothing, UV decontamination and general clean conditions can be used for the limitation of external contamination. Finally, contamination can be monitored through the use of reaction negatives as well as the use of polymorphic markers in multiplex amplification reactions (Harton *et al*, 2011c).

Allele drop out occurs commonly during amplification of small DNA quantities. It is the failure of amplification of one of the two alleles in a heterozygote sample resulting in the appearance of a homozygote (Piyamongkol *et al*, 2003). Since ADO could be detrimental in the diagnosis, if undetected, the use of polymorphic markers linked to the mutation-causing gene was introduced. The mutation site and the loci of the linked markers can be amplified simultaneously in a multiplex PCR reaction. With the haplotype of the alleles determined, amplification of an affected sample with ADO on the mutation site can be detected when the marker allele in phase with the mutation is present (Rechitsky *et al*, 1998).

1.2.3 Preimplantation genetic screening

Preimplantation genetic screening (PGS) is performed in order to increase the implantation and pregnancy rates in a specific group of patients by analysing the chromosomal status of oocytes or embryos. In this way, embryos that are aneuploid are not selected for transfer and miscarriages as well as abnormal pregnancies are avoided (Munné *et al*, 1993). PGS is offered in couples of advanced maternal age (AMA), usually older than 37 or 38 years of age, couples that have gone through recurrent miscarriage (RM), which is established after three consecutive pregnancy losses, those that have had three or more repeated IVF failures (RIF) and finally those couples with severe male factor infertility, for example cases of non-obstructive azoospermia (NOA) or obstructive azoospermia (OA) with a normal karyotype, (Donoso *et al*, 2007). These groups of infertile patients have a high incidence of chromosomally abnormal embryos (Rubio *et al*, 2005). Indeed, in embryos from couples undergoing PGS, there is a significantly higher number of arrested aneuploid and mosaic embryos than euploid embryos (Rubio *et al*, 2007). A large number of PGS cycles are performed, as indicated by the latest data

collection of the ESHRE PGD Consortium. The number of PGS cycles with oocyte collection for the period between December 2008 and October 2009 was 3401 (with AMA as the most common indication), a much higher number than the overall number of PGD cycles for monogenic disorders and structural chromosomal abnormalities (Goossens *et al*, 2012).

PGS can be performed post polar body, cleavage stage embryo and blastocyst biopsy. There are benefits and drawbacks for each stage of biopsy. Analysis of the embryos for aneuploidy screening on day three, at cleavage stage, causes concerns especially due to mosaicism. Biopsy of one cell from a cleavage stage mosaic embryo may not be representative of the chromosomal status of the whole embryo. In a mosaic embryo, biopsy of a diploid blastomere will lower the proportion of these blastomeres and lead to the transfer of an embryo with a higher proportion of aneuploid cells. On the other hand, biopsy and analysis of an aneuploid blastomere will increase the number of diploid cells and therefore hamper possible viability of an embryo, which will be discarded due to the result (Harper *et al*, 2009). Comparison on the outcome of PGS analysis post polar body and blastocyst biopsy indicated a higher pregnancy rate after blastocyst biopsy (69.2%) than after polar body biopsy (21.4%) in a group of patients with repeated IVF failure (Fragouli *et al*, 2010).

Based on a valid theory, that transfer of euploid embryos will result in higher success rates, many IVF clinics started offering PGS in the above mentioned couples analysing chromosomes by FISH. Initial studies showed promising results. It was shown that PGS was beneficial in increasing implantation and pregnancy rates in couples with AMA and RIF, however two to four embryos were transferred (Gianaroli *et al*, 1997; 1999). PGS also seemed to be beneficial for RIF patients when compared to fertile controls receiving diagnosis for X-linked diseases (Pehlivan *et al*, 2003). Retrospective analysis of IVF cycles with either PGS or no PGS showed an increase in successful implantation rates and decrease in miscarriage in patients with AMA (Munné *et al*, 1999, Munné *et al*, 2003) as well as decrease of spontaneous abortion in patients with RM (Garrisi *et al*, 2009). However, it has been argued that studies showing good results post PGS, lack the needed strength to prove that PGS is indeed beneficial (Shahine *et al*, 2006). Several RCTs were published to evaluate the efficacy of PGS when FISH analysis was performed. Despite the fact that there were differences in the set up of the trials, including different time of biopsy, different number of probes used and blastomeres biopsied, the day of transfer, the patient cohort and the primary outcome, all of them showed

that PGS had no positive outcomes. Some of them even showed a negative impact in the implantation, pregnancy and live birth rates. A summary of all these RCTs is found in table 1.1.

Table 1.1: RCTs to evaluate PGS										
Study	**No of couples ****	**No of probes**	**Indication**	**Stage of biopsy**	**IR (%)**		**PR (%)**		**LBR (%)**	
					PGS	No PGS	PGS	No PGS	PGS	No PGS
Stevens *et al*, 2004	40	9	AMA	Cleavage	32	44	60	88.9	-	-
Staessen *et al*, 2004	400	7	AMA	Cleavage	17.1	11.5	19.6	27.7	-	-
Blockeel *et al*, 2008	200	7	RIF	Cleavage	21.4	25.3	21.4	25.3	-	-
Hardarson *et al*, 2008	109	7	AMA	Cleavage	11.4	18.9	11.4	18.9	5.4	18.9
Jansen *et al*, 2008	101	5	Infertile***	Blastocyst	-	-	45.5	60.9	35.7	58.7
Mastenbroek *et al*, 2007	408	5	AMA	Cleavage	11.7	14.7	11.7	14.7	24*	35
Mersereau *et al*, 2008	53	7	No indication	Cleavage	20.0	25.4	20.0	25.4	29.2	39.3
Staessen *et al*, 2008	240	7	No indication	Cleavage	-	-	48.6	43.9	34.6	34.6
Meyer *et al*, 2009	47	8	No indication	Cleavage	31.7*	62.3	31.7*	62.3	28.6*	68.2
Schoolcraft *et al*, 2009	62	9	AMA	Cleavage	36.5	37.3	67.7	76.6	78	67.74
Debrock *et al*, 2010	55	7	AMA	Cleavage	15.0	16.0	15.0	16.0	9.4	14.9

Table 1.1: RCTs performed to evaluate the efficacy of PGS. *IR: Implantation rate, which is the ratio of the number of gestational sacs with a fetal heartbeat over the total number of embryos that were transferred (Staessen et al, 2004). PR: Pregnancy rate. LBR: Live birth rate. *: Statistically significant lower measures. **: Number of couples randomized. ***: This study did not present the patients' indication for PGS.*

PGS is controversial due to its important limitations that are both technical and biological (reviewed by Go *et al*, 2009). Technically, not all chromosomes can be analysed by FISH as described below and biologically, embryos at the preimplantation stage may be mosaic.

1.2.3.1 Fluorescence *in situ* hybridisation

Fluorescence *in situ* hybridisation (FISH) involves the hybridisation of labelled DNA on genomic targets, which in the case of PGS are interphase nuclei (Speicher and Carter, 2005). The most important limitation of FISH is that analysis does not cover the whole genome and is only limited in the detection of abnormalities in a few chromosomes (Stumm *et al*, 2006). Probes for the chromosomes most commonly

involved in aneuploidies, more specifically those that are detected frequently in spontaneous abortions and live births, including chromosomes 13, 15, 18, 21, 22, X and Y, are used (Munné *et al*, 1998). Analysis of chromosomes can be performed in consecutive FISH rounds (Liu *et al*, 1998). A successful FISH protocol on embryonic nuclei, analysing all 24 chromosomes has only been described for research purposes (Ioannou *et al*, 2012), however in a clinical setting, protocols with probes for up to 12 chromosomes have been performed (Colls *et al*, 2009). Moreover, the accuracy of each probe is not 100%; it varies between 92 and 99%. The accuracy of a set of multiple probes, used in consecutive FISH rounds, which is needed in PGS, is, therefore, even lower (Mastenbroek *et al*, 2008). A false result may be obtained by FISH due to further technical limitations, for example signals that are split or overlapped or even lost before the analysis (DeUgarte *et al*, 2008). Finally, the outcome of FISH could be compromised by the fixation of the biopsied sample, which is essential for this technique (Coulam *et al,* 2007). All the limitations imposed to PGS because of FISH can be overcome with the use of comparative genomic hybridisation.

1.2.3.2 Comparative genomic hybridisation

Comparative genomic hybridisation (CGH) involves the analysis of all the chromosomes in one sample. The sample and a reference DNA are differentially labelled and compete with each other for complementary hybridisation sites. Analysis of the ratios of the sample to reference fluorescence gives an indication of chromosomal gain or loss (Speicher and Carter, 2005). The amount of DNA needed for analysis by CGH, which is around 1 microgram (μg), is much higher than that found in a single cell, 5-10 picograms (pg). For this reason, WGA of the biopsied DNA is essential in order to reach the required amount of DNA (Wells *et al*, 2008).

Two different CGH techniques have been applied in PGS with different hybridisation targets. In metaphase CGH (mCGH) the hybridisation site is metaphase spreads and in array CGH (aCGH) hybridisation is performed on mapped clones of the genome, for example in the form of bacterial artificial chromosomes (BAC) or oligonucleotides (figure 1.5). mCGH was developed as a molecular technique for the analysis of all 24 chromosomes and as an alternative to G-banding (Kallioniemi *et al*, 1992). The most suitable WGA technique prior to mCGH is DOP-PCR (Wells *et al*, 1999) and with suitable optimisation it can be applied successfully in the analysis of single blastomeres from embryos (Wells and Delhanty, 2000) with a

resolution of around 40 megabases (Mb) (Voullaire *et al*, 1999). mCGH was used clinically for the analysis of all chromosomes in single blastomeres for a case of AMA/RIF (Wilton *et al*, 2001). Comparison of PGS by mCGH to PGS by FISH in patients with RIF showed that the use of mCGH led to an increase of implantation and pregnancy rates, although without statistical significance, due to the small number of cases (Wilton *et al*, 2003). Despite the fact that mCGH can be performed for analysis of embryonic samples, its clinical application is difficult as it is a lengthy process and cryopreservation of embryos is needed when cleavage or blastocyst stage analysis is performed. Moreover, it requires molecular and extensive cytogenetic expertise to analyse the results (Wells *et al*, 2008).

Figure 1.5: Comparative genomic hybridisation

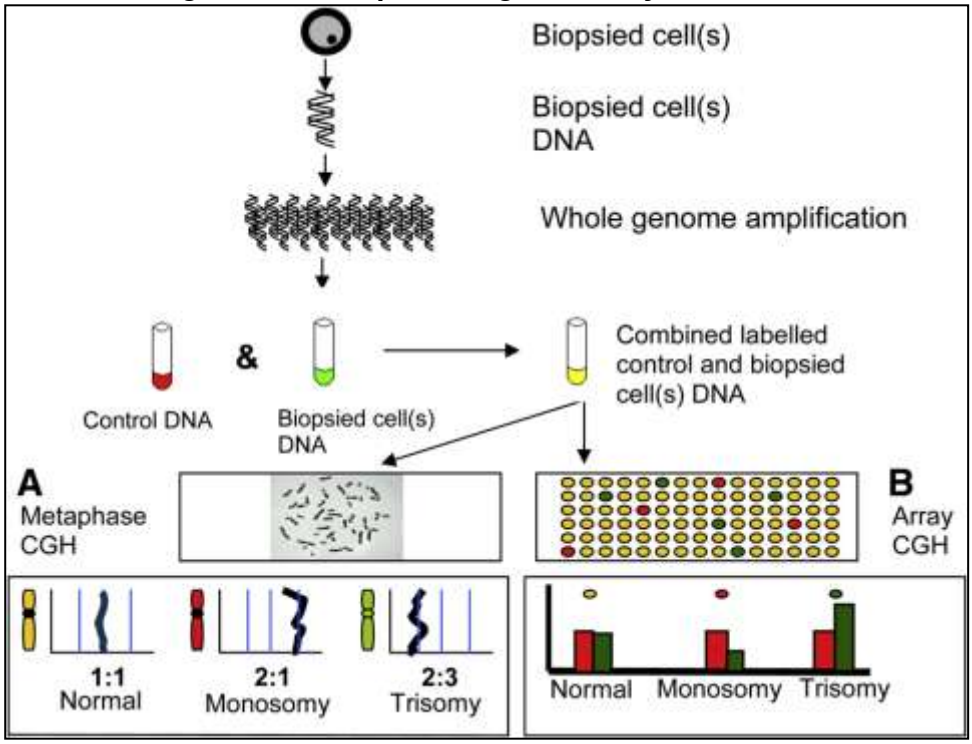


Figure 1.5: *Biopsied samples are subjected to whole genome amplification to increase the amount of DNA. The test and a reference DNA are then differentially labelled and hybridised either on metaphase spreads to perform mCGH (A) or slides containing clones representing the whole genome to perform aCGH (B). [Reprinted from Fertility and Sterility, 94 (4), Harper and Harton, The use of arrays in preimplantation genetic diagnosis and screening, 1173-1177, Copyright (2010), with permission from Elsevier]*

Array comparative genomic hybridisation (aCGH) is a technique used to detect chromosomal imbalance. It was developed as an alternative to metaphase CGH, in order to improve the resolution and detect small copy number gains and losses (Solinas-Toldo *et al*, 1997) initially for the analysis of cancer (Pinkel *et al*, 1998). It is quicker, more automated and simpler than mCGH making it ideal for use in PGS.

Successful aCGH with interpretable results can be performed in a clinical setting within 12 to 13 hours (Magli *et al*, 2011). It involves the hybridisation of labelled DNA on clones, which are fabricated on a solid platform, scanning of the platform and analysis of the results by a suitable software (Glentis *et al*, 2006). Obtaining a satisfactory result in aCGH is influenced by many factors, since a number of experimental steps are involved in the technique. A low signal to noise ratio, as well as a low standard deviation (SD) of the intensity ratios obtained from the fluorochores on all the clones are essential for a good quality result. Another important quality indicator of an aGCH experiment is the number of clones included in the analysis, as bad quality may cause some clones to fail (Vermeesch *et al*, 2005).

aCGH on single cells has been successful using a variety of WGA methods and clones on cells from tumor and other cell lines, euploid or carrying known aneuploidies. Combinations include DOP-PCR amplified samples hybridised on DOP-PCR amplified chromosome-specific libraries (Hu *et al*, 2004), MDA amplification and oligonucleotide clones (Le Caignec *et al*, 2006), GenomePlex, a type of adaptor-linker PCR amplification and BAC DNA clones (Fiegler *et al*, 2007) and finally combinations of three different WGA methods, DOP-, adaptor-linker PCR and MDA on BAC and oligonucleotide clones (Fuhrmann *et al*, 2008). BAC clones consist of DNA fragments that correspond to specific chromosomal regions of a size 150-200kb. Despite the fact that BAC arrays have fewer clones than other type of arrays, they are enough for comprehensive chromosome screening. Since sites of the genome are represented multiple times on each array, WGA artefacts like amplification failure or ADO can be supressed (Sills *et al*, 2012).

aCGH has been applied clinically for the detection of chromosomal aneuploidies in PGS. Again a variety of WGA methods and array platforms are used. The first application, after blastomere biopsy, involved the use of MDA with oligonucleotide arrays (Hellani *et al*, 2008). aCGH has also been clinically applied for the analysis of biopsied polar bodies and trophectoderm samples (Fishel *et al*, 2010, Fragouli *et al,* 2011a). When aCGH is applied during PGS on trophectoderm samples from blastocysts, the result obtained is an average of all the cells in the biopsied sample. Mosaicism is not detected in detail as in FISH, when each cell is analysed separately. The importance of this in the clinical outcome is not yet known. However, one hypothesis is that this low-level mosaicism might not be of clinical importance and that embryos of this constitution might lead to a normal pregnancy (Fragouli *et al*, 2008).

One major disadvantage of aCGH is that, in some cases, it cannot detect abnormalities in the ploidy of the analysed sample, such as haploidy or triploidy (Harper and Harton, 2010). The detection of structural chromosomal rearrangements is possible with aCGH. However, no distinction can be made between chromosomally normal and balanced carrier embryos (Alfarawati *et al*, 2011, Fiorentino *et al,* 2011). The ESHRE PGS task force will perform an RCT (Geraedts *et al*, 2011) on the efficacy of aCGH in PGS with polar body analysis. Recently, a randomised pilot study was performed in good prognosis patients to examine the outcome of single blastocyst transfer after selection of embryos by aCGH and by morphology alone. Results showed significantly higher pregnancy and ongoing pregnancy rates in the couples that had aCGH than those who had a transfer based on morphology (Yang *et al*, 2012).

1.2.3.3 Single nucleotide polymorphism arrays

Screening of the whole genome can also be performed by the use of single nucleotide polymorphism (SNP) arrays. These are oligonucleotide arrays of high density, which are able to detect copy number changes at the nucleotide level (Speicher and Carter, 2005). In addition to the ability to identify changes in copy number of chromosomes, SNP arrays can also detect monogenic disorders through haplotyping, as well as, other multifactorial inherited diseases like autism and diabetes (Harper and Harton, 2010). Analysis by SNP arrays is possible on single cells (Treff *et al*, 2010) and has been shown to have high overall predictive value (Scott *et al*, 2012b).

Clinical application of SNP arrays during PGS for AMA has been described post biopsy of both polar bodies (Scott *et al*, 2012a). SNP arrays have also been used after blastocyst biopsy and vitrification, in couples with RIF, RM and AMA. A very high implantation (67%) rate per embryo transferred and live birth rate 55.9% per oocyte retrieval for 130 cycles was reported (Schoolcraft *et al*, 2011). Finally, Treff *et al*, have described the ability to distinguish between balanced and normal chromosomes in embryos from a couple with a female translocation carrier. This was again performed in a clinical setting following blastocyst biopsy, resulting in the birth of a male with a normal karyotype (Treff *et al*, 2011).

1.3 Aims

1.3.1 Aim 1: Validation of aCGH for clinical use in PGS

The first aim of the study was to validate aCGH for the clinical use in PGS. This was performed first through the utilisation of cell lines of known chromosomal status. The efficacy of aCGH post cleavage stage biopsy was assessed on single cells isolated from the cell lines. The effect of mosaicism in a biopsied sample from a blastocyst was examined by the use of mosaic models containing euploid and aneuploid cells from the most stable cell lines. The second step of validation was to test aCGH on embryonic samples and examine concordance between a biopsied TE sample and the remaining of the blastocyst. The final step was to confirm the clinical aCGH result in PGS cases after cleavage and blastocyst stage biopsies on untransferred embryos, through the use of FISH.

1.3.2 Aim 2: Examination of aneuploidy level in embryos from couples undergoing PGD

The second aim of the study was to determine the level of aneuploidy in embryos from couples undergoing PGD for single gene disorders. One group of embryos was analysed by FISH testing all cells for a small number of chromosomes and a second group by aCGH analysing all chromosomes in whole embryos. Through the aneuploidy analysis, it was aimed to examine the effect of aneuploidy in embryonic development, the effect of age in the rate of aneuploidy and the origin of abnormalities. Comparison with the level of aneuploidy in embryos from PGS cycles was performed, aiming to identify distinct differences in the aneuploidy level in embryos from PGD and PGS cycles.

1.3.3 Aim 3: Investigation of recombination in embryos

The third and final aim was to investigate recombination in embryos. Polymorphic markers on known areas of the genome of high and low recombination were utilised to identify cross-over events in embryos from couples undergoing PGD and PGS. Recombination was calculated per family and per embryo with the aim to identify differences in the recombination between chromosomes, sexes and different age groups. The possibility of differences in recombination between embryos and the general population was also examined.

2 Materials and Methods

2.1 Materials and methods outline

The Material and Methods chapter has been organised in three main sections:

1. Sample collection and preparation: describes all the samples used and the techniques followed to collect and prepare them prior to all subsequent experiments.

2. Sample processing: describes the three main procedures used for examining the samples depending on the analysis that followed. This included amplification of DNA and hybridisation of chromosomes with fluorescent probes.

3. Sample analysis: presents the methods employed to analyse and interpret the results of the tests conducted on the samples.

The techniques used in each section to fulfil appropriate aims are shown in table 2.1. Figures 2.1, 2.2, 2.3 are flowcharts for each aim presenting the samples and techniques used.

All reagents and general laboratory consumables were from VWR, International, unless otherwise stated. All fluorescent primers were from Eurogentec, UK and all fluorescent probes were from Abbott Molecular, UK.

Consumables and solutions that needed sterilisation were autoclaved using the New Swiftlock autofill steriliser (Astell Scientific, UK). General laboratory equipment included a microcentrifuge (MicroCentaur, Sanyo, UK) and a benchtop centrifuge (Heraus, Labofuge 300, Thermo Scientific, USA). The thermal cyclers used were the Mastercycler gradient, Mastercycler ProS (both Eppendorf, UK) or the GeneAmp® PCR System 9700 (Applied Biosystems, UK).

Aim	Sample collection and preparation (Materials and Methods section)	Sample processing (Materials and Methods section)	Sample analysis (Materials and Methods section)
1	Single cell isolation (2.3.1.2) Mosaic trophectoderm and blastocyst models (2.3.1.3) Embryo disaggregation and tubing (2.3.2.3)	Whole genome amplification (2.4.1) FISH (2.4.2)	aCGH (2.5.2) Fluorescent microscope analysis (2.5.3)
2	Embryo spreading (2.3.2.4) Embryo disaggregation and tubing (2.3.2.3)	FISH (2.4.2) Whole genome amplification (2.4.1)	Fluorescent microscope analysis (2.5.3) aCGH (2.5.2)
3	DNA extraction (2.3.3.1) Embryo disaggregation and tubing (2.3.2.3)	Whole genome amplification (2.4.1) FPCR (2.4.3)	aCGH (2.5.2) STR analysis (2.5.4) Haplotype analysis (2.5.4.1)

Table 2.1: Techniques used for sample collection, preparation, processing and analysis to fulfil all aims of this thesis

Figure 2.1: Aim 1 - Validation of aCGH for clinical use in PGS

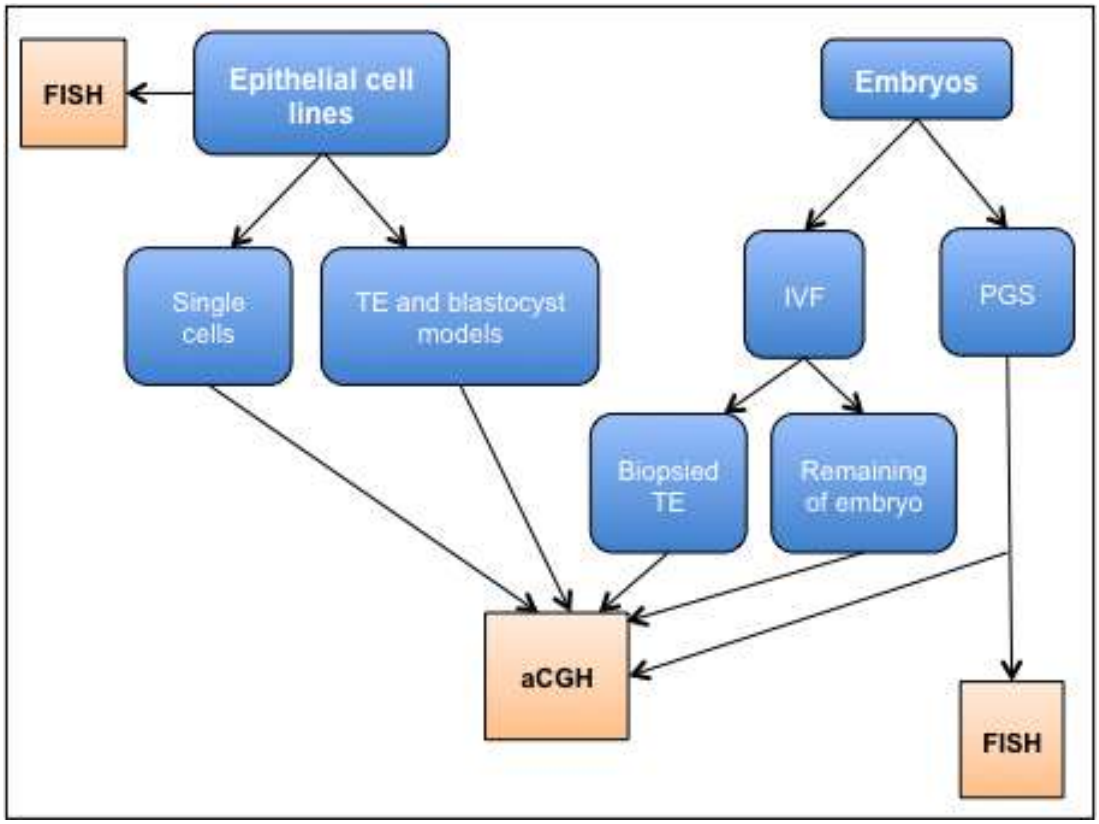


Figure 2.1: Aim 1. *Epithelial cell lines and embryos were used for the validation of aCGH to be used clinically during PGS for the detection of numerical chromosomal abnormalities.*

Figure 2.2: Aim 2 - Examination of aneuploidy level in embryos from couples undergoing PGD

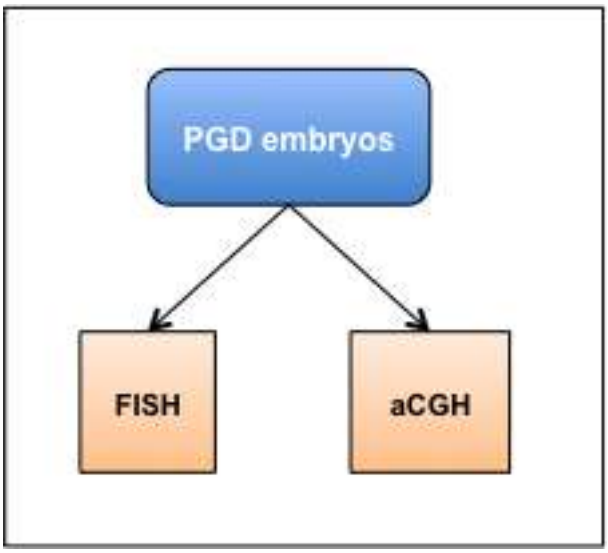


Figure 2.2: Aim 2. *Embryos from patients that had undergone PGD for single gene disorders were analysed by FISH and aCGH to determine the aneuploidy level.*

Figure 2.3: Aim 3 - Investigation of recombination in preimplantation embryos

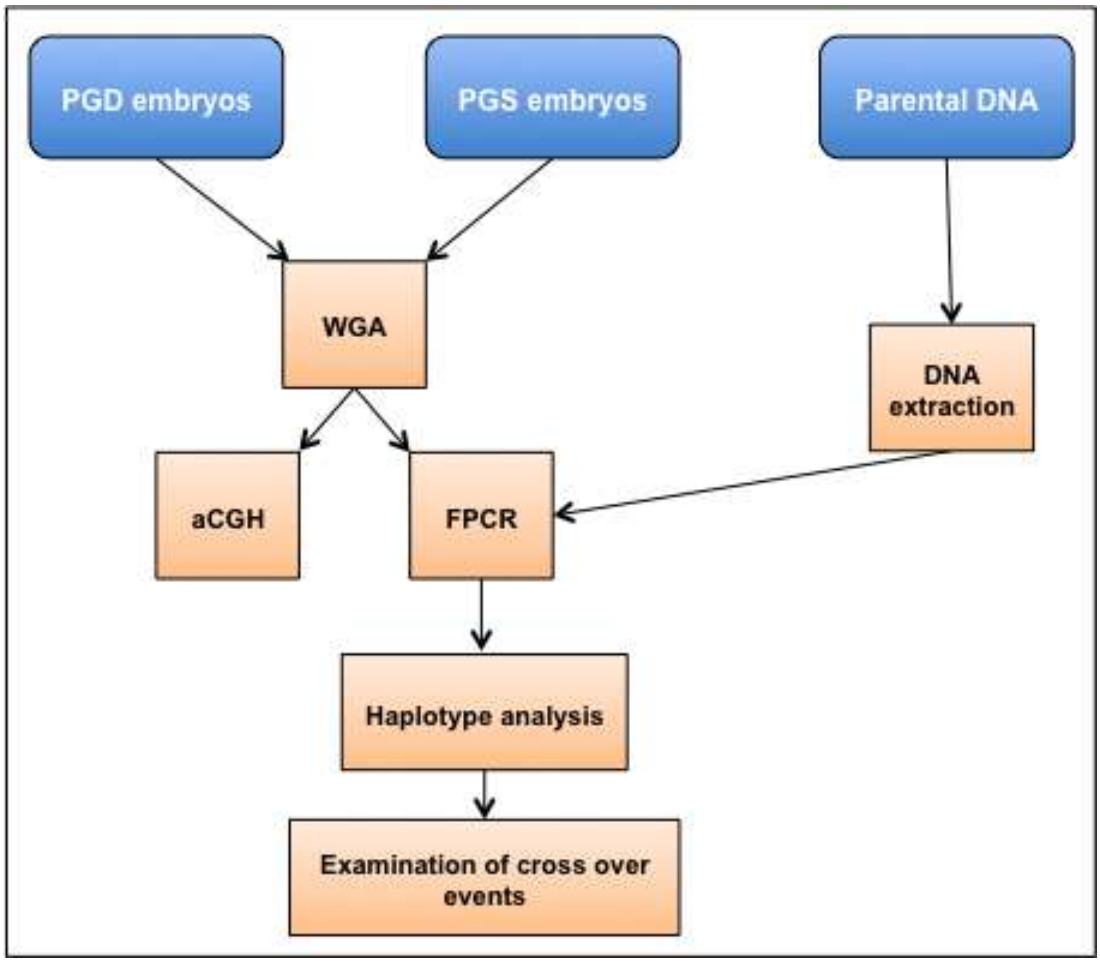


Figure 2.3: Aim 3. *Recombination and aneuploidy levels were determined by FPCR and aCGH respectively in embryos from couples undergoing PGD and PGS.*

2.2 General laboratory practice

Techniques prior to DNA amplification that needed clean conditions, like single cell isolation, preparation of mosaic models, embryo disaggregation, as well as all primer reconstitutions and setting up of PCR and WGA amplifications were performed in a clean room used by designated staff. This room was constantly maintained under positive pressure and the total volume of air was changed 20 times per hour. Dedicated lab coats were available and disposable fresh hat, overshoes and gloves were worn before entering. All consumables, apart from samples and primers, brought in the clean room were placed under ultraviolet (UV) light in the AirClean 600, PCR Workstation, AirClean Systems (Starlab, UK) for at least 10 minutes. All amplification reactions were set up under a Microflow Bio-safety cabinet class II, equipped with a UV light for decontamination. All pipettes, double-filtered, sterile, DNA- and RNA-free pipette tips and tubes were kept inside the safety cabinet and were decontaminated daily. All post amplification work was performed in areas separated from the clean room with dedicated pipettes and any other consumables needed.

2.3 Sample collection and preparation

2.3.1 Epithelial cell lines

Ovarian epithelial cell lines were used for the validation of aCGH for clinical application in PGS (Aim 1). The cell lines were provided by the Translational Lab, Institute for Women's Health, University College London and were received as pellets.

2.3.1.1 Description and pre-treatment

Overall, four different cell lines were used: TOV-21G, SKOV3, IOSE-1 and IOSE-19. The first two lines carried known aneuploidies and the last two, IOSE-1 and IOSE-19, were euploid. FISH was performed in all the cell lines, as described in section 2.4.2 of the Materials and Methods, to confirm the presence or absence of aneuploidies and to examine possible cell-to-cell variation within each line. All cell lines were from the same passage, minimising differences that may arise in the cells' constitution of one cell line from different passages.

Cell lines were received as pellets in 15 millilitre (ml) Falcon tubes. The pellets were resuspended in 15ml of Dulbecco's 1x phosphate-buffered saline (PBS, Invitrogen, UK) and centrifuged at 1300 revolutions per minute (rpm) for 15 minutes (min) in a benchtop centrifuge. The supernatant was discarded and the pellets were re-washed twice with 15ml of PBS and pelleted by centrifugation at 1300rpm for 15 min. After the final wash the pellets were resuspended in 2ml of PBS and kept at 4°C until use.

2.3.1.2 Single cell isolation from epithelial cell lines

Isolation of single cells was performed under an inverted microscope (Nikon, USA). Small, 3 microliters (μl), wash drops of Dulbecco's 1xPBS (Invitrogen, UK) containing 1% polyvinyl alcohol (PVA, Sigma-Aldrich, UK) were aliquoted on a Petri dish together with a bigger, 12μl PBS/PVA suspension drop. Three μl of cell suspension were transferred to the suspension drop on the dish. Transfer and isolation of cells was performed with a 0.2-millimeter (mm) polycarbonate microcapillary (Biohit, Finland) attached to a mouth or a hand pipette (Cook, UK). A small number of cells were transferred from the suspension drop to the first wash drop. Cells were transferred to consecutive wash drops until there was only one cell visible in the drop. The single cell was then washed in three fresh drops and finally aliquoted to an empty 0.2ml, thin walled, RNase-, DNase-, DNA- and pyrogen-free, non sterile, microfuge tube (Molecular BioProducts, UK). After tubing, all cells were pulse spun in a microcentrifuge and were kept at -20°C until use. For the validation of aCGH for PGS after biopsy of single blastomeres, all single cells were coded at this stage for blind analysis.

2.3.1.3 Mosaic trophectoderm and blastocyst model preparation

For the validation of aCGH for clinical application in PGS after biopsy of a trophectoderm sample, mosaic trophectoderm and blastocyst models were prepared using two of the above mentioned epithelial cell lines, TOV-21G, which consisted of aneuploid cells, carrying trisomy for chromosome 10 and the euploid IOSE-19. Similar to the single cell isolation procedure, PBS/PVA drops were prepared on a Petri dish. Cells were transferred in the suspension drop and were washed in fresh PBS droplets. For the mosaic TE models a total of eight cells were prepared for each 0.2 microfuge tube, with the number of euploid:aneuploid cells to be 8:0, 6:2, 5:3, 4:4, 3:5, 2:6 and 0:8, in duplicates (TE group A and TE group B). All the cells

required for each sample were transferred in the final drop and then into a fresh 0.2ml microfuge tube.

The total number of cells in the mosaic blastocyst models was 100 and 10 samples were prepared, in which the euploid:aneuploid number of cells was 100:0, 75:25, 50:50, 25:75, 0:100, again in duplicates (Blastocyst group A and Blastocyst group B, figure 2.4). Due to the high number of cells in each sample and in order to avoid transfer of excessive PBS/PVA, the cells were transferred in 0.2ml PCR microfuge tubes in groups of 10.

Figure 2.4: Mosaic trophectoderm and aneuploid models

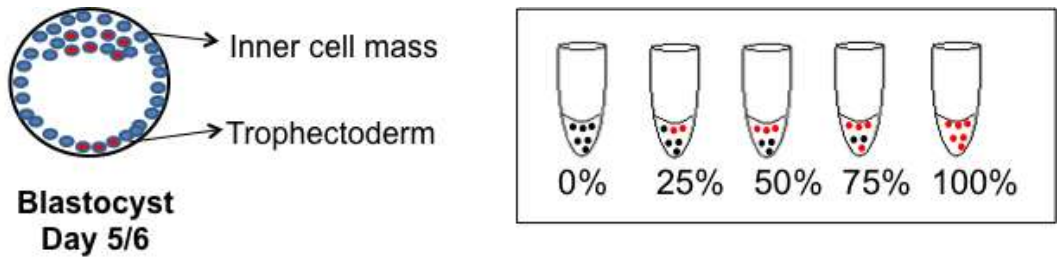


Figure 2.4: *Aneuploid (red) and euploid (black) cells were used to generate mosaic TE and blastocyst models with different levels of aneuploidy (right), mimicking a mosaic blastocyst (left).*

2.3.2 Human embryos from IVF, PGD and PGS cycles

To achieve the three aims of the project (figure 2.5) human embryos were collected from patients attending the Centre for Reproductive and Genetic Health (CRGH) who had given informed written consent to donate their embryos for research. This work was licenced by the Human Fertilisation and Embryology Authority (HFEA project reference: RO113) and ethical approval was granted by the national research ethics service (NRES), research ethics committee (REC reference number: 10/H0709/26).

For the aCGH validation (aim 1), frozen blastocysts donated for research from routine IVF patients were thawed and biopsied by the embryologists at the CRGH. The TE biopsied samples as well as the remainder of the blastocysts were tubed separately for analysis. Embryos from PGS patients were also included in this study. Once aCGH was applied clinically, embryos that were found to be aneuploid were spread onto slides (Materials and Methods section 2.3.2.4) to confirm the presence

of chromosomal anomalies seen during the diagnosis in the whole embryo by FISH (Materials and Methods section 2.4.2). The WGA product from some PGS cases was also used for the aneuploidy and recombination studies (aim 2 and 3).

Embryos from patients undergoing PGD for single gene disorders were used to determine the level of aneuploidy in these couples (aim 2) and examine recombination events (aim 3). Those were embryos that had been diagnosed as affected with a single gene disorder after PGD or were unaffected but unsuitable for transfer due to developmental arrest. The embryos were either spread whole for FISH analysis, or tubed whole for aCGH and haplotype analysis.

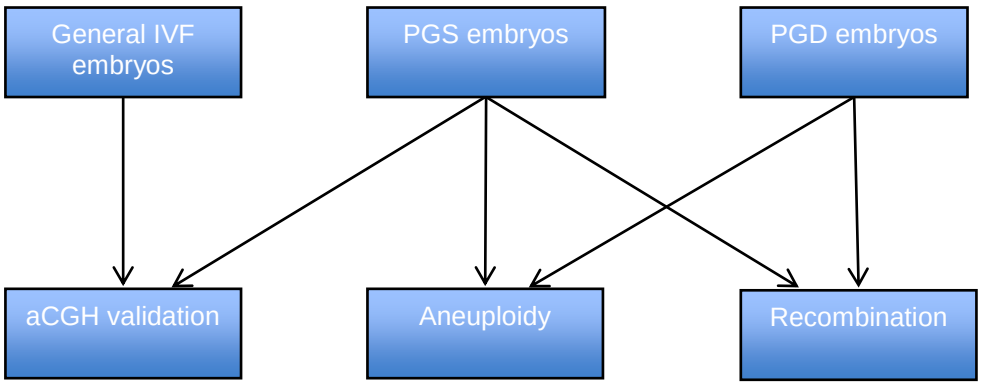


Figure 2.5: Use of human embryos in different projects

2.3.2.1 IVF cycle

All IVF cycles were performed at the CRGH and the embryologists conducted the embryo fertilisation and morphology checks as well as the embryo biopsy. Fertility checks were done in all couples to verify their suitability for the IVF procedure. An ovarian reserve test (ORT) was performed in all women at day two or day four of their cycle. Sperm parameters were checked for all men prior to IVF. Vaginal oocyte collection was performed 35 hours after the administration of human chorionic gonadotrophin (hCG). hCG injection was given when the two lead follicles had reached 18mm, following ovarian stimulation. Fertilisation of all the embryos used in this thesis was performed by intracytoplasmic sperm injection (ICSI). A single sperm was injected into each mature oocyte 41 hours after hCG administration. Sixteen to 20 hours post insemination, on day one, the embryologists checked for the formation of pronuclei in each embryo. On day two, the morphology of the embryos was scored and on day three, 68 to 72 hours post insemination, cleavage stage

biopsy of single blastomeres was performed. Blastocyst biopsy was performed on day 5 or day 6, when trophectoderm had started to hatch from the hole drilled in the zona on day three.

Cleavage stage biopsies and zona drilling for blastocyst biopsies were performed using an Octax laser (MTG, Germany). For PGD and PGS cleavage stage biopsies, two blastomeres were removed from embryos that had more than five cells and one was removed from the rest. For the biopsy, embryos were placed into calcium and magnesium (Ca^{2+}/Mg^{2+}) -free media to stop compaction of the cells (SAGE media, Biopharma, USA). For PGS embryos that were analysed by aCGH, blastocyst biopsy was performed in media that contained Ca^{2+}/Mg^{2+} (HEPES media, Invitrogen UK).

2.3.2.2 Embryo collection from the Centre for Genetic and Reproductive Health

Embryos donated from IVF, PGD and PGS patients and were used in this thesis were transferred from the CRGH to the UCL Centre for PGD in culture dishes, in an insulated box. The embryologists in the CRGH performed the scoring of the embryos at the day of collection. They were kept in an incubator at 37°C until use.

2.3.2.3 Whole embryo tubing

Embryos were tubed whole for molecular, haplotype analysis and/or molecular cytogenetic analysis by aCGH. This procedure was performed in the clean room. Petri dishes were prepared with 3μl PBS/PVA drops. Embryos were observed under an inverted microscope to confirm the developmental stage and morphology provided by the embryologists. They were then transferred from the culture dish to a PBS/PVA drop with a 0.2mm or 0.35mm microcapillary (Biohit, Finland), attached to a mouth or a hand pipette, depending on the size of the embryo. Initially, the embryo was removed from the zona completely. For biopsied embryos, this was performed by gentle pipetting of the embryo in and out of the microcapillary in order for it to be released from the zona through the biopsy hole. If the embryo had not been biopsied or was not possible to aspirate it through the biopsy hole it was transferred to a 3μl Acid Tyrode's (MediCult Ltd, UK) drop. It was then observed until the zona had dissolved and then immediately transferred to a fresh PBS/PVA drop. Finally,

the embryo was tubed whole in separate 0.2ml microfuge tubes. The tubes were pulse centrifuged and kept at -80°C until use.

2.3.2.4 Embryo spreading

The embryos were spread on slides coated with poly-L-lysine to be analysed by FISH. The slides were prepared by incubating them for 5 minutes in a coplin jar containing poly-L-lysine (Sigma-Aldich, UK) diluted 1:10 in deionised water (dH_2O). They were then left to dry at room temperature and were stored at 4°C.

For the spreading, a circle was made on the bottom of the slide with a diamond marker. The circle was filled with 10μl spreading solution, which was prepared fresh [0.01N hydrochloric acid (HCl)/0.1% Tween® 20, Sigma-Aldrich, UK]. A 10μl drop 1xPBS was placed adjacent to the spreading drop [for 1000ml, 5 tablets of PBS (Sigma-Aldrich, UK) were dissolved in 1000ml of dH_2O] and was used for washing. The embryo was transferred from the culture dish to the PBS drop with a 0.2mm or 0.35mm microcapillary under a dissecting microscope (Nikon, USA). From the PBS drop the embryo was transferred to the spreading drop and the slide was transferred on an inverted microscope (Olympus, UK). The spreading solution was agitated carefully and the embryo was observed for the cell membrane to start to lyse and the cytoplasm to be washed away. The slide was left to dry, then incubated in PBS for 5 minutes and then dehydrated in 70%, 90% and 100% ethanol for 5 minutes each (ethanol series dehydration) at room temperature. When the slide was dry the co-ordinates of the location of the spread embryo was recorded under a phase microscope with an England Finder (Optech, UK). The slides were stored for up to 2 weeks at 4°C until FISH was performed.

2.3.2.5 Blastocyst biopsy and trophectoderm tubing

Blastocyst biopsy and tubing of the TE sample of embryos donated for research from general IVF couples was performed at the CRGH for the validation of aCGH. These were frozen blastocysts and were thawed for this purpose. Biopsy was performed by the embryologists in those embryos that showed satisfactory expansion post thawing.

TE tubing was performed at the CRGH under a laminar flow class II cabinet, equipped with a UV bulb for decontamination prior to use. All tubes, ice racks and solutions needed for the tubing were kept clear from DNA contamination. Petri

dishes with a diameter of 5 centimetres (cm), containing 1xPBS/PVA drops were prepared. A new petri dish and microcapillary were used for each embryo. Biopsied samples were provided from the embryologists in the biopsy dish. The sample was located in the drop containing biopsy media under a dissecting microscope (Nikon, USA) and transferred with a 0.2mm microcapillary to a PBS/PVA drop. The TE sample was washed three times in fresh drops and transferred to a labelled 0.2ml PCR tube. A witness performed the labelling of the tubes and checking of the embryo identification.

2.3.3 Genomic DNA

Patient DNA was extracted from whole blood for analysis in the recombination study (aim 3). Blood samples were received from couples undergoing PGD and PGS at the CRGH in ethylenediaminetetraacetic acid (EDTA) tubes.

2.3.3.1 DNA extraction from whole blood using the Qiagen QIAmp® Blood Maxi Kit

The “spin protocol” was carried out according to the manufacturer’s guidelines (Qiagen, UK). All the steps were performed in a Class II safety cabinet and all supernatants and used pipettes were discarded in a beaker containing 5 PreSept tablets (Johnson + Johnson, UK) diluted in 1000ml of H_2O, for decontamination purposes. Briefly, 3-5ml of whole blood were mixed thoroughly with 500µl protease enzyme and 6ml of buffer AL to lyse the cells. The samples were incubated at a 70°C water bath for 10 minutes. Five ml of 100% ethanol were added and the samples were shaken vigorously to ensure sufficient binding to the membrane of the QiAmp Maxi column inserted in 50ml centrifuge tubes, where the samples were then transferred. The samples were centrifuged at 3000rpm in a benchtop centrifuge (Heraus, Labofuge 300, Thermo Scientific, USA) for 3 minutes. After removal of the filtrate, the DNA bound on the membrane was washed once with 5ml of buffer AW1 and once with 5ml of buffer AW2, by centrifugation at 5000rpm for 1 minute and 15 minutes respectively. The filtrate was removed between the washes. Any remaining ethanol on the membranes was evaporated by incubation at 70°C for 10 min, without the tube lids. Finally, DNA was collected by adding 600µl of elution buffer AE on the membrane and centrifuging once at 5000rpm for 2 minutes. Six hundred µl of elution buffer were added again on the filter, the samples were incubated at room temperature for 5 minutes and then centrifuged at 5000rpm for 5 minutes. The

DNA was washed with elution buffer twice in order to achieve maximum yield. Samples were then stored at 4ºC. This technique of DNA extraction produced samples with a DNA concentration of around 80ng/μl.

2.4 Sample processing

2.4.1 Whole genome amplification

Whole genome amplification (WGA) was performed for representative amplification of the whole genome and was used for further analysis by aCGH and/or haplotyping. GenomePlex® (Sigma-Aldrich, UK) was used for the amplification of DNA from single cells isolated from epithelial cell lines for the clinical validation of aCGH at single blastomeres. SurePlex (BlueGnome Ltd, UK) was used for all other samples that were amplified by WGA (mosaic TE and blastocyst models, embryos). Both of these WGA methods were based in a similar technology.

2.4.1.1 GenomePlex®

The GenomePlex® single cell whole genome amplification (WGA-4) kit (Sigma-Aldrich, UK) was used, with the modification described by Fiegler *et al* (2007). The amplification procedure of the single cells was performed by BlueGnome Ltd, UK.

2.4.1.2 SurePlex

SurePlex (BlueGnome Ltd, UK) provided representation of 70-90% of the genome resulting in an amplified sample of 2-5μg and it was performed following the manufacturer's protocol. The sample was first lysed, followed by the pre-amplification step, during which DNA libraries of short, overlapping amplimers were constructed with self-inert degenerative primers that were annealed at multiple sites of the genome. In the final amplification step multiple fragments spanning each site were created (figure 2.6).

Figure 2.6: WGA amplification with SurePlex (BlueGnome Ltd, UK)

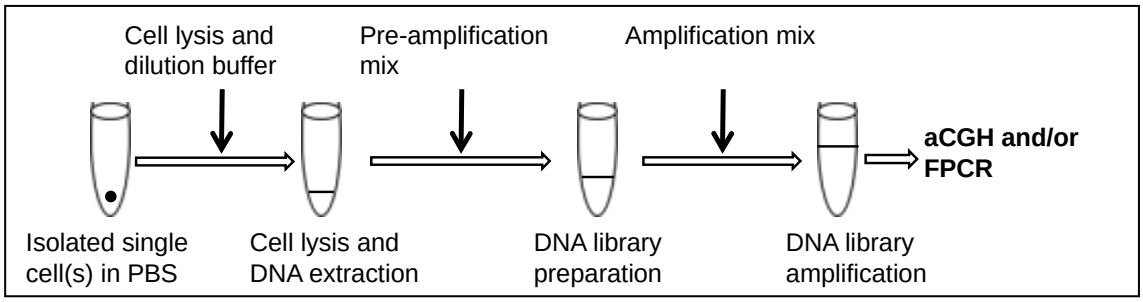


Figure 2.6: *Small amount of DNA was amplified reaching a 70-90% representation of its whole genome at a quantity of 2-5μg*

The samples were first pulse centrifuged to ensure all contents were at the bottom of each tube. It was assumed that the volume of PBS/PVA with the isolated cell in the tube was 2μl. Three μl of Cell extraction buffer were added in each sample to make up to 5μl of total volume. For the cell lysis and DNA extraction, 4.8μl of Extraction enzyme dilution buffer and 0.2μl of Cell extraction enzyme were aliquoted in each tube. The samples were then incubated at 75°C for 10 min followed by 4 min at 95°C in a thermal cycler. For the preparation of the library of short, overlapping amplimers, 4.8μl of SurePlex pre-amp buffer and 0.2μl of SurePlex pre-amp enzyme were added in each sample and were placed in the thermal cycler for the pre-amplification procedure seen in table 2.2.

Table 2.2: SurePlex pre-amplification program		
Temperature	**Time**	**Cycles**
95°C	2 min	1 cycle
95°C	15 sec	12 cycles
15°C	50 sec	
25°C	40 sec	
35°C	30 sec	
65°C	40 sec	
75°C	40 sec	
4°C	Hold	1 cycle

Table 2.2: Pre-amplification by SurePlex. *DNA libraries were prepared in this procedure in a thermal cycler.*

When pre-amplification was complete, the tubes were pulse centrifuged and 25μl SurePlex amplification buffer, 0.8μl SurePlex amplification enzyme and 34.2μl nuclease-free water were added in each sample. The DNA of the samples was then amplified in a thermal cycler following the program seen in table 2.3.

Table 2.3: SurePlex amplification program		
Temperature	**Time**	**Cycles**
95°C	2 min	1 cycle
95°C	15 sec	14 cycles
65°C	1 min	
75°C	1 min	
4°C	Hold	1 cycle

Table 2.3: Amplification by SurePlex. *Constructed DNA libraries of amplimers were amplified during this program in a thermal cycler.*

Amplified products were kept at -20°C until use. If more than two samples were amplified at the same time then reaction mixes were prepared for the extraction, pre-amplification and amplification procedures, containing the required volume of each reagent and the required volume of the reaction mix was added to each sample to minimise aliquoting.

2.4.2 Fluorescence *in situ* hybridisation (FISH)

The chromosomal constitution of embryos post PGD and PGS was analysed by FISH as part of the aCGH validation (aim 1) and the examination of the level of aneuploidy in couples undergoing PGD (aim 2). A variety of fluorescent probes was used in sequential rounds of FISH. A general outline of the procedure is given below and details of all the probes used and conditions are listed in table 2.4.

2.4.2.1 Slide preparation

After spreading on poly-L-lysine coated slides the embryos were digested to remove the cytoplasm and make the nuclei accessible for the fluorescent probes to hybridise. The digestion solution, 0.01HCl [49ml dH_2O, 0.5ml 1M HCl (for a 50ml Coplin jar)] was prepared in a 50 or 100ml Coplin jar (depending on the number of slides) and warmed up to 37°C in a water bath. Once the solution had reached the desired temperature, 0.5ml of pepsin (10mg/ml – Sigma-Aldrich, UK) were added and mixed using a Pasteur pipette. The slides were then placed in the Coplin jar and

incubated for 15 minutes in the 37°C water bath. The HCl/Pepsin solution was poured and the slides were washed once with dH_2O and once with 1xPBS. The samples were then fixed on the slides by incubation for 10 minutes in 1% paraformaldehyde/PBS [49ml 1xPBS, 1.34ml paraformaldehyde [to make 500ml 1% paraformaldehyde: 5cm of sodium bicarbonate ($NaHCO_3$) in a one litre (L) bottle were measured, 500ml formaldehyde were added and the solution was left to saturate overnight)] at 4°C. The slides were then rinsed again once with 1xPBS and twice with dH_2O.

2.4.2.2 1st round FISH

The slides were first dehydrated through an ethanol series and were left to air dry on a rack in a vertical position. The probe mix was prepared containing the desired fluorescent probe(s), the suitable buffer and dH_2O in a 0.5ml autoclaved microfuge tube. Depending on the probe(s) used, denaturation of the probe(s) and the slides was performed simultaneously (co-denaturation) or separately (sep-denaturation). All work with the FISH fluorescent probes was performed under darkened conditions. If co-denaturation was used, 13mm round glass cover slips were arranged and five μl of probe mix were aliquoted on each slip. Each slide was inverted on each cover slip at the position of the marked area of the sample. The slides and probes were then denatured at 75°C for 5 minutes. They were then placed in a dark humid chamber (humidity was created by a wet tissue at the bottom of the chamber) and if overnight hybridisation was performed the cover slips were sealed with Fixogum rubber cement (QBiogene, UK).

For the probes that required sep-denaturation, the probe mix was prepared in a 0.5ml tube as described above and denatured at 75°C for five minutes in a denaturation oven. It was then kept at a 37°C water bath until use. The slide denaturation solution was prepared by mixing 70μl of deionised formamide, 20μl of dH_2O and 10μl of 20xSaline sodium citrate [SSC – 175.3g of 3M Sodium Chloride (NaCl), 88.2g of 3M Sodium Citrate, dissolved in 1000ml of dH_2O and adjusted with 1M HCl or 1M sodium hydroxide (NaOH) to pH 7.0] for each slide. This 70% deionised formamide solution was then pipetted onto 55x22mm glass cover slips, 100μl for each slide and the slides were inverted on them. Denaturation of the slides was performed at 75°C for five minutes. The cover slips were removed and the slides were dehydrated in 70% ice-cold ethanol for five minutes, followed by

dehydration at 90% and 100% ethanol for three minutes each. The denatured probe mix was aliquoted on 13mm round glass cover slips and applied on the slides as described above and hybridisation was initiated straight after. Post hybridisation the slides were washed to removed any unbound probe as described in section 2.4.2.4.

2.4.2.3 Reprobing of slides, 2nd round of FISH

For the majority of experiments, a second round of FISH was performed to increase the number of chromosomes tested. Following hybridisation (Materials and Methods 2.4.2.2) and analysis (Materials and Methods 2.5.3) the coverslips were removed from analysed slides that needed to be reprobed. They were incubated for five minutes in a Coplin jar filled with 1xPBS at room temperature on a rocking plate under bright light to remove the 1st round probes. The slides where then dehydrated through an ethanol series and left to air-dry in a vertical position. Co- or separate denaturation of the slides and the probe(s) was then performed as described in section 2.4.2.2.

2.4.2.4 Post-hybridisation washes

All post-hybridisation washes were performed in the dark in a suitably warmed water bath. Cover slips sealed with rubber cement were carefully removed with a pair of tweezers. All 1st round slides were washed three times for three minutes in a pre-warmed formamide/2xSSC solution. The concentration of the formamide and the temperature depended on the probe mix. Details for these conditions are found in table 2.4. All slides, regardless of the probe mix, were then washed three times for three minutes each in 2xSSC at 40°C.

Second round slides were again washed with a formamide/2xSSC solution, once for five minutes followed by one wash with 2xSSC. The washed slides were dehydrated through an ethanol series and left vertically to air-dry. Cover slips of 55x22mm size were positioned and 10µl of Vectashield 4',6-diamidino-2-phenylindole [DAPI: 1ml Vectashield + 6µl of 0.2mg/ml DAPI (both from Vector labs, UK)] were aliquoted for each hybridisation area to stain the nuclei. Slides were inverted on the cover slips and kept at 4°C until analysis.

Table 2.4: Fluorescent probes used in this thesis for FISH						
Probe mix	**Chromosome**	**Type of probe**	**Volume of each probe (μl)**	**Buffer**	**Denaturation**	**Post-hybridisation washes**
LSI 13/LSI 21	13 and 21	Locus specific	0.6	LSI	Sep	50% formamide/2xSSC, 40°C
CEP X/CEP Y/ CEP 18	X, Y and 18	Centromeric	2.5	CEP	Co	60% formamide/2xSSC, 40°C
CEP 10 (SA)	10	Centromeric	0.5	CEP	Co	40% formamide/2xSSC, 41°C
CEP 12 (SG)	12	Centromeric	0.1	CEP	Co	50% formamide/2xSSC, 40°C
TelVysion 14q (SO)	14	Telomeric	0.6	LSI	Co	40% formamide/2xSSC, 40°C
TelVysion 1q (SO) **CEP4 (SA)** **TelVysion 5p (SG)**	1 4 5	Telomeric Centromeric Telomeric	0.6 0.5 0.6	CEP	Co	50% formamide/2xSSC, 41°C

Table 2.4: Information on the probe mixes, colours of the fluorescent probes and conditions used during FISH. *Each probe name is presented, its chromosomal location, the type, the volume used in a total of 5μl of probe mix, the type of buffer used, denaturation (sep: separate slide and probe denaturation, co: simultaneous slide and probe denaturation) and post-hybridisation washes. Probes for chromosomes 13, 21, X, Y and 18 were part of the AneuVision kit. LSI: Locus specific identifier probe, CEP: chromosome enumeration probe, SG: spectrum green, SO: spectrum orange, SA: spectrum aqua.*

2.4.3 Fluorescence polymerase reaction

Fluorescence polymerase chain reaction (FPCR) was used to amplify polymorphic loci in genomic DNA from couples as well as WGA products from embryos (aim 3). Haplotyping was then performed in order to detect cross over events and determine the recombination rate in embryos from PGD and PGS couples. In total, 14 loci were amplified corresponding to 14 different short tandem repeat (STR) polymorphic markers on five different chromosomes.

2.4.3.1 Polymorphic marker selection

Selection of markers was performed by Dr Nick Maniatis and Dr Winston Lau of the Department of Genetics, Evolution and Environment at UCL. Four genomic regions of high recombination on chromosomes 1, 5, 16 and 19 and one region of no recombination on chromosome 17 were selected on the basis of genetic maps. Information from two types of genetic maps was used in order to identify the five selected regions. These were the high-resolution linkage disequilibrium (LD) maps based on the SNP data from the International HapMap project (Maniatis *et al*, 2002) and the most recent recombination map (cM) based on family data (Kong *et al*, 2010).

High-density LDU maps for the whole genome were constructed using four different populations from the PHASE II data of the HapMap project (International HapMap Consortium, 2007). On LDU maps, recombination hotspots were represented as “steps” and recombination silent spots as “blocks”. Figure 2.7 shows the LDU map from the CEU (Utah residents with Northern and Western European ancestry) HapMap II data for the recombination silent spot on chromosome 17. All maps were very similar across all four populations (CEU, Chinese, Japanese, Yoruba) for all five selected regions.

The LDU map provided information about current and historic recombination at a very fine resolution. However, the most recent recombination map in cM (Kong *et al*, 2010) was also used in this study. Although the cM map was of lower marker resolution, compared to LDU maps, it was based on family data and hence provided evidence of current recombination that could be directly compared to our embryo recombination data. This cM map was based on CEU families and, as seen in figure 2.7, a very close relationship with the LDU map was shown.

Figure 2.7: The LD structure of the *NF1* region

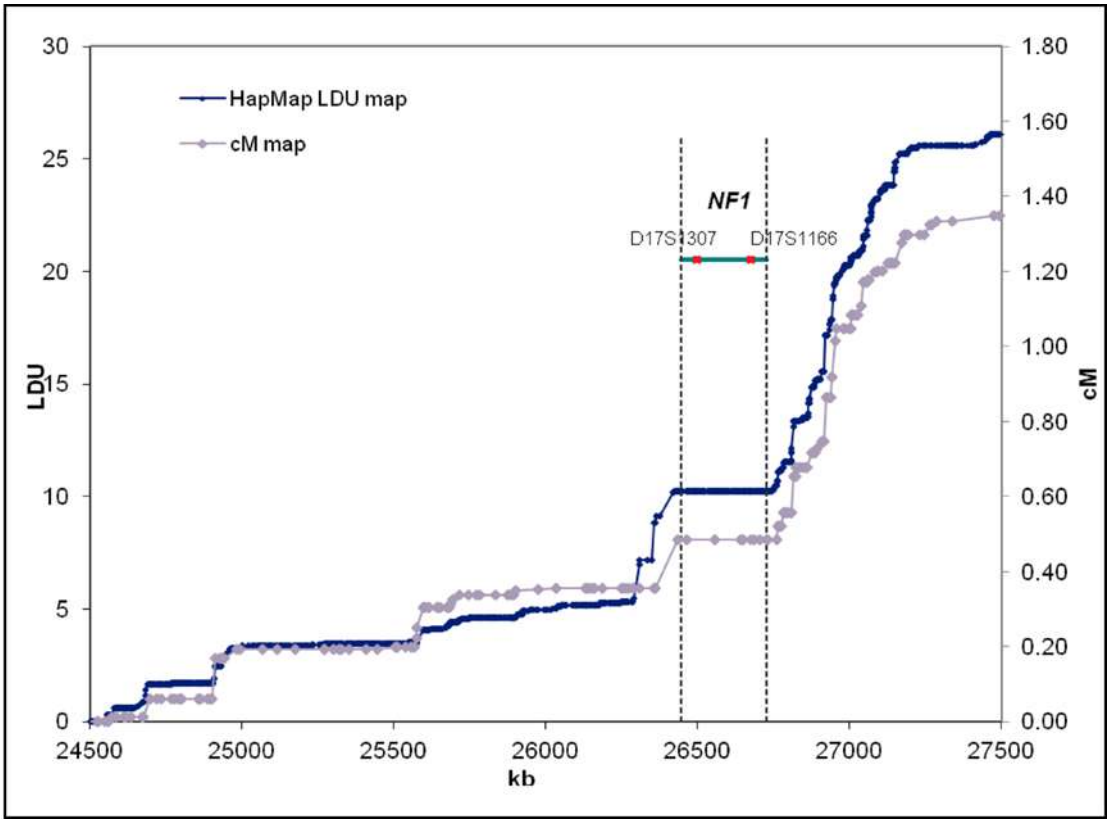


Figure 2.7: *The LD map of the region is shown by plotting HapMap LDUs (Y axis) against kb (X axis). The family based recombination map for the same region is shown by plotting sex-averaged cM (secondary Y axis) against kb (X axis). The vertical lines show the start and end of the gene and the red points within the gene are two of the five STR on that region markers that were used to estimate recombination frequency using embryo data.*

2.4.3.2 Primer design

For the purpose of primer design information on markers was retrieved in Ensembl (Ensembl release 60, www.ensembl.org, NCBI build 37). The sequence of each marker was identified through BLASTN, of the marker primers provided by Ensembl, in the Basic Local Alignment Search Tool (BLAST) programme (http://www.ensembl.org/Homo_sapiens/blastview). Primers were then designed with the aid of Primer3 software via a web interface (http://frodo.wi.mit.edu/). All primers were 20-28 bases long with similar melting temperatures of around 60°C, low self-complementarity. Primer product size ranged from Sets of forward and reverse primers were designed specific for each locus, with the forward primer labelled with a fluorescent dye an at the 5'- end to allow fragment analysis post FPCR. Each set of primers was initially tested on genomic DNA in a singleplex reaction using either High Fidelity (HiFi) polymerase (Roche Diagnostics, UK), or *Taq* polymerase (Qiagen, UK). Optimisation was achieved by performing a gradient

PCR in a thermocycler (Mastercycler gradient, Eppendorf, UK) and testing different annealing temperatures, ranging +/-4°C of the determined melting temperature provided by the supplier company. Once optimisation for each primer set was achieved markers were amplified, if possible, in multiplex reactions, using the Qiagen® Multiplex PCR kit (Qiagen, UK) or in singleplexes.

All PCR reactions were performed in 0.2ml microfuge tubes. All mastermixes were prepared in 0.5ml PCR or 1.5ml individually packed, sterile microfuge tubes (VWR, UK).

All primers arrived in lyophilised pellets, in 40nm quantities. Each primer was reconstituted by adding the required volume of nuclease-free water (Promega, UK) or 1xTE [Tris(hydroxymethyl)aminomethane (Tris)/EDTA] buffer (Promega, UK) to make up to 50, 100 or 200 micromolar (μM) concentration. Stock aliquots of the primers were kept at -80°C. Working aliquots of each primer were prepared at 50μM concentration and kept at -20°C, in order to minimise the number of freeze/thaws of the stock aliquots. Details of each primer sequence and fluorescent dye are found in table 2.5.

Table 2.5: Primer sets used in this thesis			
Marker	**Primer sequence (5'- to 3'- end)**		**Fluorescent label**
D1S495	Forward	CTGCAGAGAAAGGGAACCTG	FAM
	Reverse	CTTTGCAGAGGAGGCAAACT	
D1S486	Forward	GTTGCAGTGAGCTGAGATCG	DO
	Reverse	GTCTCCTCCTTGGTGCATGT	
D5S1991	Forward	GAAAAGCAAGGTGCCAAATC	FAM
	Reverse	TCTTCCATCCCAACTCCAAC	
D5S2081	Forward	ATCTCCGGAAGGAAAAGGAA	FAM
	Reverse	AATTTCATCCTGGCATCCTG	
D16S492	Forward	GCTGTGAGTAGCGACAGTGC	FAM
	Reverse	GTCCCAGCCTCTCTGCTCTA	
D16S3053	Forward	AGCTGATAGCGTCCACAGGT	FAM
	Reverse	ATGGAGCAGGGTATCACCAA	
NF1int1	Forward	CAAAGTGCTGGGATTACAGCATGAG	FAM
	Reverse	TATACATTCTGAAATGATTACCACG	
D17S1307	Forward	ATAGGAGACCTGCTGCCTTT	YY
	Reverse	AGGGCAGAGAAACCTAAGGA	
NF1int17	Forward	CTCTTGTGAGTTATTGTATGCGG	FAM
	Reverse	CTGAGAGTCAAGGGTGGAAGAC	
NF1int29	Forward	CTTTCCTCTAAACAAACAGAGTCAG	YY
	Reverse	CAGTGCACTCCCAGTGCTGGTG	
D17S1166	Forward	TAACAATTGTGGAACTGCAGCAATTATT	YY
	Reverse	CCCATACCTAGTTCTTAAAGTCTGT	
D19S219	Forward	TTGCTGGGTCATTCAGTTTG	FAM
	Reverse	AGCGAGAATCCGTCTCAAAA	
D19S207	Forward	GAGGGGAACTATAGCCACCA	DO
	Reverse	AGGCAGAGGTTGCAGTGAGT	
D19S412	Forward	GTTGCAGTGAGCTGAGATCG	FAM
	Reverse	GTCTCCTCCTTGGTGCATGT	

Table 2.5: Details of primers used for the detection of cross-over events. *The sequences (from the 5'- to the 3'- ends) and the fluorescent labels at the 5'- end of each forward primer (FAM – 6-Carboxyfluorescein, YY – Yakima Yellow, DO – Dragonfly Orange) are shown.*

2.4.3.3 PCR using High Fidelity polymerase

Singleplex PCR reactions were prepared using the Expand High Fidelity (HiFi) PCR system (Roche Diagnostics, UK). Each PCR reaction contained 0.2μM of each primer (forward and reverse), 0.4mM deoxyribonucleotide triphospathes (dNTPs, Promega, UK), 2.5μl of Expand High Fidelity buffer [containing 1.5mM magnesium chloride ($MgCl_2$)] and 1.5 units of Expand High Fidelity polymerase. All PCR reaction volumes for amplification of either genomic DNA or WGA products were made up to 24μl with nuclease-free water (Promega, UK) and 1μl of DNA or WGA product was added. The program followed is found in table 2.6.

Table 2.6: Singleplex PCR program using High Fidelity polymerase

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	1 cycle
Denaturation	96°C	15 sec	10 cycles
Annealing	58 or 60°C	45 sec	
Elongation	72°C	1 min	
Denaturation	94°C	15 sec	30 cycles
Annealing	60°C	45 sec	
Elongation	72°C	1 min	
Final elongation	72°C	7 min	1 cycle
Hold	4°C	Hold	1 cycle

Table 2.6: PCR amplification by the Expand High Fidelity PCR system.

2.4.3.4 PCR using the *Taq* PCR master mix

The Qiagen *Taq* PCR master mix was used to prepare some of the singleplex PCR reactions (Qiagen, UK). The kit included a ready-made mix, the 2x *Taq* PCR master mix that contained the *Taq* DNA polymerase, Qiagen PCR buffer [20mM Tris-HCl, 100mM potassium chloride (KCl), 1mM DL-Dithiothreitol (DTT), 0.1 mM EDTA, 0.5% (v/v) Nonidet® P-40, 0.5% (v/v) Tween® 20, 50% (v/v) glycerol; pH 8.0] with 3mM Magnesium chloride ($MgCl_2$) and 400mM of each dNTP. Each PCR reaction was prepared in a final volume of 25µl, containing 12.5µl of 2x *Taq* PCR master mix, resulting in a final concentration of 2.5 units of *Taq*, 1x Qiagen PCR buffer and 200mM of each dNTP, 0.2µM of each of the primers (forward and reverse), 1µl of genomic DNA or WGA product and sufficient volume of nuclease-free dH_2O to make up to 25µl. The program followed for each reaction is found in table 2.7.

Table 2.7: Singleplex PCR program using *Taq* polymerase

Step	Temperature	Time	Cycles
Initial denaturation	94°C	3 min	1 cycle
Denaturation	96°C	30 sec	10 cycles
Annealing	60°C	45 sec	
Elongation	72°C	1 min	
Denaturation	94°C	30 sec	30 cycles
Annealing	60°C	45 sec	
Elongation	72°C	1 min	
Final elongation	72°C	10 min	1 cycle
Hold	4°C	Hold	1 cycle

Table 2.7: DNA amplification using the Taq PCR master mix

2.4.3.5 Multiplex PCR using the Qiagen multiplex kit

If possible, different sets of primers were combined in a single, multiplex PCR reaction, for the simultaneous amplification of multiple loci. The Qiagen® Multiplex kit was used for that purpose (Qiagen, UK). The kit included the 2x Qiagen Multiplex PCR Master Mix (HotStarTaq® DNA polymerase, Multiplex PCR buffer and dNTP mix) and 5x Q-Solution, a PCR additive to make the reaction more stringent, which changed the melting behaviour of DNA and improved reactions, where primers used were of high GC content. A suitable 10x primer mix was prepared for each reaction containing each primer in equal concentration of 2μM. Each reaction was of a final volume of 25μl and included, 12.5μl of Qiagen Multiplex PCR Master Mix, 2.5μl of Q solution, 2.5μl of 10x primer mix, 1μl of genomic DNA or WGA product and 6.5μl of nuclease-free dH_2O. The program followed for all multiplex reactions using this kit is shown in table 2.8.

Table 2.8: Multiplex PCR program using the Qiagen multiplex kit

Step	Temperature	Time	Cycles
Initial denaturation	95°C	15 min	1 cycle
Denaturation	96°C	30 sec	10 cycles
Annealing	60°C	90 sec	
Elongation	72°C	90 sec	
Denaturation	94°C	30 sec	30 cycles
Annealing	60°C	90 sec	
Elongation	72°C	90 sec	
Final elongation	72°C	10 min	1 cycle
Hold	4°C	Hold	1 cycle

Table 2.8: DNA amplification with the Qiagen multiplex kit.

2.5 Sample analysis

2.5.1 Agarose gel electrophoresis

WGA products were run on 2% agarose gels to confirm amplification. The gel was prepared by melting 1g agarose (Sigma-Aldrich, UK) in 50ml of 1xTBE buffer (10xTBE: 3.7gr EDTA, 108gr Tris, 45g boric acid, in 1L of dH_2O). 1.5µl of ethidium bromide (10mg/ml, Sigma-Aldrich, UK) were added in order to visualise the bands under UV light. The gel was poured onto a mini-gel mould and was left to set at room temperature. The set gel was transferred to a tank containing 50ml of 1xTBE. Five µl of each product were mixed with 0.8µl of loading buffer (40% w/v sucrose, 4mM bromophenol blue, 4mM xylene cyanol) and were loaded on the gel wells. The Hypeladder IV (1.5µl, Bioline, UK, figure 2.8), 100 base pair (bp) molecular weight marker was used to confirm the presence of the WGA smears at the right size. The gel was left to run at 80V for 15 minutes in 50ml of 1xTBE. After electrophoresis the gel was visualised under UV light using the MultiImage Light Cabinet (Flowgen, UK).

Figure 2.8: Hyperladder IV (Bioline, UK)

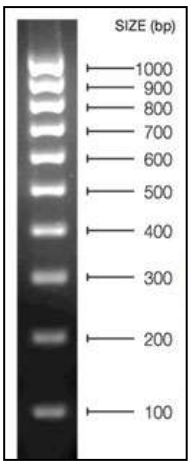


Figure 2.8: *Molecular weight marker used for the sizing of WGA products on 2% agarose gels.*

2.5.2 Array comparative genomic hybridisation using the 24sure microarray platform

aCGH was performed to achieve complete enumeration of the chromosomes in various samples. Tests were undertaken to validate aCGH for clinical application in PGS and for research purposes in PGD embryos. All aCGH experiments were performed using the 24sure V2 array platform (BlueGnome Ltd, UK) with various changes in the conditions to optimise the result in our laboratory setting. 24sure V2 arrays consisted of 2674 Roswell Park BAC clones, DOP-PCR amplified and random spotted in duplicates onto Surmodics glass sides, in two hybridisation areas. The performance of each BAC clone had been highly validated by whole chromosome reverse painting on arrays, end sequencing, FISH and over aCGH experiments. The 2674 clones had been chosen to have low noise (control experiment hybridisation to hybridisation variation) and to be outside any regions of known number polymorphisms (data from Database of Genomic variants and BlueGnome customer post-natal aCGH database, provided by BlueGnome, UK).

The 24sure kit included the control DNA and reagents needed for the labelling of the WGA products and controls and for the hybridisation on the array slides. In summary, the test DNA (our samples) and the control DNA were differentially labelled, co-precipitated and resuspended and finally applied on the array slide for hybridisation. The slides were then washed, dried and scanned to produce an image, which was analysed using the BlueFuse Multi software v2.2 (BlueGnome, Ltd, UK). Details of each step are outlined below.

2.5.2.1 Labelling

The labelling reactions were all prepared in 0.5ml, autoclaved microfuge tubes. All steps were performed on ice. All the reagents were thawed, briefly vortexed and pulse centrifuged. For each labelling reaction the following reagents were added:

- 5μl of reaction buffer
- 5μl of primer solution
- 5μl of dCTP-labelling mix
- 1μl of Cyanine 3 (Cy3) for sample DNA (blue) or 1μl of Cyanine 5 (Cy5) for control DNA (red)

In each sample DNA tube, 8μl of the amplified product were aliquoted and in each control DNA tube 8μl of SureRef DNA (BlueGnome Ltd, UK) were added (figure 2.9a).

The samples were then denatured in a prewarmed thermal cycler for 5 min at 94°C and transferred immediately on ice, where they were further incubated for 5 min. In all the tubes 1μl of Klenow enzyme (BlueGnome Ltd, UK) was added and the tubes were then vortexed and spun to collect all components in the bottom of each tube. The labelling reaction was performed for 3 to 18 hours in a thermal cycler at 37°C.

2.5.2.2 Combination and ethanol precipitation

The labelled control and sample DNAs were combined into labelled 1.5ml, autoclaved microfuge tubes (figure 2.9b). Human COT DNA (0.4mg, BlueGnome Ltd, UK) was added in all the combined samples at a volume of 25μl in order to block repetitive sequences in the genome, mainly found around the centromeres and in the telomeres of each chromosome and prevent non-specific hybridisation. In the combined samples, 7.5μl of sodium acetate (Sigma-Aldrich, UK) were added in each sample to aid DNA precipitation. Finally, 187.5μl of absolute ethanol (Analar, VWR, UK) were added and the tubes were inverted twice to mix and placed in -80°C for 10 min.

2.5.2.3 Hybridisation

The combined labelled DNAs were centrifuged for 10 min at full speed (13000 rpm). After centrifugation the pellets should have had a strong purple colour (figure 2.9c). The supernatant was discarded and 500μl of 70% ethanol were added to wash the pellets. The tubes were inverted to mix and centrifuged for 5 min at full speed. The supernatant was again discarded and the tubes were inverted gently on a tissue to remove more ethanol. As it was essential to remove as much of the ethanol as possible, the tubes were again pulse centrifuged and the remaining ethanol was removed with a small pipette, carefully not to touch or move the pellet. The pellets were air dried for exactly 2 min at room temperature, as over-drying the pellets made resuspension more difficult. Resuspension was performed by adding 21μl of prewarmed hybridisation buffer in a heating block set at 75°C. The pellets were vigorously agitated in order for them to be completely dissolved. Once the solution

was clear of the pellets, the tubes were incubated for 10 min at 75°C for denaturation.

Figure 2.9: Labelling, combination and precipitation of WGA products for aCGH

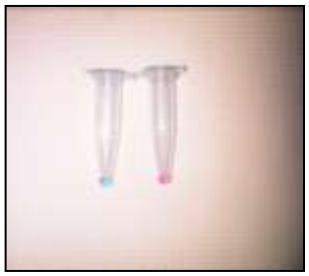
a)  b)  c) 

Figure 2.9: *a) Differentially labelled sample (blue, left) and control (red, right) DNAs prior to combination. b) Combined labelled and control DNA prior to co-precipitation. c) Precipitated labelled and control DNAs prior to resuspension. A strong purple pellet is seen on the left and a blue pellet on the right indicating poor labelling or precipitation.*

In order to make sure that the samples were correctly placed on the slide, the hybridisation template provided with the kit was used (figure 2.10a). Each array slide had two array areas and therefore two samples were applied on each slide. The 22x22mm hybri-slips (Sigma, UK) were placed on each hybridisation area. Eighteen μl of the labelled DNA were carefully applied on each hybri-slip and the array was lowered with the barcode facing down. The DNA was pipetted slowly on the cover slip in order to avoid the formation of bubbles. If any bubbles were formed the solution was aspirated back and returned to the tube, which was pulse centrifuged to remove the bubbles and the DNA was applied again on the hybri-slip. The position of each sample on the slide as well as the slide's barcode was reported, as they were the only indicators of each sample.

The 24sure array slides with the labelled DNAs were placed in a hybridisation chamber (figure 2.10b), which was prepared with a tissue saturated with 6ml of 2xSSC/50% formamide (7.5ml formamide, 1.5ml 20xSSC and 6ml of dH_2O). The chambers were tightly closed and sealed with parafilm and incubated at a 47°C water bath for 3 to 16 hours.

Figure 2.10: aCGH with 24sure microarray slides

a)  b) 

Figure 2.10: *a) Hybridisation template with the labelled DNA (purple drop) aliquoted on the cover slip before application on the array slide. b) Hybridisation chamber with the saturated tissue. Two ordinary microscope slides were used for the image, which were of the same size as a microarray slide.*

2.5.2.4 Post-hybridisation washes

Once hybridisation was completed the slides were washed to remove any un-hybridised DNA and dried. Two different types of washing were used. Initially, the slides were washed using more stringent conditions, with a formamide wash step. Quicker and easier steps of washes with no formamide and a high-temperature step were followed later on. Both methods are described in the next sections.

2.5.2.4.1 Method 1: Formamide washes

The solutions used for the formamide washes were:

- 1xPBS/0.05% Tween20: 1800ml dH_2O, 200ml 10xPBS, 1ml Tween20 (final volume 2000ml)
- 2xSSC/50% formamide: 40ml 20xSSC (pH 7.0), 200ml formamide, 160ml dH_2O (final volume 400ml)
- 1xPBS: 900ml dH_2O, 100ml 10xPBS (final volume 1000ml)

Following hybridisation the slides were moved to a 100ml Coplin jar filled with 1xPBS/0.05% Tween20 to remove the cover slips, at room temperature (RT). They were removed by holding the slides at the barcode and with gentle agitation in the jar. With the cover slips removed the slides were then transferred to a 25-position steel staining rack and into a 500ml glass staining dish, containing 400ml of 1xPBS/0.05% Tween20 and a 2.5cm magnetic stir bar. The dish was placed on a

stirrer with the lid replaced and was covered with aluminium foil to keep in the dark. The steps of the washes are summarised in table 2.9.

Table 2.9: Formamide washes of hybridised array slides					
Wash	**Volume**	**Temperature**	**Time**	**Agitation**	**Buffer**
1	500ml	RT	10min	Stirrer	1xPBS/0.05% Tween20
2	500ml	RT	10min	Stirrer	1xPBS/0.05% Tween20
3	500ml	RT	10min	Stirrer	1xPBS/0.05% Tween20
4	100ml	42°C	30min	Hand stirring	2xSSC/50% formamide
5	500ml	RT	10min	Stirrer	1xPBS/0.05% Tween20
6	500ml	RT	5 min	Stirrer	1xPBS
7	500ml	RT	5 min	Stirrer	1xPBS

Table 2.9: Stringent conditions of washing aCGH slides.

The rack with the slides remained in a buffer at all times and transfer to the consecutive wash steps was done immediately upon completion of each wash. The slides on the rack were facing away from the stir bar to avoid contact of the bar with the arrays. Wash 4 was performed in a water bath and the Coplin jar was gently swirled by hand every 10 minutes.

2.5.2.4.2 Method 2: Formamide-free washes

Prior to washing the slides, the solutions were warmed to the appropriate temperature. These solutions were:

- 2x SSC/0.05% Tween 20: 100ml 20x SSC (pH 7.0), 899.5ml dH_2O, 0.5ml Tween 20 (final volume: 1000ml)
- 1x SSC: 25ml 20x SSC (pH 7.0), 475ml dH_2O (final volume: 500ml)
- 0.1x SSC: 5ml 20x SSC (pH 7.0), 995ml dH_2O (final volume: 1000ml)

The 0.1xSSC solution was poured in the ClearHyb (BlueGnome, UK) washing chamber, which was set at 60°C. The temperature of the solution was checked with a thermometer before the start of the washes. At room temperature, 100ml of 2x SSC/0.05% Tween 20 were added to a 100ml Coplin jar, in which the slides were positioned and the cover slips were removed as described above. The washing steps are summarised in table 2.10.

Table 2.10: Formamide-free washes of hybridised array slides					
Wash	**Volume**	**Temperature**	**Time**	**Agitation**	**Buffer**
1	400ml	RT	10 min	Stirrer	2x SSC/0.05% Tween 20
2	400ml	RT	10 min	Stirrer	1x SSC
3	500ml	60°C	5 min	None (ClearHyb)	0.1x SSC
4	100ml	RT	1 min	Stirrer	0.1x SSC

Table 2.10: Less stringent washing conditions of aCGH slides using the ClearHyb.

Once the washes were completed the slides were dried by centrifugation. They were placed in 50ml Falcon tubes with the barcode at the bottom of the tube and spun at 1200rpm for 3 min in a benchtop centrifuge (Heraus, Labofuge 300, Thermo Scientific, USA). The slides were finally removed from the tubes using forceps and placed in a dark slide box until scanning.

2.5.2.5 Scanning

Three different scanners were used in this project for the arrays slides. The ScanArray Express (Perkin Elmer, UK), the InnoScan 700 (Innopsys SA, France) and the Agilent, High-Resolution Microarray Scanner (Agilent Technologies, UK). All slides were scanned at a resolution of 10μm. All scanners were equipped with suitable lasers to excite the two different fluorescent dyes Cy3 and Cy5 at appropriate wavelengths 532nm and 635nm respectively. When the ScanArray Express scanner was used the voltage value of the photomultiplier tube (PMT) was adjusted manually after a quick scan of each slide. For the other two scanners the PMT value was adjusted automatically. Scanning produced TIFF (Tagged Image File Format) images of all the arrays, which were stored until the analysis.

2.5.2.6 Analysis

The scanned images of the 24sure array slides were analysed and interpreted using the BlueFuse Multi v2.2 (BlueGnome, UK). aCGH images of each result were available from this software showing the $\log_2$ ratio of the test over the control DNA for each chromosome. The cut-off value (threshold) of the $\log_2$ ratio for chromosome gains was set at 0.3 and for losses at -0.3. For the majority of the analyses chromosome gains and losses were considered but for the analysis of the mosaic

models, the average $\log_2$ratio and the standard deviation (SD) of all the clones for chromosome 10 were calculated.

The software that produced the aCGH result also provided a list of values indicating the quality of each experiment. These indicators were checked for every experiment to assess its efficacy. The indicators were:

- % of clones included: This was the percentage of clones that were analysable by the software. The higher the percentage the better the quality of the experiment. Ideally, it should have been >90%.

- Signal to background ratio (SBR) channel 1/channel 2 (Ch1/Ch2): The signal to background ratio for channels 1 and 2, which represented the two fluorescently labelled DNAs. Channel 1, was the test DNA labelled with Cy3 and channel 2 was the reference DNA, labelled with Cy5. An experiment with a signal to background ratio of one indicated similar intensities of the clones and the background, which resulted to poor analysis. A good experiment should have had a ratio over two.

- Mean spot amplitude (MSA) Ch1/Ch2: This value gave an indication of how bright the clones were for each of the two channels. For an analysis to be successful this value should have been over 1000.

2.5.2.7 Chromosomal classification of embryos analysed by aCGH

Embryos from couples that had undergone PGD that were used in the aneuploidy study, to determine the aneuploidy level and were tested by aCGH, were characterised as:

- Euploid: There was no change detected on the $\log_2$ ratio for any of the chromosomes.

- Aneuploid: A gain or loss in up to two chromosomes was detected.

- Complex aneuploid: A gain or loss in three or more chromosomes was detected.

Mosaicism was not scored by aCGH, since all embryos were tubed as whole and therefore no individual cell assessment was performed as in FISH.

2.5.3 Fluorescence microscope analysis

Analysis of chromosomes in human embryos was performed using an epifluorescence microscope: Olympus BX 40 (Olympus, UK). The microscope was equipped with a Sensys Photometrics camera for capturing of images and was connected with a computer running the Smartcapture software for image acquisition (both Digital Scientific, UK). Nuclei were first located under the blue filter, as they were stained with DAPI, using the coordinates taken immediately after spreading. The colour of the filters was changed depending on the probes that were used and each nucleus was scored.

The criteria used for the signal scoring where those suggested by Hopman *et al* (1988). Signals that appeared to be split were considered to be one. For signals to be scored as two distinct signals, the distance between them should have been at least the width of one. Nuclei that seemed to be covered with cytoplasm and did not produce clear bright signals were not considered.

2.5.3.1 Chromosomal classification of embryos analysed by FISH

The criteria that were used for the classification of the embryos from couples that had undergone PGD in the aneuploidy study were similar to those used by Mantzouratou *et al* (2007). Embryos were classified as:

- Diploid: When more than 90% of cells showed no aneuploidies.
- Aneuploid: When more than 90% of cells showed the same abnormality due to a meiotic error.
- Diploid mosaic: When embryos consisted of a distinct diploid cell line, and a proportion of cells with one or more abnormalities (aneuploid and/or chaotic).
- Aneuploid mosaic: When embryos had an aneuploid cell line and cell lines with one or more different abnormalities.
- Chaotic: When all the nuclei examined were abnormal, carrying a variety of abnormalities.
- Haploid: When all nuclei had one copy for all the chromosomes tested.

2.5.4 Fragment analysis of fluorescent PCR products

FPCR products were analysed by capillary electrophoresis using the ABI Prism 310, 3100 and 3730 genetic analysers (Applied Biosystems, UK). All reagents and software used for these procedures were from Applied Biosystems, UK.

All genomic DNA FPCR products were diluted with nuclease free water before the analysis in a 1:10 dilution due to their high concentration; FPCR products amplified from WGA products were not diluted. The products were mixed with formamide and a molecular size standard to allow sizing of the peaks. Genescan-500 ROX™ size standard was used for the analysis in the 310 and 3100 prisms, whereas Genescan-500 LIZ™ was used for the 3730.

Preparation of the products for the analysis was similar and according to the analyser that was used. One microliter of FPCR product was mixed with 12μl of Hi-Di formamide and 0.3μl of the appropriate size standard, depending on the analyser used. The samples were then denatured at 95°C for five minutes in a thermal cycler.

Products analysed in the 310 Prism were subjected to single capillary electrophoresis. The conditions followed during electrophoresis were five seconds of injection time at 15000V in the single capillary. Separation of the fragments was performed at 60°C for 30 minutes using the Performance Optimised Polymer 4 (POP-6™) and 1x Genetic Analyser Buffer with EDTA. The data were analysed using the Genescan® software.

Products analysed in the 3100 or 3730 prisms were subjected to a 16- or 96-capillary injection for five seconds at 1000V or five seconds at 2000V respectively. Separation was performed at 15000V for 24 minutes at 60°C using POP-6 ™ polymer if the 3100 was used and POP-7™ when the 3730 genetic analysed was used. The data from both were analysed by the Genemapper® analysis software, version 3.5 (Applied Biosystems, UK).

2.5.4.1 Haplotype analysis of parental and embryo DNA for detection of recombination

FPCR was used to amplify microsatellite markers on five different chromosomes to detect recombination on parental DNA and WGA amplified products from embryonic DNA. For each family (mother, father and embryos) it was essential to determine the segregation of the markers on the parental chromosomes correctly. Haplotype analysis was not possible in cases where the parents were not informative for the markers and heterozygosity of at least one of the markers on each locus for both parents was essential. The marker alleles in phase with each other were determined by the combination of parental alleles that resulted in the smallest recombination fraction in the embryos for that family. Allele drop out (ADO) was observed in some embryonic samples. Cases that produced a haplotype that could have been a result of either recombination or ADO were excluded from the analysis.

2.6 Statistical analysis

Distribution of embryos among different groups was compared using the Fisher's exact test. Means were compared by t-test. StatPlus:mac LE.2009 software for Microsoft Excel was used for statistical analysis. When p was found to be less than 0.05 the finding was considered to be of statistical significance.

3 Results

3.1 Validation of aCGH for clinical application

Array comparative genomic hybridisation (aCGH) was validated as a technique to detect aneuploidy in embryos during preimplantation genetic screening (PGS). The steps that were followed for the validation were, testing aCGH primarily on single cells from aneuploid and euploid cell lines, for PGS after cleavage stage embryo biopsy of single blastomeres. This was followed by examining the effect of mosaicism on aCGH post biopsy of a trophectoderm (TE) sample from a blastocyst. This was achieved by mixing euploid and aneuploid cells in different ratios to produce TE and blastocyst mosaic models, which were analysed by aCGH. Prior to clinical application the optimum conditions to get a quick and efficient aCGH result were determined through a series of tests. A clinical "dry-run" of aCGH post TE biopsy was performed by analysing biopsied TE samples from frozen-thawed blastocysts from routine IVF patients and confirming the result on the remainder of the blastocysts. Following the application of aCGH in PGS, confirmation of the clinical result on cleavage stage embryos or blastocysts was carried out by FISH as follow up analysis on untrasferred embryos using probes for the abnormalities detected by aCGH.

3.1.1 Analysis by aCGH

In this section, two examples of aCGH profiles, using the 24sure by BlueGnome, UK, which was the platform used in this project, are provided (figure 3.1) in order to describe the aCGH result. The profile was provided as a graph with the X-axis indicating all 22 autosomes, with the sex chromosomes at the end of the axis. The Y-axis represented the $\log_2$ ratio of the clone fluorescence of the test over the reference DNA, a diploid male. Each green spot represented a clone. Spots along zero $\log_2$ ratio indicated that the fluorescence of the two DNA samples was the same and therefore there was no gain or loss of genetic material in the test. Chromosomal gain was detected when the $\log_2$ ratio of the clones for the chromosome was over the 0.3 threshold (green line) as seen for chromosome X in figure 3.1A and chromosome 4 in 3.1B. Chromosomal loss was detected when the ratio was below the -0.3 threshold (red line), shown in figure 3.1A for chromosome 18. For female samples, apart from the gain of chromosome X, a loss of chromosome Y needed to be seen as well. Since this was a nullisomy the ratio was expected to be well below -0.3, at around -0.8. In males the $\log_2$ ratio for both sex chromosomes was expected to be zero since there was no difference from the normal diploid male reference DNA (figure 3.1B).

Figure 3.1: Analysis by aCGH

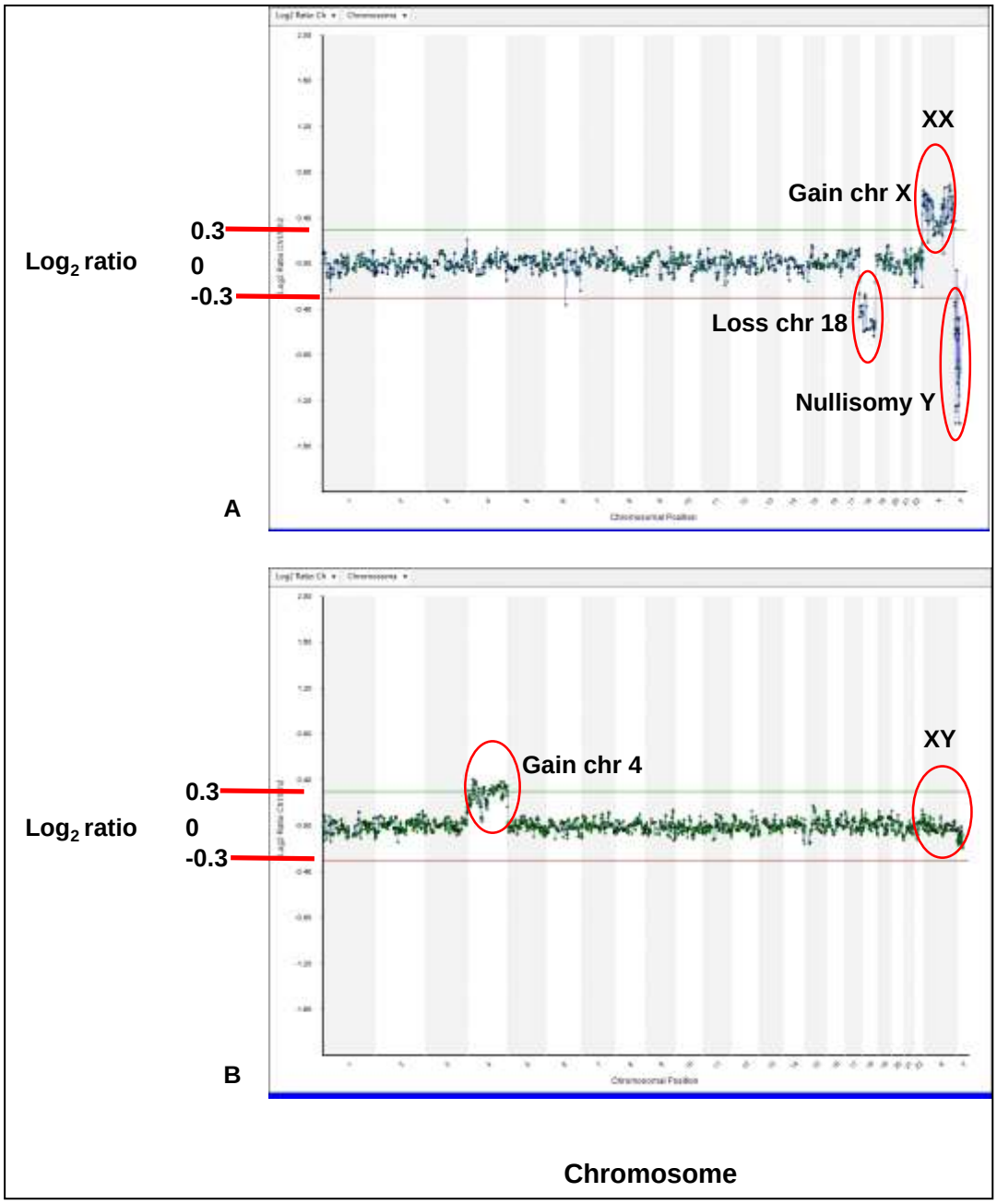


Figure 3.1: *A: Female with loss of chromosome 18. Gain of chromosome X and loss of chromosome Y indicated the sex. B: Male with gain of chromosome 4. No change in the ratio of the sex chromosomes indicated the sex. In both profiles the thresholds for chromosomal gains and losses were indicated on the Y-axis. The X-axis represented all the autosomes and the sex chromosomes.*

3.1.2 Description of cell lines

Cells from ovarian epithelial cell lines were used for the validation of aCGH in PGS. These were the aneuploid SKOV3 and TOV-21G and euploid IOSE-1 and IOSE-19. Cells in all experiments were retrieved from a single passage of each of the lines in order to minimise cell-to-cell variation. Prior to the aCGH experiments, FISH was performed in order to verify the karyotype of each of the cell lines.

3.1.3 FISH on cell lines to verify the karyotype

Probes for chromosomes 13, 18, 21, X and Y were used for all the cell lines in two consecutive FISH rounds as described in the Materials and Methods section 2.4.2. An additional probe of the known aneuploidies was used in a third FISH round for the two aneuploid cell lines; chromosome 10 for TOV-21G and chromosome 12 for SKOV3. Analysis on a minimum of 50 nuclei was performed for each cell line. As these were all ovarian epithelial cell lines, a female sex was expected and that was confirmed in lines IOSE11, IOSE19 and TOV-21G on an average of 96% of cells. In the aneuploid cell line TOV-21G trisomy for chromosome 10 was detected in 97% of the nuclei. Only one copy for chromosome X was detected in 90% of the nuclei analysed from cell line SKOV3. This cell line carried a segmental deletion that included the centromere and as the probe for chromosome X was centromeric, only one signal was seen. A euploid result was observed for chromosomes 13, 18 and 21 for all cell lines in an average of 98% of analysed cells. Figure 3.2 presents the FISH result of nuclei from the two aneuploid cell lines.

Figure 3.2: FISH result of the two aneuploid cell lines, TOV21G and SKOV3

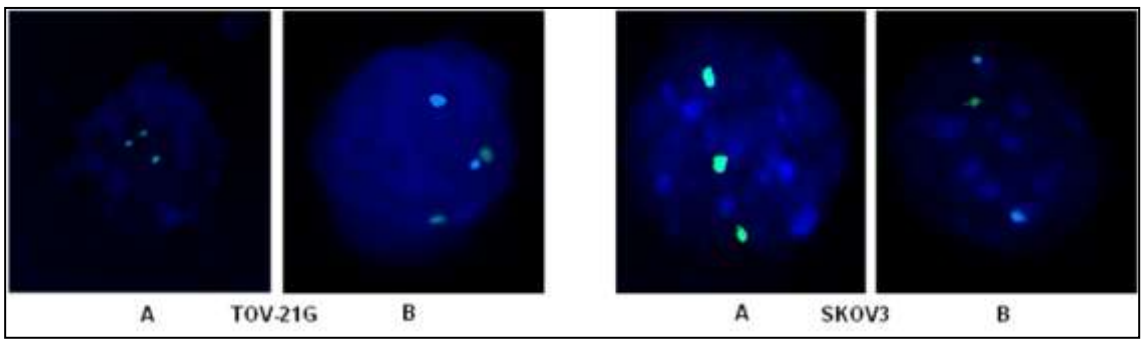


Figure 3.2: *TOV-21G (A): A nucleus with three copies of chromosome 10 in spectrum aqua. TOV-21G (B): A female nucleus diploid for chromosome 18 (spectrum green and spectrum aqua respectively). SKOV3 (A): Trisomy for chromosome 12 (spectrum green). SKOV3 (B): A nucleus with monosomy X due to a segmental deletion and diploid for chromosome 18. [Reprinted from Fertility and Sterility, 97 (4), Mamas et al, Detection of aneuploidy by array comparative genomic hybridization using cell lines to mimic a mosaic trophectoderm biopsy, 943-947, Copyright (2012), with permission from Elsevier]*

3.1.4 aCGH on single cells

Isolation of single cells from the four cell lines was performed at the UCL Centre for PGD. WGA by GenomePlex and aCGH by 24sure was performed by BlueGnome. Immediately after isolation the cells were coded using random numbers. Analysis was done blind without prior knowledge of the origin of each cell. In total 40 single cells were isolated. Five cells were isolated from each of the euploid cell lines, IOSE-1 and IOSE-19, 18 cells from TOV-21G and 12 from SKOV-3.

To assess the efficacy of WGA, all amplified samples were run on a 2% agarose gel prior to hybridisation on the array. Good quality amplified products were expected to produce a bright smear with maximum intensity at around 400 base pairs, corresponding to the average size of the fragments produced. WGA was successful for 95% (38/40) of single cells analysed (Table 3.1), as measured by the presence of a WGA product by electrophoresis on a 2% agarose gel. The two cells that failed to amplify were not analysed further. Of the 38 successful amplifications, four products were observed as weak by comparison of ethidium bromide staining intensities with other single cells after electrophoresis (Figure 3.3). These were included in the analysis to maximise the data.

Figure 3.3: 2% agarose gel electrophoresis of some WGA single cell products

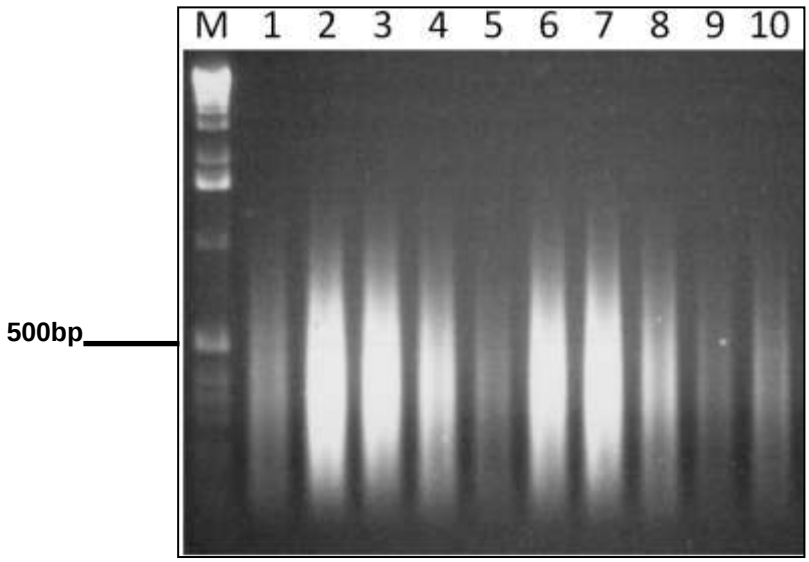


Figure 3.3*: M: 100bp ladder. 1 to 10: coded WGA products from single cell samples. Product present in wells 1, 5, 9 and 10 were observed as weak.*

Of the 38 successful amplification products, 92% (35/38) produced a result when analysed by aCGH using the 24sure platform (Table 3.1). A successful analysis was determined by the presence of an appropriate X and Y chromosome $\log_2$ ratio after cohybridisation of the WGA products of cells from the female cell lines with a male genomic control DNA (Figure 3.4). As described in the Results section 3.1.1, a female sample should have produced an X chromosome $\log_2$ ratio over the 0.3 threshold, representing a gain and a Y chromosome $\log_2$ ratio at around -0.8, representing a nullisomy. The three cells that could not be analysed by aCGH had produced a weak smear on the agarose gel. Upon decoding of the cells, it was revealed that the expected result was attributed to 100% (9/9) of the normal cells, scored as euploid female and 100% (30/30) of abnormal cells as aneuploid. Examples of single cell analysis by aCGH are found in figure 3.4. Strong concordance of the observed abnormalities was seen amongst the aCGH results of each abnormal cell line. Cells from TOV-21G showed no variability and all showed the expected gain of chromosome 10. Each SKOV-3 cell displayed the gain of chromosome 12, whilst 91% (10/11) also carried the large segmental loss of material between the terminus of the short arm of chromosome X and Xq21.3.

Table 3.1: Single cells used for the validation of aCGH

CELL LINE	EUPLOID	ANEUPLOID	NO AMPLIFICATION	NO Y CHROMOSOME RATIO	SUM
IOSE-11	4	0	0	1	**5**
IOSE-19	5	0	0	0	**5**
TOV-21G	0	15	2	1	**18**
SKOV-3	0	11	0	1	**12**
TOTAL	**9**	**26**	**2**	**3**	**40**

Table 3.1: aCGH result of the single cell analysis. *The number of cells that did not produce a WGA or an aCGH result is indicated.*

Figure 3.4: Examples of aCGH outcome on single cells

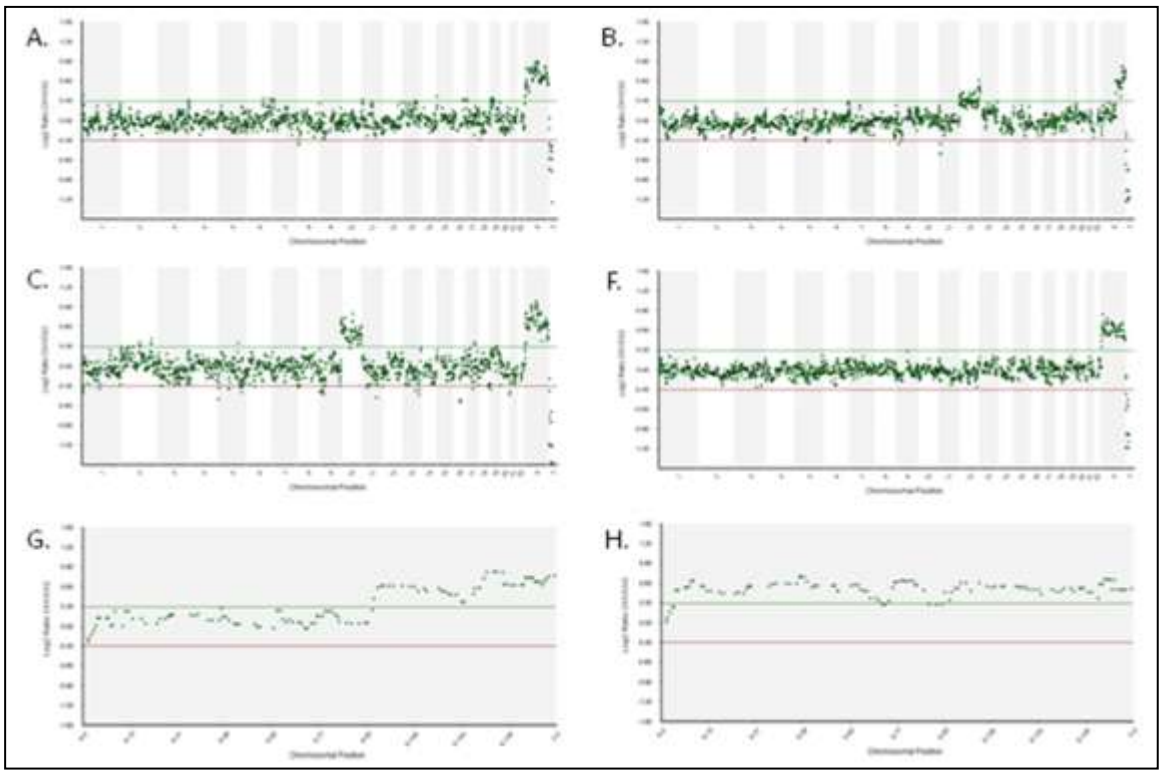


Figure 3.4: *A+F: Example of cells from each of the euploid cell lines IOSE-11 and IOSE-19. B: SKOV-3 cell line (aneuploid, gain of chromosome 12 and loss of Xpter to Xq21.3). C: TOV-21G cell line (aneuploid, gain of chromosome 10). G: Zoom in for X chromosome of an SKOV-3 cell, displaying the segmental deletion between the terminus of Xq to Xq21.3. H: Zoom in for X chromosome of a euploid IOSE-11 cell where no segmental changes were seen.*

3.1.5 aCGH on mosaic trophectoderm and blastocyst models

From the FISH and aCGH results on single cells from each cell line it was shown that TOV-21G was the most stable amongst the two aneuploid lines. For this reason it was selected to prepare the mosaic models along with the IOSE-19 euploid line. As described in the Material and Methods section 2.3.1.3, 24 mixed cell samples were prepared, 14 representing mosaic TE models with a total of eight cells each and 10 blastocyst models with a total of 100 cells each. Seven TE samples were prepared containing different ratios of aneuploid cells ranging from 0 to 100% in duplicates (TE group A and TE group B). Similarly, five blastocysts samples were prepared again in duplicates (Blastocyst group A and Blastocyst group B). All samples were amplified using the Sureplex WGA kit (BlueGnome, UK) and showed a bright smear on the agarose gel and all produced an interpretable aCGH result. In

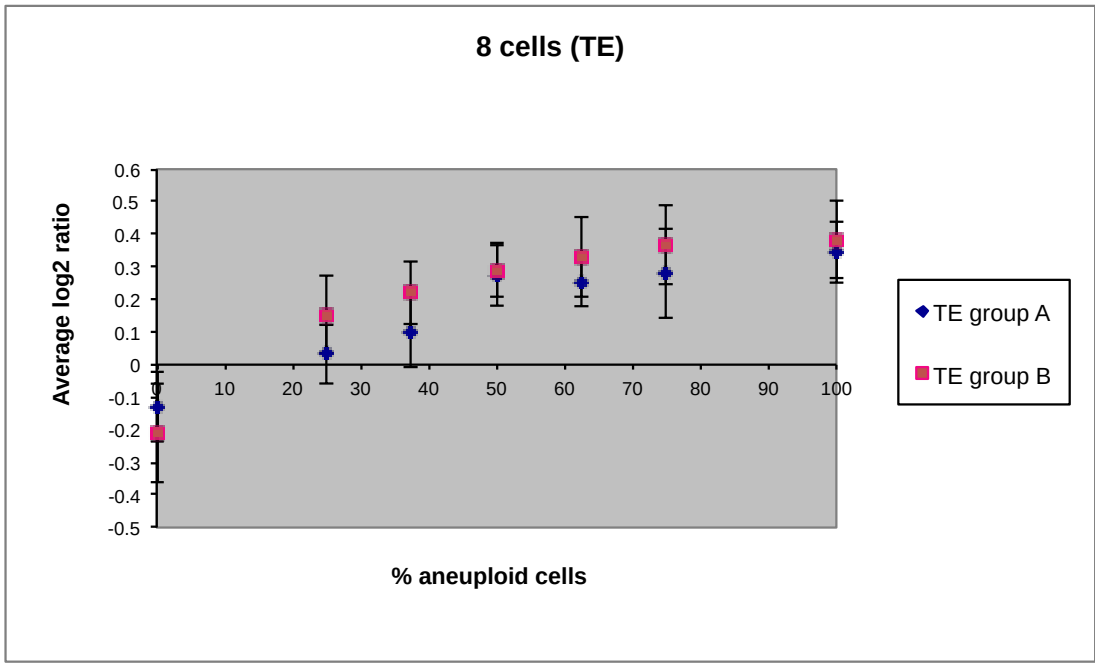
order to determine the effect of mosaicism on aCGH the average $\log_2$ ratios of the sample to control DNA of the clones for chromosome 10 were calculated.

Theoretically, the $\log_2$ ratio should have increased as the proportion of aneuploid cells in the sample increased. Variation in the ratios among the clones for chromosomes 10 was represented by the SD. Low SD values indicated that the values of the clones were similar. SD also indicated the quality of the array. Low SD indicated a good quality result with low experimental noise. The percentage of the clones included was also noted as a quality indicator of the array experiments. This also acted as a means of comparison between the analysis of a small (eight) and large (100) number of cells in the starting material. The values of the average ratios for all samples are found in table 3.2 and figure 3.5 presents graphs with the $\log_2$ ratio of all the samples.

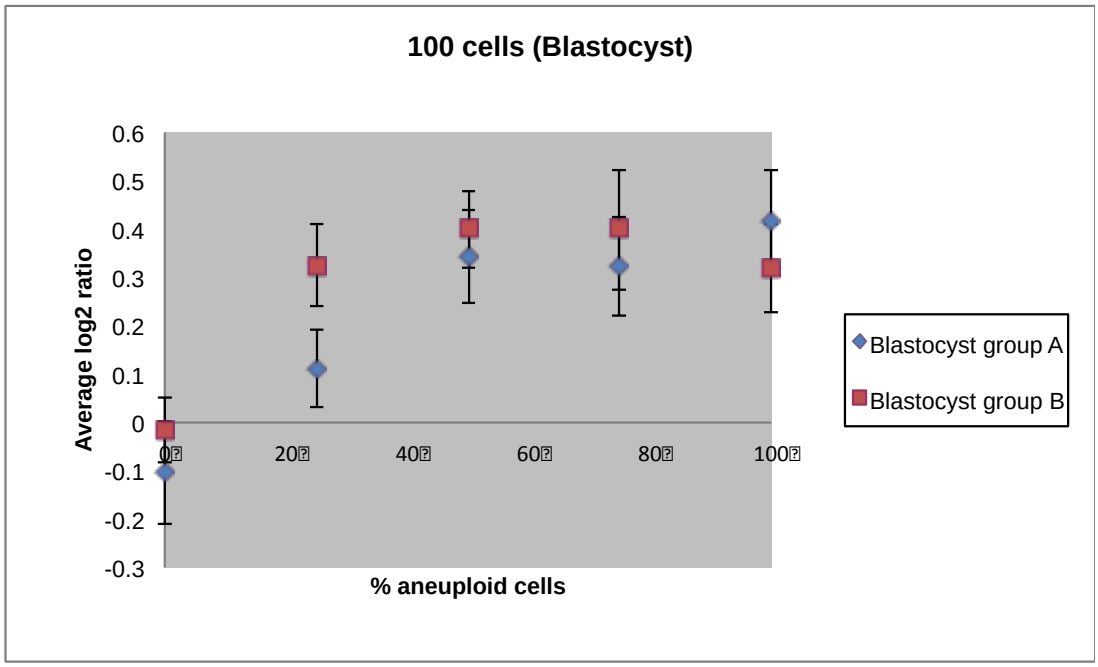
Table 3.2: Log2 ratios for chromosome 10 of the mixed samples				
8-cell samples (TE)				
Sample: euploid/aneuploid (% aneuploid cells)		**Average log_2 ratio**	**±SD**	**Clones included (%)**
1: 8/0 (0)	A	-0.13	0.11	91.64
	B	-0.21	0.15	88.63
2: 6/2 (25)	A	0.03	0.09	90.46
	B	0.15	0.12	64.38
3: 5/3 (37.5)	A	0.10	0.11	89.48
	B	0.22	0.10	90.89
4: 4/4 (50)	A	0.28	0.09	89.14
	B	0.29	0.08	68.47
5: 3/5 (62.5)	A	0.25	0.08	96.66
	B	0.33	0.12	73.19
6: 2/6 (75)	A	0.28	0.14	79.09
	B	0.37	0.12	69.34
7: 0/8 (100)	A	0.34	0.09	88.30
	B	0.38	0.12	64.72
100-cell samples (Blastocyst)				
8: 100/0 (0)	A	-0.10	0.11	94.30
	B	-0.02	0.07	74.97
9: 75/25 (25)	A	0.11	0.08	72.58
	B	0.32	0.08	79.80
10: 50/50 (50)	A	0.34	0.10	63.98
	B	0.40	0.08	75.89
11: 25/75 (75)	A	0.32	0.10	87.69
	B	0.40	0.13	63.54
12: 0/100 (100)	A	0.42	0.10	96.63
	B	0.32	0.09	67.69

Table 3.2: The log_2 ratios of the mosaic samples prepared for aCGH analysis. *The average log_2 ratios along with the standard deviation (SD) for the clones on chromosome 10 as well as the clones included in the analysis of each aCGH experiment for all the TE and blastocyst models are shown.*

Figure 3.5: Shift of the log_2 ratio in mosaic samples of clones on chromosome 10



A



B

Figure 3.5: *For the 8-cell samples (A) a shift from normality was detected even when the proportion of aneuploid cells was 25%. A gain was detected when the proportion of aneuploid cells were over 50%. For the 100-cell samples (B) a clear increase of the ratio was observed even at a proportion of 25% with clear gain detected at and above 50% of aneuploid cells [Reprinted from Fertility and Sterility, 97 (4), Mamas et al, Detection of aneuploidy by array comparative genomic hybridization using cell lines to mimic a mosaic trophectoderm biopsy, 943-947, Copyright (2012), with permission from Elsevier].*

An increasing trend of the ratio was observed for all samples when the number of aneuploid cells increased. The cut-off value for a chromosomal gain was determined by the BlueFuse Multi software at 0.3 ratio. For the TE models, the $\log_2$ ratio ranged from -0.21 for 0% aneuploid cell to 0.38 for 100% aneuploid cells. Although an increase was observed in both groups, there were some differences between them. All samples of the TE group B showed a higher value than the samples of TE group A apart from the 0% aneuploid cells samples. This could be attributed to variation within the cells and/or inclusion of anucleate cells. The ±SD of the samples also varied and reached up to 0.15 for one of the samples including 0% of aneuploid cells probably due to experimental noise of the aCGH. Both samples with 50% aneuploid cells had $\log_2$ ratios close to the 0.3 threshold (0.28 and 0.29). All samples with more than 50% aneuploid cells were over the threshold with the exception of one of the two samples with 62.5% and 75% aneuploid cells. Figure 3.6 presents examples of four mosaic TE models with 0, 25, 50 and 100% aneuploid cells.

Figure 3.6: Examples of aCGH outcome of TE mosaic models

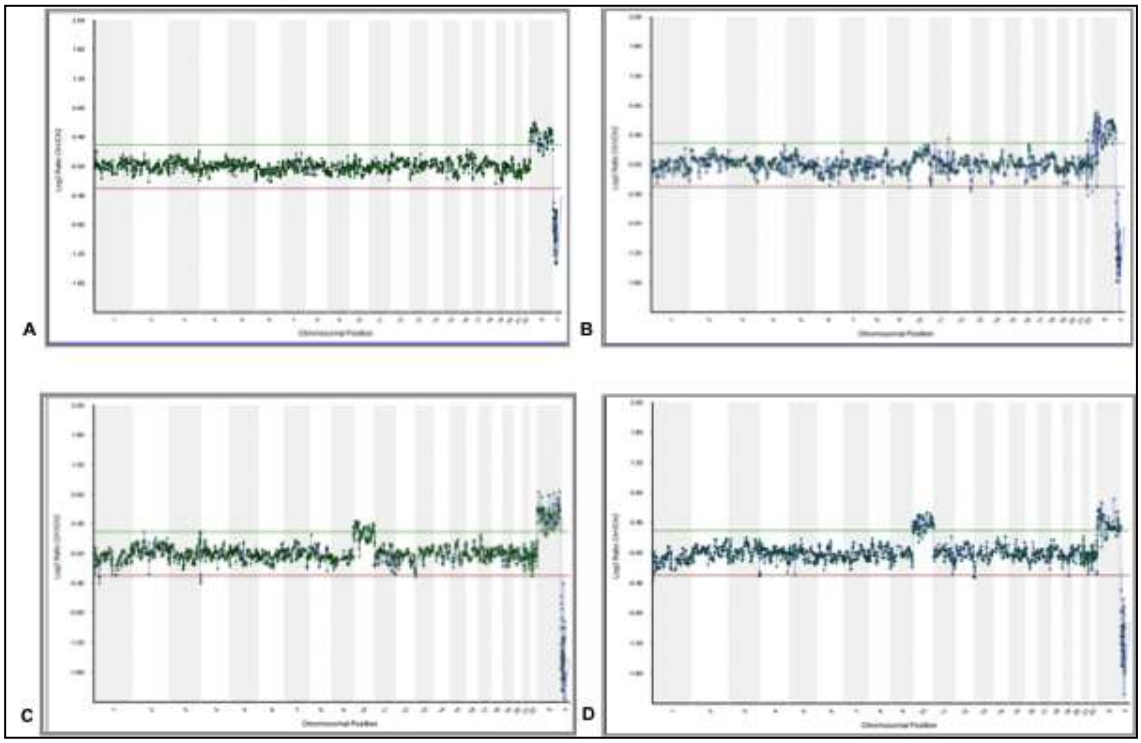


Figure 3.6: *A: Sample containing no aneuploid cells, showing a euploid 46,XX result. B: Sample containing 25% aneuploid cells, in which a small shift in the ratio towards a gain is observed. C: 50% aneuploid cells and the ratio is on the 0.3 threshold. D: Cells containing only aneuploid cells. The ratio is higher than the threshold. [Reprinted from Fertility and Sterility, 97 (4), Mamas et al, Detection of aneuploidy by array comparative genomic hybridization using cell lines to mimic a mosaic trophectoderm biopsy, 943-947, Copyright (2012), with permission from Elsevier]*

For the blastocyst model, both samples containing no aneuploid cells were below the threshold. For the two samples containing 25% cells one was below the threshold, whereas the repeat showed a high value of 0.32. All samples containing 50%, 75% and 100% aneuploid cells had $\log_2$ ratios ranging from 0.32 to 0.40. The standard deviation for this set was lower than the TE models ranging from 0.08 to 0.1, representing lower experimental noise in the aCGH result. Generally, an increasing trend was seen in the samples of the Blastocyst group A, with, however, the $\log_2$ ratio of the sample with 50% aneuploid cells being higher than the sample containing 75%. A rapid increase was observed in the samples of the Blastocyst group B between the completely normal sample and that containing 25% of aneuploid cells and finally a small decrease in the $\log_2$ ratio of the completely abnormal sample when compared to that containing 75% of aneuploid cells.

When observing the clones included for each aCGH experiment it was noted that that the average of clones included for the 8-cell sample groups was 81.74 (range: 64.38 – 96.66) and for the 100-cell sample groups, 77.71 (range: 63.54 – 96.63). Although not statistically significant (t-test, p=0.40) the clones included in the analysis of 100 cells was lower. This might be due to poor amplification resulting from the high volume of PBS in the starting material following the isolation of 100 cells in the same tube.

3.1.6 aCGH experimental quality

As described in the Materials and Methods section 2.5.2.6 during the analysis of the aCGH result, the software provided indicators for the quality of each experiment. These were the percentage of the clones included, the signal to background ratio (SBR) and the mean spot amplitude (MSA). The ideal values of these indicators in an efficient experiment were >90% clone inclusion, an SBR of over two and an MSA of over 1000. In the following sections a series of tests to decrease the experimental time and increase the efficacy of aCGH are described. Comparison of the tests was performed using these indicators.

As described by the manufacturer, the labelling and hybridisation times could vary between two to 18 and three to 16 hours respectively. In a clinical PGS setting the time of diagnosis is critical and ideally a result should be reached as soon as possible. A series of tests were performed to identify the quickest and most efficient

combination of labelling and hybridisation. This was done first by labelling the same whole genome amplified sample twice, for three hours and then hybridising one labelled product for three hours and the other for 16 hours. The sample that was run was a whole untransferred embryo, which was found to be aneuploid post PGS. Reanalysis of the embryo showed a loss of chromosome 10. The aCGH profiles as well as the values of the indicators for both samples are found in figure 3.7 (A+B). The MSA and SBR for both experiments were at acceptable levels and showed small differences. The percentage of the clones analysed, however, was much lower after hybridisation for three hours (56%) when compared to the 16-hour hybridisation (75%).

Figure 3.7: Long and short hybridisation

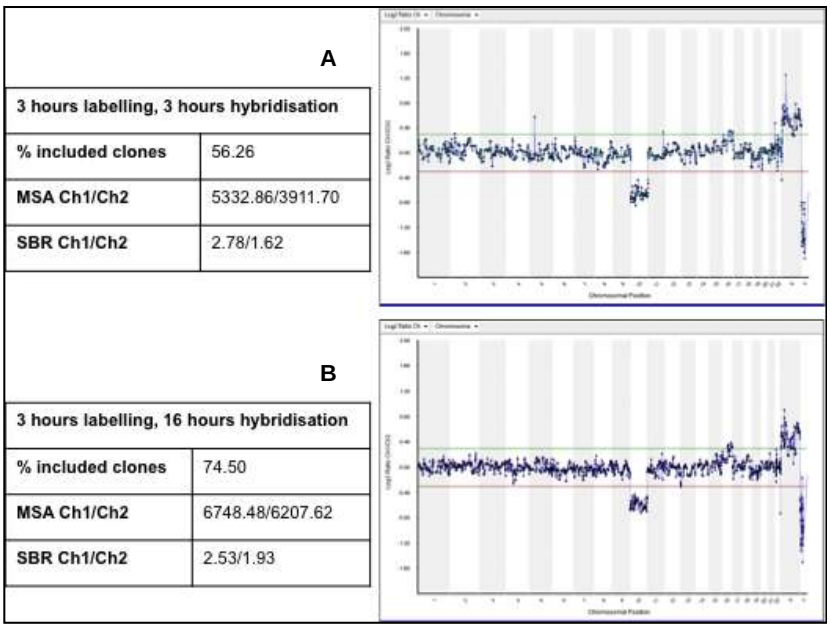


Figure 3.7: *The same whole genome amplified sample was labelled twice. One labelled product was hybridised for three hours (A) and the other for 16 hours (B).*

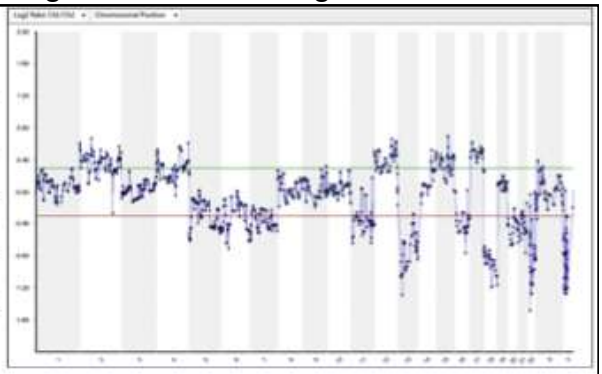
The next set of experiments tested short and long labelling times and their effects on the aCGH result. Again the same whole genome amplified sample, this time a single blastomere, was labelled for three and 18 hours. The two samples were then hybridised for three hours. A short hybridisation time was preferred over the long 16-hour one in order to reduce the overall time of the experiment. The single cell was found to be highly aneuploid, showing a shift in almost all the chromosomes (figure 3.8A+B). The values of the indicators of the sample labelled and hybridised for three hours were comparable to the whole embryo, which was subjected to the same labelling and hybridisation times described above. On the other hand, the sample that was labelled for 18 hours showed very satisfactory values. The percentage of

the clones analysed was ideal, 95%. Similarly, the MSA and SBR values were both very high. The combination of a long labelling with a short hybridisation was shown to be the most appropriate to use during a PGS case when time for achieving a good result is critical.

Figure 3.8: Long and short labelling

A

3 hours labelling, 3 hours hybridisation	
% included clones	58.36
MSA Ch1/Ch2	3318.09/7685.60
SBR Ch1/Ch2	1.29/3.29



B

18 hours labelling, 3 hours hybridisation	
% included clones	94.97
MSA Ch1/Ch2	10674.90/17440.95
SBR Ch1/Ch2	5.33/6.65

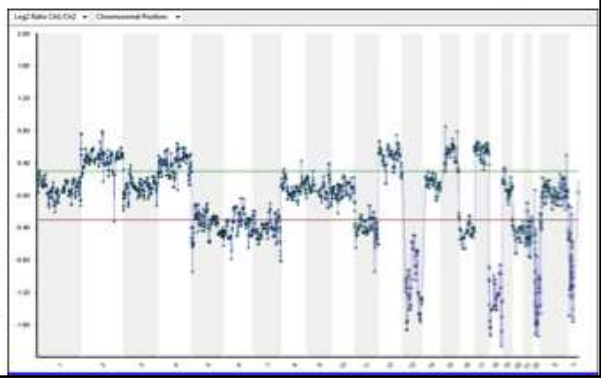


Figure 3.8: *The same whole genome amplified sample was labelled once for three hours (A) and once for 18 hours (B). Both samples were hybridised for three hours.*

3.1.7 aCGH on trophectoderm samples biopsied from frozen-thawed blastocysts

Trophectoderm (TE) was biopsied from four thawed blastocysts, to further validate the ability to detect chromosomal imbalance by aCGH post blastocyst biopsy. This also acted as a “dry-run” of a clinical PGS case, in which aCGH was going to be used for the analysis. Four blastocysts, which were donated to research, from routine IVF patients, were thawed and TE biopsy was performed by the embryologists. The biopsied TE cells and their corresponding blastocysts were run on 24sure arrays. aCGH was successful in all samples, confirmed by smears on an agarose gel. As seen in figure 3.9 smears for all the samples were more intense at around 400bp, the average size of fragments produced by SurePlex. The TE

samples (wells 1 to 4) were not as bright as the remainder of the blastocyst (wells 5 to 8) since the starting material was less.

Figure 3.9: Agarose gel electrophoresis of biopsied TE and remainder of blastocysts

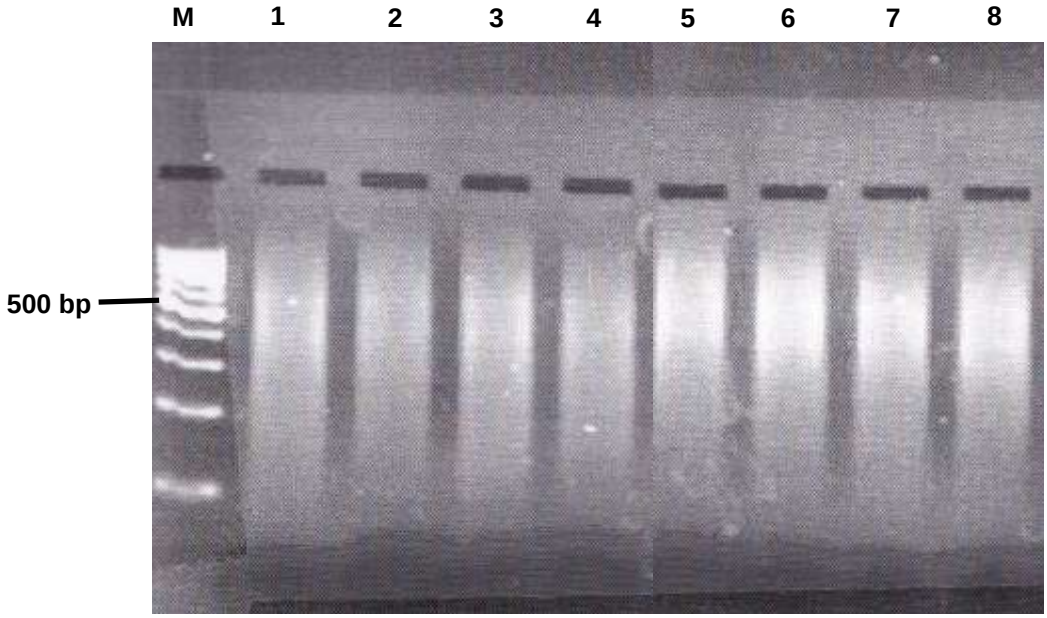


Figure 3.9: *M: 100bp molecular weight marker, indicating the 500bp band used for comparison with the bright intensities of the smears at around 400bp. Wells 1-4: Amplified biopsied TE samples. Wells 5-8: Amplified blastocysts.*

Validation was performed by first confirming the sex of the embryo seen in TE cells in the remainder of the blastocysts and then by any aneuploidies that were detected. Embryos A and B were found to be euploid female and male, respectively, after TE analysis, which was confirmed in the blastocysts. Embryo C was a male showing a loss for chromosome 21 in both samples. Finally, embryo D showed a shift in the $\log_2$ ratio for both the sex chromosomes away from zero indicating a possible female sample. However, this was not comparable to the dynamic change in ratio between the sex chromosomes that was expected in a female sample (around 0.3 for chromosome X and -0.8 for Y) but was equally reduced, thus indicating a possible 69,XXY triploid sample. Figure 3.10 presents the TE and blastocyst results of the embryos analysed.

Figure 3.10: Trophectoderm and blastocysts aCGH profiles

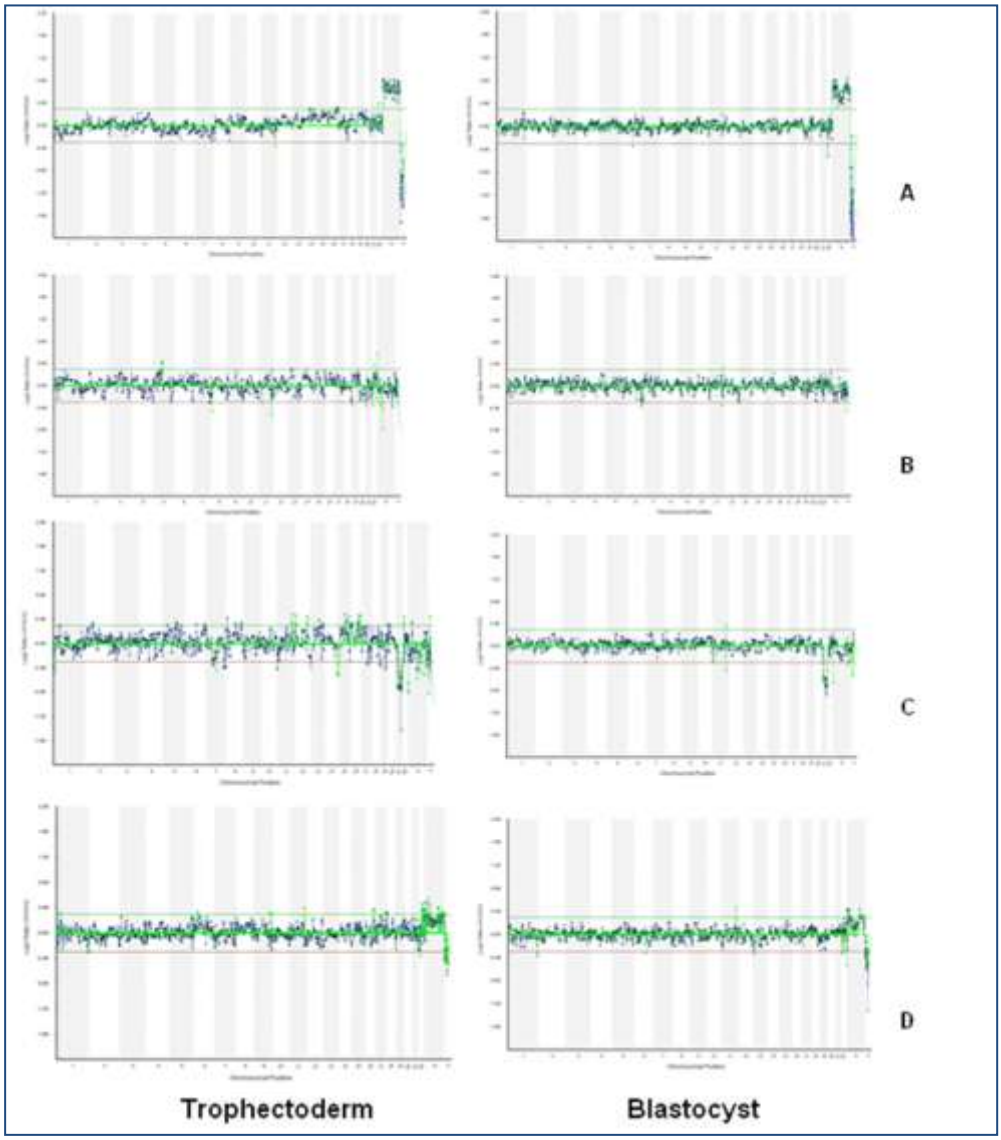


Figure 3.10: *Trophectoderm samples are presented on the left and blastocyst samples on the right of the figure. Embryos A and B were shown to be euploid female and male respectively. Embryo C was male with a loss of chromosome 21. Embryo D was 69,XXY as determined by the smaller ratio change between the sex chromosomes.*

3.1.8 FISH confirmation of abnormalities detected by aCGH in PGS

In order to complete the validation of aCGH, FISH was performed as follow up analysis of embryos that were first clinically analysed by aCGH during PGS. Probes were selected according to the abnormalities detected during the case to confirm their presence in the whole embryo. Follow-up was performed on two embryos from two cases of PGS on TE biopsied samples and six embryos from three cases of PGS after biopsy of single blastomeres from cleavage stage embryos. Table 3.3 presents the results of the FISH follow-up for all embryos.

Table 3.3: FISH follow-up analysis of embryos analysed by aCGH for PGS						
Couple	**Embryo**	**Biopsy**	**aCGH result**	**Day 5 morphology**	**No of nuclei analysed**	**FISH result**
A1	A1.1	Blastocyst	45,XY,-14	Blastocyst	51	67% nuclei disomy 14 33% nuclei monosomy 14
A2	A2.1	Blastocyst	48,XY,+1q22-qter,+4,+5	Blastocyst	34	85% nuclei trisomy 5 57% nuclei trisomy 4 88% nuclei trisomy 1q Remaining nuclei disomy for all chromosomes
A3	A3.1	Cleavage	44,XY,-6,-18	Morula	36	88% nuclei monosomy 18 12% nuclei disomy 18
	A3.2	Cleavage	51,XX,+2,+8,+12,+15,+18	Morula	7	Chromosome 18: 57% trisomy, 43% disomy
A4	A4.1	Cleavage	46,XY	Morula	58	57.4% nuclei disomy for chromosomes 13, 15, 18, 21 and 22 Remaining nuclei chaotic
	A4.2	Cleavage	44,XY,-15,-21	Morula	35	Chromosome 21: 33% monosomy, 54% disomy Chromosome 15: 24% monosomy, 29% disomy, 35% trisomy
A5	A5.1	Cleavage	45,XX,-21	Morula	43	Chromosome 21: 76% monosomy, rest disomy
	A5.2	Cleavage	45,XY,-17	Arrested	5	All nuclei showed monosomy 17

Table 3.3: Results of the follow-up by FISH after PGS using aCGH. *The type of biopsy used for each embryo, the aCGH result, morphology on day five, the number of nuclei analysed by FISH as well as the FISH result are indicated.*

Embryos from couples A1 and A2 were biopsied at the blastocyst stage, during which six to ten cells were removed from the trophectoderm. For embryo A1.1 (figure 3.11 A) a shift was observed in the ratio of chromosome 14 towards a loss. The average $\log_2$ ratio for that chromosome was -0.20 (±0.06), indicating mosaicism. Indeed, FISH with a telomeric probe on the q arm of chromosome 14 revealed that in the whole of the embryo only 33% of the nuclei showed monosomy with the rest (67%) being diploid for that chromosome.

Embryo A2.1 (figure 3.11 B) presented a shift in the ratio in the long arm from chromosome 1, from 1q22 to the terminus. The average ratio observed was 0.24 (±0.05) showing a mosaic gain. FISH, however, showed that 88% of the nuclei in the whole embryo carried that aneuploidy, a high percentage of cells, which if that was the same in the TE sample, it should have resulted in a higher shift of the $\log_2$ ratio. In the same embryo chromosome 4 had an average ratio of 0.18 (±0.07) and only 57% of the analysed nuclei by FISH showed trisomy for that chromosome. The third abnormality detected in this embryo was a gain of chromosome 5 with the highest ratio amongst the affected chromosomes of 0.28 (±0.05). Trisomy for chromosome 5 was seen in 85% of the nuclei in the whole embryo.

Figure 3.11: FISH follow-up of PGS after blastocyst biopsy

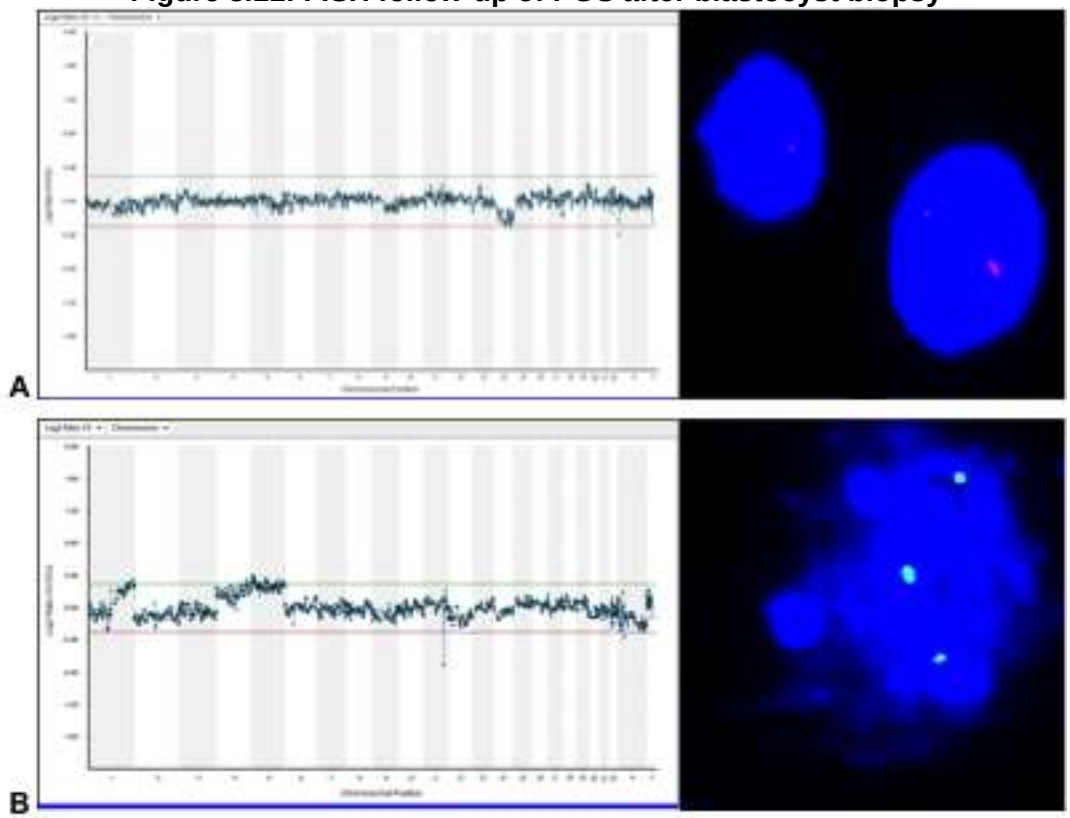


Figure 3.11: *A: Embryo A1.1. aCGH showed a shift towards a loss for chromosome 14. FISH showed 67% of the nuclei being diploid and the rest carrying monosomy 14. B: Embryo 2.1. aCGH showed a segmental gain of 1q22 to the q terminus, 4 and 5. The nucleus on the right showed trisomy for chromosome 5.*

PGS on single blastomeres biopsied from cleavage stage embryos was performed for couples A3, A4 and A5. Mosaicism in the biopsied sample was not an issue for these diagnoses, since only one cell was analysed. Mosaicism in the embryo however was very critical since the biopsied cell might have not represented the chromosomal status of the whole embryo. FISH to check only chromosome 18 was performed in the embryos of couple A3, which were both morulae on day five, due to unavailability of the rest of the probes. Embryo A3.1 showed loss for that chromosome and it was confirmed in 88% of the nuclei. Embryo A3.2 showed gain for 18, detected upon follow-up in 57% of the nuclei.

Embryo A4.1 was found to be euploid by aCGH but was not transferred as the couple had a surplus of normal embryos and was not of good quality. It reached the blastocyst stage on day five and FISH with probes for five chromosomes revealed that 57.4% of the nuclei were diploid for these chromosomes, whereas the rest showed a chaotic chromosomal complement. The second embryo of this couple showed loss for chromosomes 15 and 21. FISH revealed 24% monosomic, 35%

trisomic and 29% diploid nuclei for chromosome 15 with the rest showed a low level of chaoticism. The presence of both monosomic and trisomic nuclei revealed that mitotic non-disjunction occurred in the embryo for that chromosome. The loss of chromosome 21 was confirmed in only 33% of the nuclei, 54% were diploid and the rest showed different abnormalities.

Loss of the same chromosome was also confirmed in 76% of the nuclei of embryo A5.1 (figure 3.12 A). All the nuclei analysed from embryo A5.2 (figure 3.12 B) showed a loss for chromosome 17, which was detected by aCGH. However, this was an arrested at cleavage stage embryo and only five nuclei were analysed.

Figure 3.12: FISH follow-up of PGS after cleavage stage biopsy

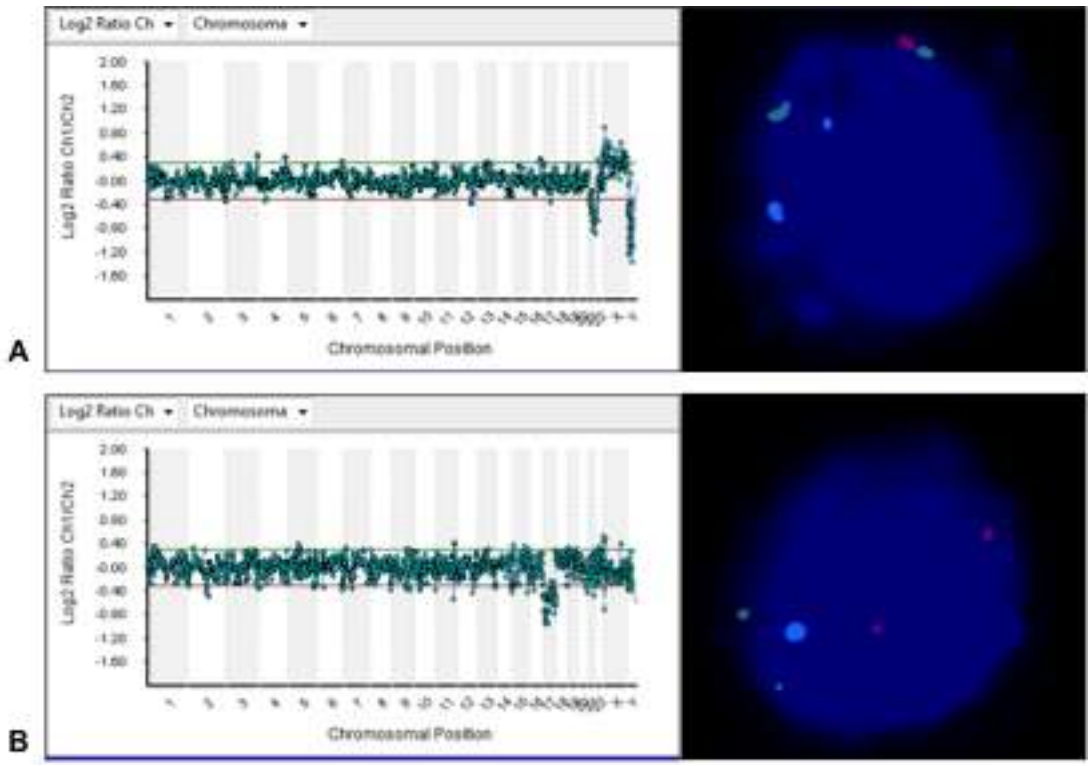


Figure 3.12: *A: Embryo A5.1. aCGH showed a loss for chromosome 21. Probes for chromosomes 13, 17 and 21 were used. Monosomy 21 is seen in spectrum orange. B: A5.2. The loss of chromosome 17 was confirmed by FISH in spectrum aqua in all nuclei analysed from that arrested embryo.*

3.1.9 Summary of results for section 3.1: aCGH validation for clinical application

- The chromosomal status of single cells was successfully analysed by aCGH. Euploidy and known aneuploidies were identified in cells from euploid and aneuploid cell lines with a 100% concordance.
- The effect of mosaicism in the aCGH result was made apparent with the analysis of mosaic TE and blastocyst models. A shift from normality was observed when 25% of aneuploid cells were present in a sample and at 50% of aneuploid cells the ratio reached the abnormality threshold.
- A series of tests showed that long labelling for 18 hours with a short, three-hour hybridisation provided the best and quickest aCGH result.
- Concordance in the chromosomal analysis by aCGH was also observed between biopsied TE samples and the remainder of their corresponding blastocysts.
- Confirmation on whole embryos of the aCGH result after PGS on cleavage stage embryos and blastocysts by FISH revealed that abnormalities detected by aCGH did not always represent the chromosomal status of the embryo mainly due to mosaicism.

3.2 Aneuploidy in embryos from couples undergoing PGD

The level of aneuploidy was examined in embryos from couples undergoing PGD by FISH and aCGH. These were couples of young age with no known fertility problems, seeking IVF only for the purpose to perform PGD. This analysis highlighted differences in the limitations between the two techniques when applied to investigate the chromosome number. Mechanisms that lead to aneuploidy were also considered, especially after FISH analysis. Finally, the level of aneuploidy in embryos from couples undergoing PGD that were considered fertile, was compared with that in embryos from couples undergoing PGS. Couples performing PGS cycles were infertile with increased chances of aneuploidy in their embryos.

3.2.1 Patient description

FISH analysis was performed on 86 embryos (PGD-FISH group) from 19 couples that went though 24 cycles of PGD. The average female age was 32.5 (±4.2) and the average male age was 34.9 (±5.4). aCGH was used to analyse 53 embryos (PGD-aCGH group) from six couples that underwent nine PGD cycles. The average female age for this group of couples was 31.5 (±4), whereas the male age was 33.3 (±4.3). The indication for PGD in both groups were mutations in genes causing a variety of monogenic disorders, namely Myotonic Dystrophy type 1 (DM1, gene: *DMPK*), Familial Adenomatous Polyposis (FAP, gene: *APC*), breast-ovarian cancer-1 (BROVCA1, gene: *BRCA1*), Neurofibromatosis type 1 (NF1, gene: *NF1*), Crouzon syndrome (gene: *FGFR2*) and non-syndromic deafness (gene: *Cx26*). Of all couples analysed, only one presented fertility issues, through a poor ovarian reserve test (ORT) probably due to advanced maternal age. Five had previous pregnancies that were either terminated due to the presence of the mutation in prenatal diagnosis, or resulted in the birth of unaffected children. The rest had no children. Tables 3.4, provides the couple information for the PGD-FISH and PGD-aCGH groups.

Table 3.4: Information on couples from PGD cycles with embryo chromosomal analyses						
PGD-FISH group						
Couple ID	**FA**	**MA**	**Reproductive history**	**Disease**	**No of cycles**	**No of embryos**
B1	33	41	No children	Crouzon	1	1
B2	31	34	No children	NF1	2	9
B3	32	29	No children	FAP	1	4
B4	34	36	1 TOP	DM1	1	2
B5	35	36	1 child +2 TOP	DM1	1	1
B6	31	38	No children	DM1	1	3
B7	38	36	Poor ORT	DM1	3	5
B8	39	42	1 m/c	DM1	1	6
B9	30	31	No children	DM1	2	3
B10	27	28	No children	BROVCA1	1	5
B11	30	31	1 child +1 TOP	FAP	1	6
B12	35	38	No children	DM1	1	1
B13	27	25	No children	BROVCA1	2	11
B14	30	38	1 child	FAP	1	4
B15	40	47	1 child	Deafness	1	3
B16	29	37	1 m/c	FAP	1	1
B17	36	32	No children	DM1	1	3
B18	35	36	No children	Crouzon	1	9
B19	25	29	No children	DM1	1	9
Average	**32.5 (±4.2)**	**34.9 (±5.4)**		**Total**	**24**	**86**
PGD-aCGH group						
Couple ID	**FA**	**MA**	**Reproductive history**	**Disease**	**No of cycles**	**No of embryos**
B20	28	29	No children	FAP	1	8
B21	27	27	No children	NF1	1	8
B22	33	34	No children	DM1	3	11
B23	36	36	No children	NF1	1	2
B24	29	37	No children	FAP	1	2
B25	36	37	No children	NF1	2	22
Average	**31.5 (±4)**	**33.3 (±4.3)**		**Total**	**9**	**53**

Table 3.4: Couples of the PGD-FISH and –aCGH groups. *For each couple the female and male age, reproductive history, the genetic disorder diagnosed by PGD, the number of PGD cycles after which FISH or aCGH follow up analysis was performed as well the number of embryos analysed are indicated. FA: Female Age, MA: male age. TOP: Termination of pregnancy. ORT: Ovarian reserve test. m/c: miscarriage*

3.2.2 Results from embryos analysed by FISH

The Aneuvision kit was used for all the embryos, for the analysis of chromosomes 13, 18, 21, X and Y in two FISH rounds. An interpretable result was obtained from 66 (82.5%) of the 86 embryos. Figure 3.13 shows an example of two nuclei that were found to be diploid for all chromosomes on both rounds (A+C), one example of a nucleus that showed monosomy for chromosome 13 (B) and finally a male nucleus with monosomy 18 (D).

Figure 3.13: Blastomere nuclei hubridised with fluorescent probes

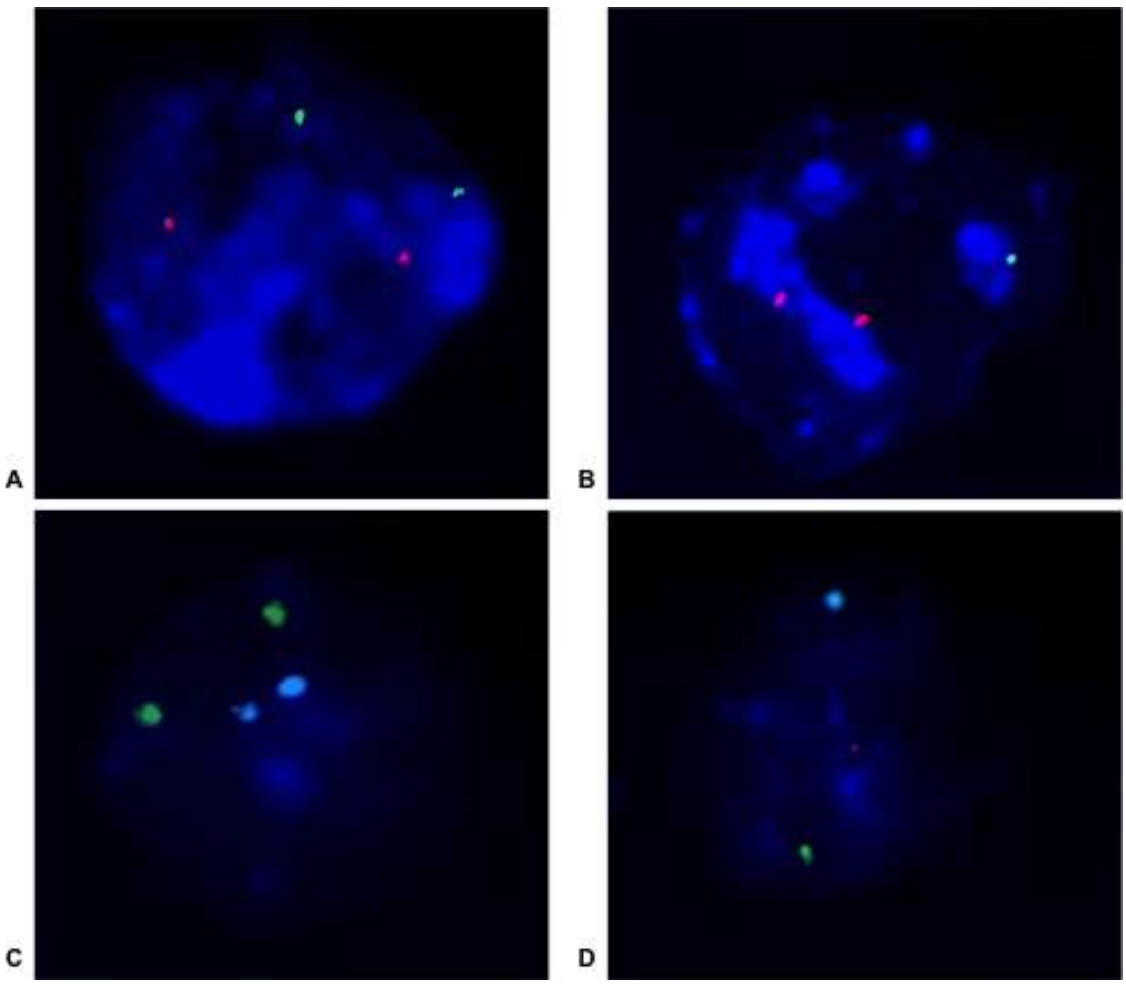


Figure 3.13: *A) Nucleus diploid for chromosome 13 (green) and 21 (orange). B) Nucleus with monosomy for chromosome 13. C) Female nucleus (X chromosome is hybridised with a green fluorescent probe) diploid for chromosome 18 (aqua). D) Male nucleus (one green signal for X and one orange for chromosome Y) with monosomy 18.*

A detailed classification of the embryos is described in the Material and Methods section 2.5.3.1. Briefly, embryos consisting of uniform cell lines were diploid, aneuploid or haploid, when 90% of cells were normal, carried the same chromosomal aneuploidies or had one copy of all the chromosomes tested respectively. Mosaic embryos consisted of different cell lines. Chaotic were those embryos in which each cell had a different chromosomal complement. Table 3.5 and figure 3.14 present the overall FISH results of all the embryos analysed. The most predominant classification amongst the embryos was diploid/chaotic mosaic (41%). Of these only two had less than 50% diploid cells. All the diploid/aneuploid/chaotic mosaic embryos had more than 50% cells that were diploid. The detailed results for each embryo chromosomal classification, along with the developmental stage, number of pronuclei, the number of nuclei analysed in each round and the number of diploid nuclei seen are found in table 6.1 of Appendix A.

Table 3.5: Chromosomal classification of embryos analysed by FISH	
Number of embryos with a result	66/86 (82.5%)
Diploid	12 (18%)
Haploid	3 (5%)
Aneuploid	4 (6%)
Diploid/chaotic mosaic	27 (41%)
Diploid/aneuploid/chaotic mosaic	6 (9%)
Aneuploid/chaotic mosaic	8 (12%)
Chaotic	6 (9%)

Table 3.5: Chromosomal status of embryos in the PGD-FISH group. *The number and percentages of embryos with each chromosomal complement are indicated.*

Figure 3.14: Percentages of the different chromosomal classifications identified by FISH in embryos from PGD cycles

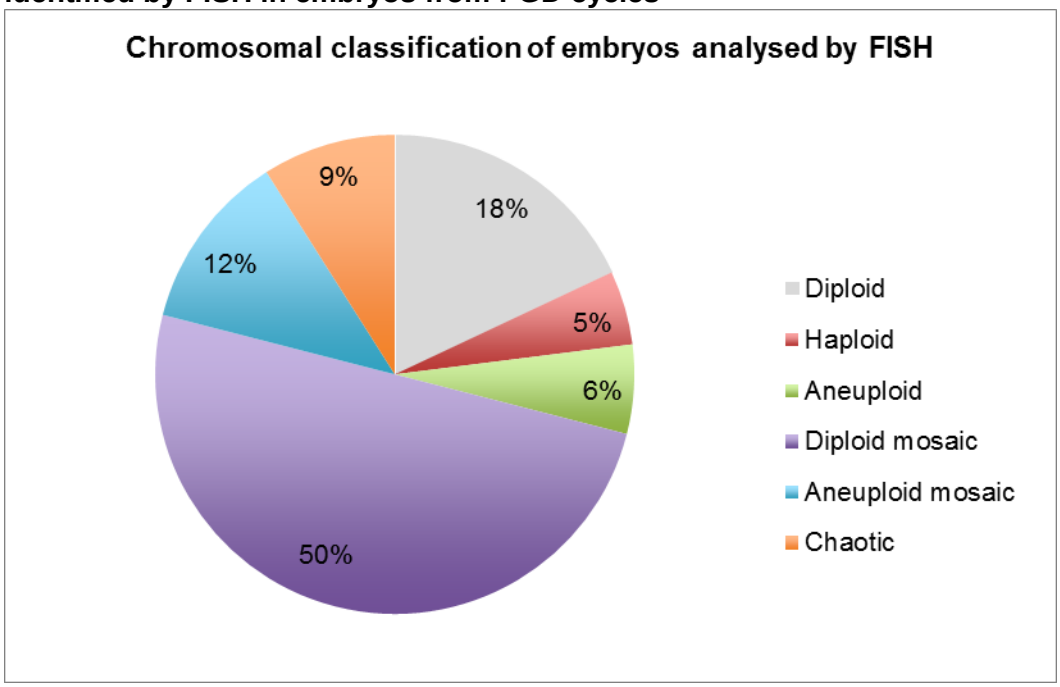


Figure 3.14: *Diploid/chaotic and diploid/aneuploid/chaotic mosaic embryos were grouped as diploid mosaics representing 50% of the embryos analysed.*

3.2.2.1 Chromosomal status of abnormally fertilised embryos

Among the 86 embryos analysed, 13 were scored as abnormally fertilised 16 to 20 hours post insemination due to abnormal number of pronuclei (PN). No pronuclei were seen in nine embryos (0PN) and three in four embryos (3PN). Table 3.6 presents the results of the FISH analysis for this small group of embryos. Both embryos with 3PN that produced a result had a diploid cell line. Of the seven 0PN embryos that gave a result, 3 were diploid/chaotic mosaic, one was haploid, two were chaotic and one was aneuploid/chaotic mosaic, which showed chromosome gain for chromosome 13. A deviation from the expected number of pronuclei post insemination might have been due to abnormal fertilisation but also due to the fact that embryologists had missed the normal two-pronuclei status. The absence of pronuclei might also have been due to delay of pronuclei appearance and the presence of three pronuclei might have been due to accelerated development of the embryo. For this reason, results of abnormally fertilised embryos are reported here separately, but they were included in the overall results of all embryos.

Table 3.6: Chromosomal classification of abnormally fertilised embryos	
Number of abnormally fertilised embryos with a result	9/13 (69%)
0PN embryos with a result	7
Haploid	1
Chaotic	2
Diploid/chaotic mosaic	3
Aneuploid/chaotic mosaic	1
3PN embryos with a result	2
Diploid	1
Diploid/chaotic mosaic	1

Table 3.6: Chromosomal status of abnormally fertilised embryos in the PGD-FISH group.

3.2.3 Results of embryos analysed by aCGH

aCGH was used to analyse the total chromosomal complement of 53 embryos. The 24sure microarray platform (BlueGnome, UK) was used. A result was obtained from 52 embryos (98%). The classification of the embryos according to their chromosome status was different to that of embryos analysed by FISH due to the differences between the two techniques. Contrary to FISH, where each cell was scored individually, in aCGH a cumulative result of all the cells in each embryo was obtained. Therefore, it was not possible to score mosaicism with the level of detail that is possible in FISH.

The $\log_2$ ratio of the fluorescence of the male reference DNA over the sample DNA for each chromosome was given after analysis of the array by the BlueFuse Multi software. Clear gains and losses should have resulted in $\log_2$ ratios that were above the 0.3 and below the -0.3 thresholds respectively. However, mosaicism in the form of diploid and aneuploid cells in the same sample could have altered these ratios. As shown in the Results section 3.1.5 that describes the mixing of euploid and aneuploid cells to mimic trophectoderm samples and whole blastocysts, a mosaic sample that consisted >50% of aneuploid cells, was represented by a $\log_2$ ratio above the threshold. Representation of mosaicism in aCGH was also confirmed when FISH follow-up was used to confirm the aCGH as described in section 3.1.8. On the other hand, errors that were caused by mitotic non-disjunction in a mosaic sample could not be detected by aCGH as the presence of reciprocal gains and

losses would not alter the $\log_2$ ratio. Moreover, one of the consequences of bad experimental quality is the reduction of the shift of the $\log_2$ ratio of an aneuploidy. Detailed analysis of mosaicism by aCGH is only possible after embryo disaggregation and separate analysis of each individual blastomere. Since all embryos, in this study were tubed whole, no attempt was made to score mosaicism. Chromosomal gains and losses were considered when there was a deviation from zero for the majority of the clones for each chromosome.

Embryos were characterised as euploid when there was no deviation from zero for any of the chromosomes. Aneuploid were those with a gain or loss for up to two chromosomes and complex aneuploid were those where three or more chromosomes were affected. Figure 3.15 shows four examples of aCGH profiles of euploid, aneuploid and complex aneuploid embryos.

Figure 3.15: aCGH profiles of embryos

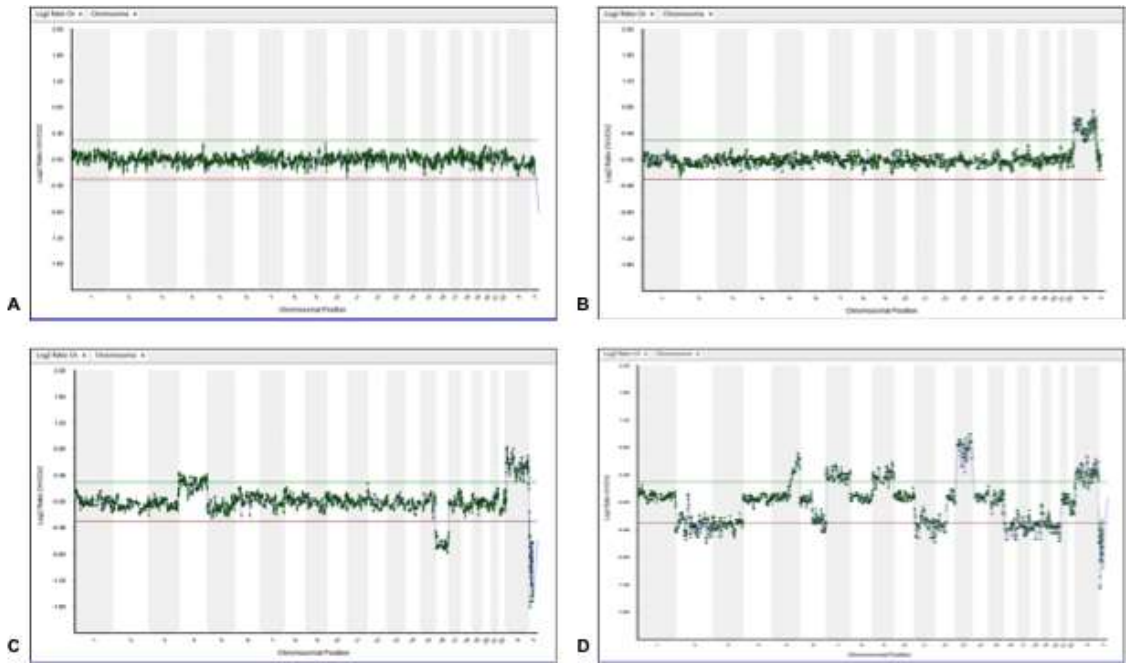


Figure 3.15: *A) A euploid male embryo, B) an aneuploid embryo with a gain of chromosome X, C) an aneuploid embryo with gain of chromosome 4 and loss of chromosome 16 and D) a complex aneuploid embryo with gain of chromosomes 7, 9, 13, loss of chromosomes 2, 3, 11, 16, 17, 18, 19 and 20, partial gain of 5q arm and partial loss of 6q and 12p arms.*

The detailed results for each embryo, the number of pronuclei, developmental stage, chromosomal classification and complement are found in table 7.1 of Appendix B. As seen in table 3.7 and figure 3.16, 38% of embryos analysed by aCGH were found to be euploid, whereas 37% were aneuploid. Twenty five per cent of embryos carried abnormalities affecting more than three chromosomes and were characterised as complex aneuploid.

Table 3.7: Chromosomal classification of embryos analysed by aCGH	
Number of embryos with a result	52/53
Euploid	20 (38%)
Aneuploid	19 (37%)
Complex aneuploid	13 (25%)

Table 3.7: Chromosomal status of embryos in the PGD-aCGH group. *The number and percentages of embryos with each chromosomal complement are indicated.*

Figure 3.16: Percentage of the different chromosomal classifications in embryos from PGD cycles analysed by aCGH

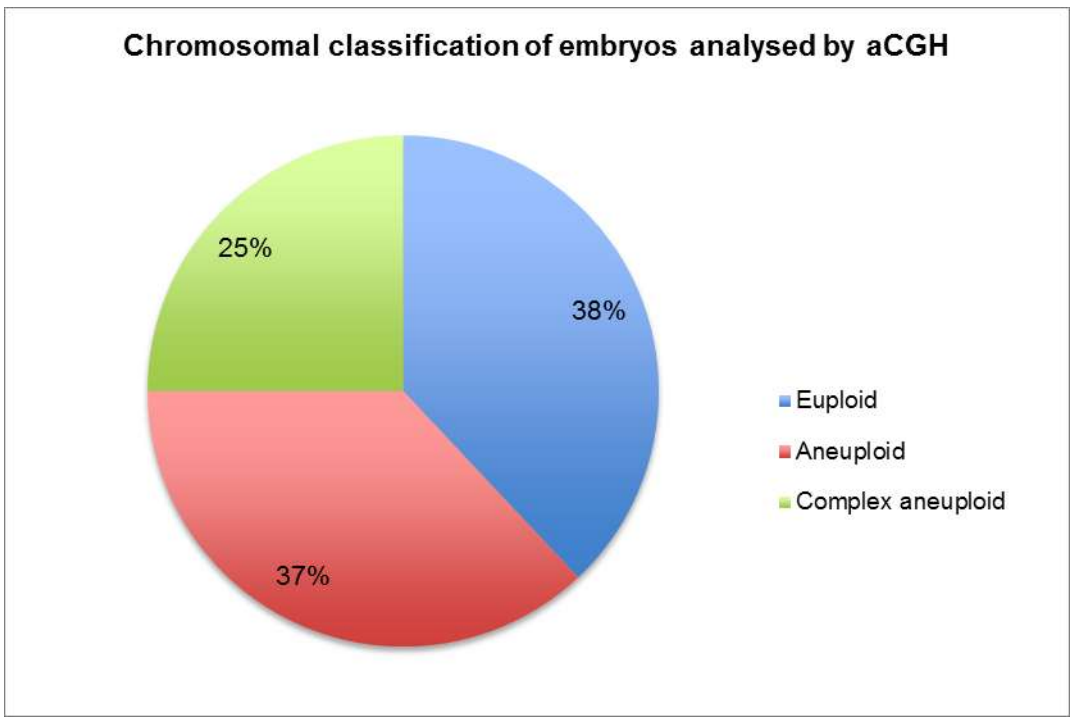


Figure 3.16: *Euploid embryos showed no abnormalities, aneuploid embryos carried abnormalities affecting one or two chromosomes and complex aneuploid embryos carried three or more affected chromosomes.*

3.2.4 Chromosomal status and development

All embryos with chromosomal analysis in this section were untrasferred embryos from PGD cycles on day five or six of development. They were arrested at cleavage stage, morulae or blastocysts. Of the 86 embryos analysed by FISH, 36 were arrested at cleavage stage, 33 were morulae and 17 were blastocysts. Of the 53 embryos analysed by aCGH, 18 were arrested, 20 morulae and 15 blastocysts. Figure 3.17 presents the chromosomal status of embryos at different developmental stages analysed by FISH (3.17A) or aCGH (3.17.B). All blastocysts analysed by FISH were found to be diploid mosaic. Diploid, aneuploid, diploid mosaic and aneuploid mosaic morulae were seen, however the distribution of diploid mosaic morulae was significantly higher than any other chromosomal type within that group of embryos ($p<0.05$, Fisher's exact test). All chromosomal classifications were seen within the arrested embryos, apart from diploid mosaic. It is important to note that all chaotic and haploid embryos had arrested at the cleavage stage.

All three different chromosomal classifications were seen among all embryos analysed by aCGH. Some differences were noted among the distribution of embryos in the chromosomal classifications. Euploid blastocysts were significantly more than complex aneuploid, aneuploid morulae were significantly more than aneuploid arrested and blastocysts and finally, complex aneuploid arrested embryos were significantly more than complex aneuploid blastocysts (all $p<0.05$, Fisher's exact test).

Figure 3.17: Grouping of embryos from PGD cycles according to chromosomal status and developmental stage

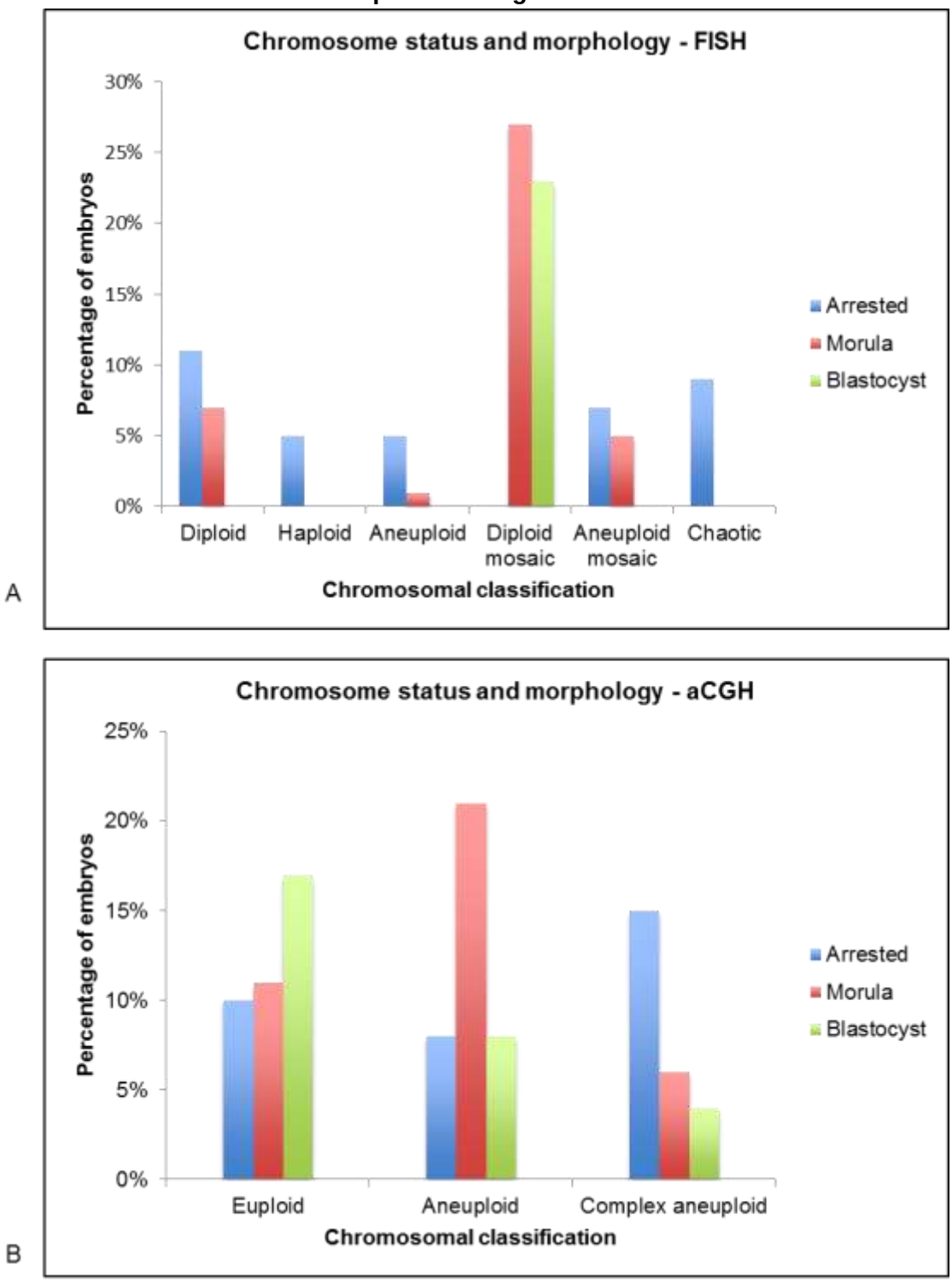


Figure 3.17: *A) Distribution of embryos analysed by FISH, B) distribution of embryos analysed by aCGH.*

3.2.5 Chromosomal status and female age

Despite the fact that the average female age in both groups was relatively low (32.5 and 31.5 for the PGD-FISH and -aCGH groups respectively) the age among the females ranged from 27 to 40 in the PGD-FISH group and 27 to 36 in PGD-aCGH group. In order to investigate any possible effect of maternal age in the embryonic chromosomal status, the embryos were categorised in two sub-groups. The first was of women who were 27 to 34 years old and the second was of women who were 35 years or above. Tables 3.8 and 3.9 show the distribution of embryos according to their chromosomal status in the two age sub-groups in the PGD-FISH and PGD-aCGH groups respectively. When comparing the two age groups the only significant difference was noted in the number of diploid embryos, which was higher in the older age sub-group of the PGD-FISH group. On the other hand, the percentage of diploid and aneuploid mosaic embryos was higher in younger women, haploid embryos were only seen in this group and the rest of the classifications occurred in similar numbers in both age groups. In the PGD-aCGH group no significant differences were noted, nevertheless, euploid and aneuploid embryos were more common in the younger group but complex aneuploid embryos occured at a higher percentage in the older group.

When examining the developmental stage of the embryos in addition to the age and the chromosomal status no significant differences were observed. As seen in the graphs of figure 3.18, in the PGD-FISH group arrested embryos were more evenly distributed in all chromosomal classifications in younger women, whereas in older the majority of arrested embryos were diploid. Similarly in the PGD-aCGH group no significant differences were seen, however in the younger group among the euploid embryos none of them were arrested, whereas in the older group the total number of euploid arrested embryos was higher than the sum of the euploid morulae and the blastocysts.

Table 3.8: Chromosomal status and age in the PGD-FISH group			
	27-34 years	**≥35 years**	***p* value**
Diploid	3/44 (7%)	9/22 (41%)	0.0015*
Haploid	3/44 (7%)	0	0.5452
Aneuploid	3/44 (7%)	1/22 (4.5%)	1.000
Diploid mosaic	24/44 (54%)	9/22 (41%)	0.4339
Aneuploid mosaic	7/44 (16%)	1/22 (4.5%)	0.2520
Chaotic	4/44 (9%)	2/22 (9%)	1.000

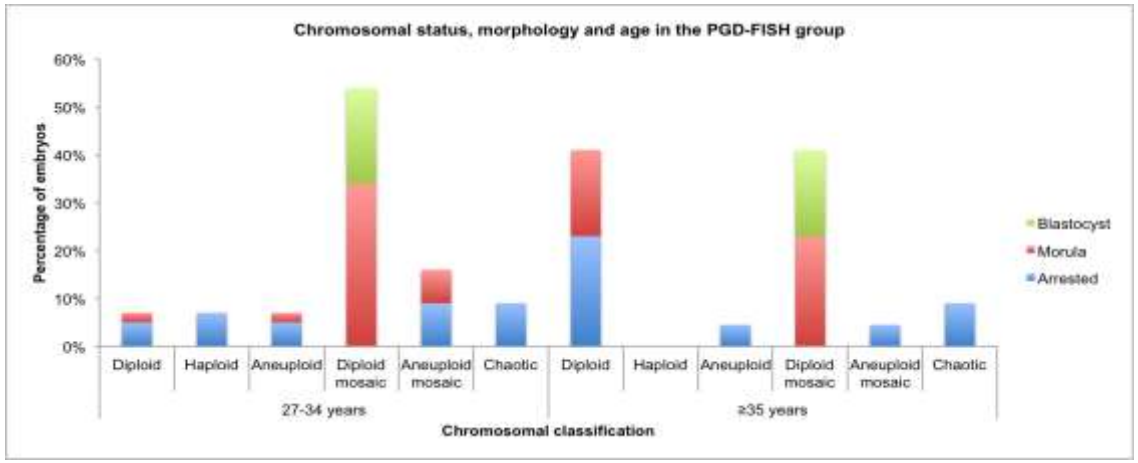
Table 3.8: Distribution of embryos analysed by FISH according to chromosomal status in young and older women. *The p value was calculated by the Fisher's exact test. *The only significance was seen for the diploid embryos, which were more in the older age group.*

Table 3.9: Chromosomal status and age in the PGD-aCGH			
	27-34 years	**≥35 years**	***p* value**
Euploid	11/28 (39%)	9/24 (33%)	1.0000
Aneuploid	12/28 (43%)	7/24 (33%)	0.3911
Complex aneuploid	5/28 (18%)	8/24 (33%)	0.2201

Table 3.9: Distribution of embryos analysed by aCGH according to chromosomal status in young and older women. *No statistical significance was observed between the two age groups.*

Figure 3.18: Chromosomal status, morphology and maternal age in embryos from PGD cycles

A



B

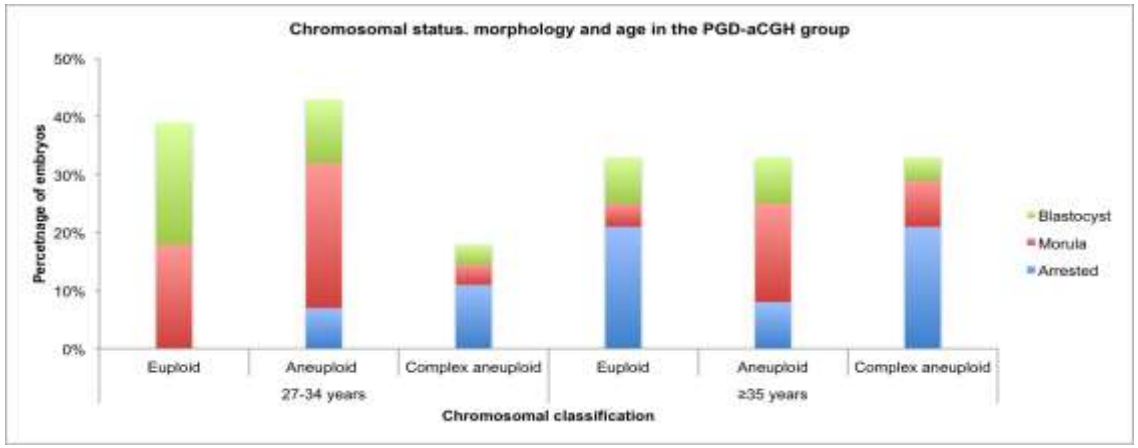


Figure 3.18: *The distribution of embryos according to their chromosomal status in women of 27 to 34 years and women over 35 years analysed by FISH (A) or aCGH (B).*

3.2.6 Analysis of chromosomal errors

Depending on the technique used to analyse the chromosomes different conclusions could be drawn about the mechanisms leading to the errors in preimplantation embryos. With FISH due to the detailed examination of each cell, the mechanisms that lead to aneuploidy and mosaicism could be uncovered, but only for the chromosomes examined. On the other hand, aCGH gave an important insight on the fact that almost all the chromosomes could be affected by aneuploidy in the embryos.

3.2.6.1 Chromosomal analysis by FISH

Of the total of 66 embryos that produced a result, chromosomal errors could be analysed in detail in those that were aneuploid and those that were chaotic and mosaic and mechanisms that led to their chromosomal status could be determined. Monosomies and trisomies of the chromosomes tested were detected in aneuploid embryos and were errors that occurred in meiosis leading to uniform abnormalities. A variety of mechanisms led to mitotic errors in mosaic and chaotic embryos, namely mitotic non-disjunction, chromosome loss or chromosome gain. A mechanism was attributed to an abnormality only when it affected more than 20% of the nuclei analysed. Table 3.10 presents the meiotic and mitotic errors identified for the chromosomes tested by FISH. In total, thirteen meiotic errors were identified and none of them involved the Y chromosome. Most meiotic errors were seen for chromosome 21. Monosomy was observed at least once for all the chromosomes tested, apart from Y, whereas a trisomy was only seen three times for chromosome 21 and once for 13, 18 and X. Amongst the mechanisms leading to a mitotic error, chromosome loss was observed more frequently whereas chromosome 13 showed the most mitotic errors in total.

Table 3.10: Meiotic and mitotic errors as detected by FISH							
	Meiotic errors			Mitotic errors			
Chromosome	**Monosomy**	**Trisomy**	**Total**	**CG**	**CL**	**MND**	**Total**
13	3	1	**4**	3	5	6	**14**
18	1	1	**2**	2	8	2	**12**
21	2	3	**5**	4	2	5	**11**
X	1	1	**2**	5	3	1	**9**
Y	0	0	**0**	1	1	0	**2**
Total	7	6	**13**	15	19	14	**48**

Table 3.10: Meiotic and mitotic errors detected by FISH. *The number of errors detected for each of the five chromosomes tested by FISH is shown. CG: Chromosome gain. CL: Chromosome loss. MND: Mitotic non-disjunction.*

3.2.6.2 Chromosomal analysis by aCGH

Testing all the chromosomes by aCGH showed that aneuploidy could affect all the autosomes and chromosome X as seen in figure 3.19, which shows the cumulative gains and losses detected at different developmental stages. Chromosome 18 showed the highest number of errors, which were nine. Chromosomes 16, 17, 19 and 22 showed eight errors each, whereas chromosome X showed only two errors. In total 60 errors were detected in arrested embryos, 29 in morulae and 34 in blastocysts. Within the blastocysts chromosome 22 showed the highest number of errors (five) and in the morulae four errors were seen for chromosome 16. In arrested embryos five errors were detected for chromosomes 17, 18 and 19.

When splitting the errors in gains and losses in all embryos, several observations could be made (figure 3.20). For all the chromosomes that showed aneuploidy 55 were losses and 68 were gains. Chromosomes five, fourteen and X showed no losses. More gains were seen amongst the small chromosomes, apart from chromosome 22 that showed six losses and two gains. Chromosome 17 showed the highest number of losses, which were six.

Figure 3.19: Overall chromosomal errors in embryos from PGD cycles

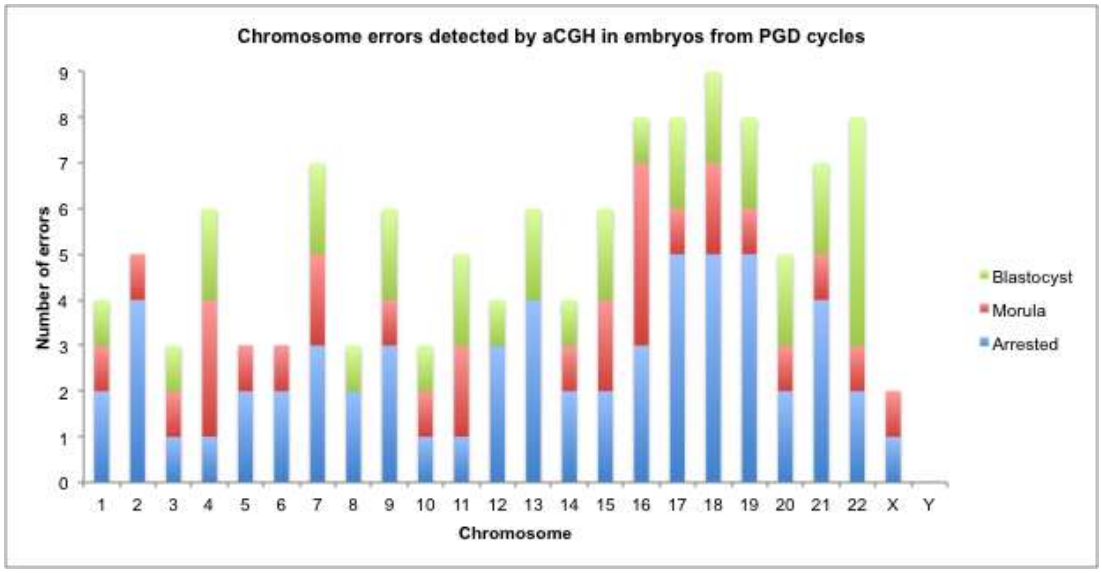


Figure 3.19: *Errors seen for each chromosome at different stages of embryo development are presented.*

Figure 3.20: Chromosomal gains and losses seen in all embryos of PGD cycles

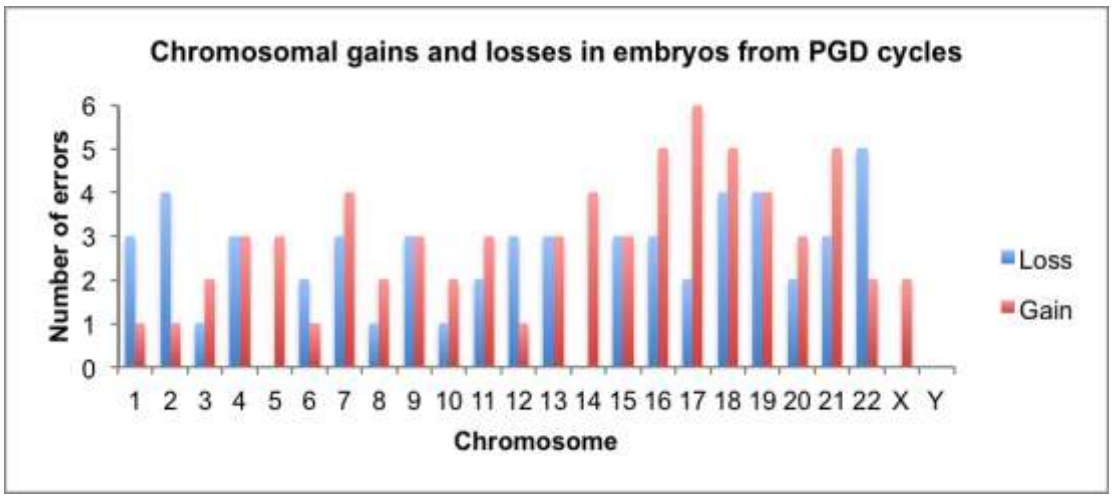


Figure 3.20: *The overall number of gains detected for all chromosomes was higher than the number of losses.*

Partial chromosomal changes could also be detected by aCGH. Only those changes that spanned more than 10Mb of one chromosome were considered as aneuploidies, as smaller changes could have been an artefact of unclear aCGH profiles. Ten partial changes were detected in eight embryos. Three embryos carried only these partial changes whereas the rest showed additional whole chromosome changes. Only one gain was seen for the long arm of chromosome 5. The rest were losses of either the long or short arms of several chromosomes. Of these eight embryos, two were blastocysts, three were morulae and three were arrested. The embryos that showed partial changes are listed in table 3.11.

Table 3.11: Partial chromosomal changes detected by aCGH	
Embryo	**Partial chromosomal change**
B20.1.3	-10q22.3-qter (54Mb)
B21.1.1	-6q14.1-qter (60Mb)
B21.1.2	-8pter-p22 (17Mb)
B21.1.6	+5q23.1-qter (63Mb), -6q14.3-qter (82Mb), -12pter-12q21;.2 (61Mb)
B22.3.3	-18q11-q13.2 (31Mb)
B22.3.5	-5q33.3-qter (29Mb)
B25.1.5	-10p15.2-p11.21 (32Mb)
B25.1.9	-2q34-qter (27Mb)

Table 3.11: List of embryos that showed partial chromosomal changes. *Embryos B21.1.1, B21.1.2 and B22.3.5 carried only these partial aneuploidies. All the rest had additional whole chromosome changes.*

3.2.7 Comparison of aneuploidy in embryos from PGD and PGS cycles

The aneuploidy level in embryos from PGD cycles as detected by FISH and aCGH was compared with the level of aneuploidy seen in embryos from couples that went through PGS and were considered infertile. All patients had attended the Centre for Reproductive and Genetic Health and therefore PGD and PGS cycles were performed under the same clinical setting.

3.2.7.1 FISH analysis

Comparison was performed with already published data from a study performed in our centre (Mantzouratou *et al*, 2007). Follow up analysis was performed on untransferred embryos from PGS cycles. For the comparison all mosaic types were grouped in diploid and aneuploid mosaics. It is important to highlight that in the PGS study out of the 50 mosaic embryos with a diploid cell line only five had a majority of diploid cells. As seen in figure 3.21 the distribution of diploid and diploid mosaic

embryos in the PGD-FISH group was significantly higher than in the PGS-FISH group, whereas chaotic embryos were more common in the PGS-FISH group ($p<0.0001$). No significant differences were seen among the distribution of haploid, aneuploid and aneuploid mosaic embryos between the two groups.

Figure 3.21: Distribution of embryos according to chromosomal status after FISH analysis in PGD and PGS cycles

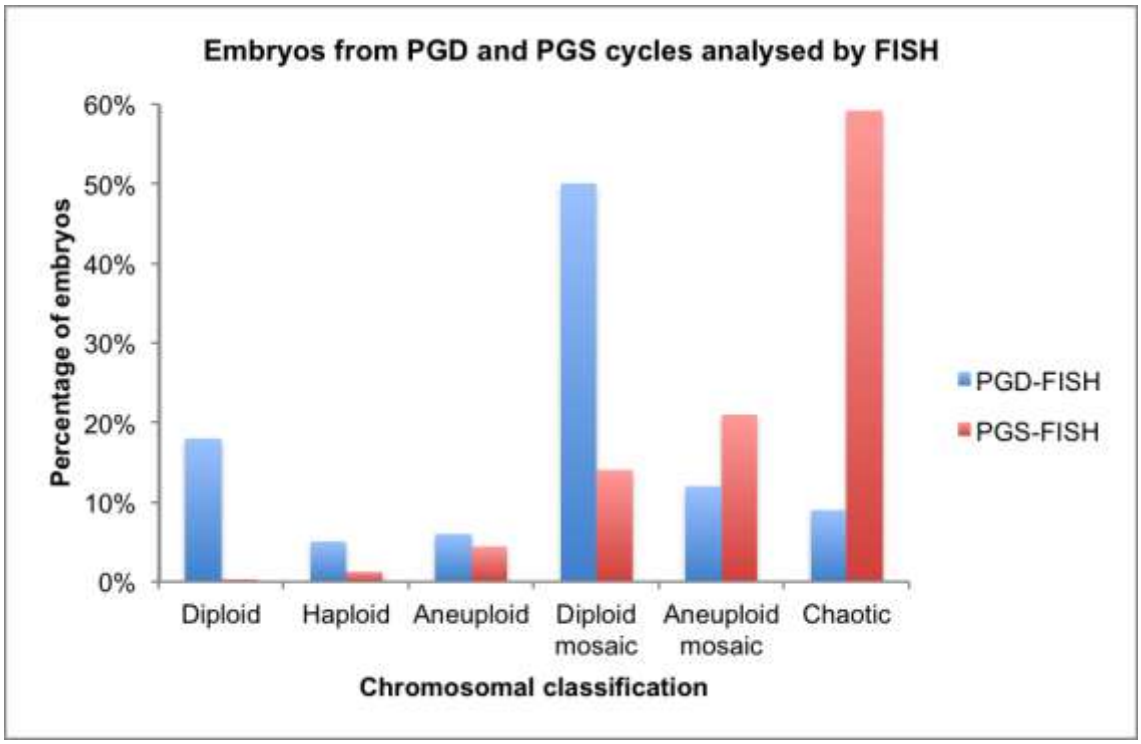


Figure 3.21: *Significant differences were seen in the diploid, diploid mosaic and chaotic embryos.*

3.2.7.2 aCGH analysis

Comparison of the aneuploidy level in embryos analysed by aCGH was performed with embryos from patients that went through PGS analysis after blastocyst biopsy. For this reason only the blastocysts from the PGD-aCGH group were included in the comparison. These were 37 blastocysts that gave an interpretable aCGH result from 14 cycles of ten couples that went through PGS for indications including advanced maternal age, recurrent miscarriage and repeated IVF failure. Details of the couples are found in table 3.12.

Table 3.12: Information of couples from PGS cycles with blastocyst biopsy					
Couple ID	**FA**	**MA**	**Indication**	**No of cycles**	**No of blastocysts**
B26	37	36	RIF	2	5
B27	38	42	RIF	1	1
B28	41	47	RIF/AMA	1	3
B29	36	38	RM	1	1
B30	44	50	AMA	1	2
B31	43	41	RM/AMA	4	5
B32	41	44	AMA	1	7
B33	37	40	RM	1	3
B34	44	44	AMA	1	4
B35	34	39	RIF	1	6
Average	39.5 (±3.6)	42.1 (±4.2)		Total	37

Table 3.12: Couples that went through PGS post blastocyst biopsy. *Information on female and male age, the indication for PGS, number of cycles and blastocysts for each couple are shown. (FA: female age, MA: male age, AMA: advanced maternal age, RIF: repeated IVF failure, RM: recurrent miscarriage)*

The detailed chromosomal classification and complement of each of these blastocysts is found in table 7.2 of Appendix B. Upon comparison no statistical significance was seen between the two groups, however, as shown in the graph in figure 3.22, the distribution of euploid blastocysts was higher in the PGD-aCGH group, while aneuploid and complex aneuploid blastocysts were higher in the PGS-aCGH group.

Figure 3.22: Distribution of blastocysts analysed by aCGH according to their chromosomal status in the PGD and PGS groups

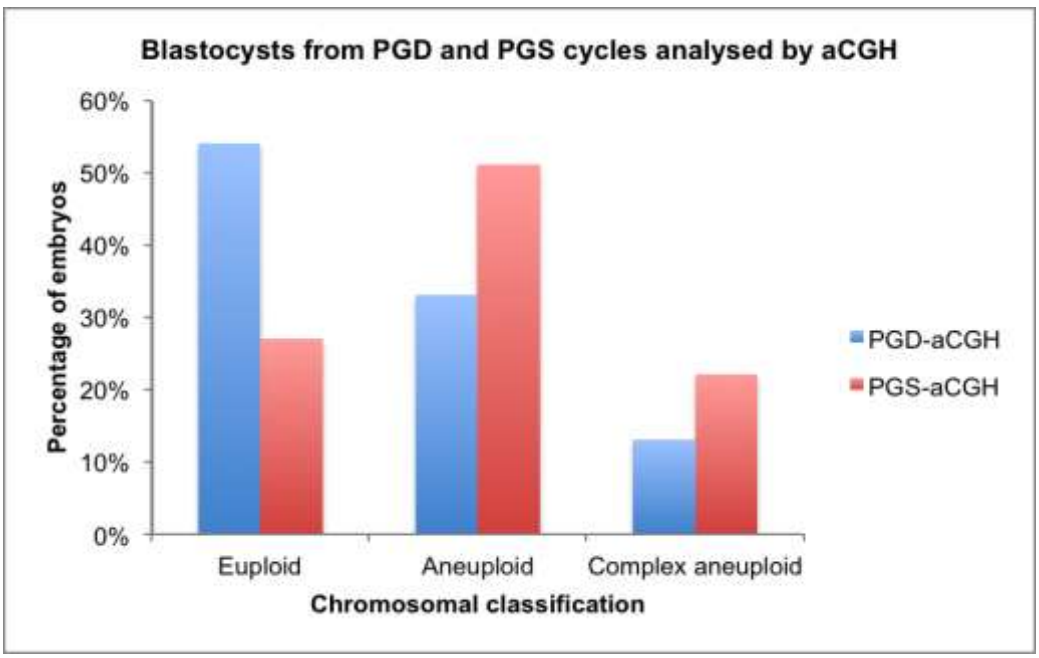


Figure 3.22: *More euploid blastocysts were observed in the PGD group and more aneuploid and complex aneuploid in the PGS group, however, no significant differences were noted.*

3.2.8 Summary of results for section 3.2: Aneuploidy in embryos from couples undergoing PGD

- Chromosomal analysis by FISH using probes to detect abnormalities on five chromosomes showed that diploid mosaic embryos were more common in couples undergoing PGD. Chaotic, haploid and aneuploid embryos were the least common. Blastocysts were characterised only as diploid mosaic with more than 50% of diploid cells. The chaotic and haploid chromosomal constitutions were only seen in arrested embryos.

- Chromosomal analysis by aCGH revealed that euploid and aneuploid embryos were almost equally distributed and a small proportion were complex aneuploid. The majority of blastocysts were euploid, whereas the majority of arrested embryos were complex aneuploid.

- No significant differences were seen among the distribution of all chromosomal classifications between couples of young and older maternal age, apart from diploid embryos, characterised by FISH, which were significantly more in embryos from couples with maternal age >35 years.

- Thirteen meiotic and 48 mitotic errors were observed in the embryos analysed by FISH. Monosomy was the most common meiotic error and chromosome loss the most common mitotic error.

- No distinction between meiotic and mitotic errors could be made by aCGH. Chromosome 18 was found to be most commonly involved in errors. Chromosome loss was most frequently seen for chromosome 22 and chromosome gain was most frequently seen for chromosome 17.

- Comparison of the aneuploidy level as determined after FISH analysis with that reported from PGS cycles revealed that the distribution of diploid mosaic embryos was significantly higher in the PGD group, whereas the distribution of chaotic embryos was significantly higher in the PGS group.

- Comparison of the aneuploidy level in blastocysts from the PGD group analysed by aCGH with that of blastocysts from PGS cycles revealed no statistically significant differences. However, euploid blastocysts where more common in the PGD than the PGS group.

3.3 Investigation of recombination in preimplantation embryos

Recombination was investigated in preimplantation embryos and their parents. This was performed by amplifying microsatellite polymorphic markers in five different chromosomes on whole genome amplified embryonic DNA and non-amplified genomic DNA from the parents. It was possible to identify cross-over events occurring in the oocyte and/or the sperm after establishing phase for each set of markers in the embryos. Aneuploidy analysis was also performed on the same WGA embryonic products by aCGH.

3.3.1 Couple and embryo description

In total 77 embryos were collected from six couples undergoing PGD and four couples that opted for PGS. The six families that were used for the recombination investigation after PGD for a single gene disorder (C1 – C6) were also used for the aneuploidy analysis by aCGH, described in section 3.2.1 (couples B20 – B25). Embryos from four couples (C7 – C10) were also analysed for recombination after PGS in order to detect any differences in the recombination frequency of couples of young maternal age and no fertility issues (PGD group) and those that were infertile (PGS group). Family data for both groups are presented in tables 3.13 and 3.14. The average female age for the PGD group was 31.5 years (±4), the average male age was 33.3 (±4.3) and the couples were treated for a variety of single gene disorders as presented in table 3.13. For the PGS group the average female and male age were 38.8 (±2.6) and 38.8 (±3) respectively. The indication for PGS for each couple is presented in table 3.14.

Table 3.13: Family data of the PGD group			
Family ID	**FA**	**MA**	**Disease**
C1	28	29	FAP
C2	27	27	NF1
C3	33	34	DM1
C4	36	36	NF1
C5	29	37	FAP
C6	36	37	NF1
Average	31.5 (±4)	33.3 (±4.3)	

Table 3.13: Families of the PGD group used in the recombination analysis. *The female (FA) and male age (MA) as well as the diseases for which PGD was performed are indicated. (FAP: Familial adenomatous polyposis, NF1: Neurofibromatosis type 1, DM1: Myotonic Dystrophy type 1).*

Table 3.14: Family data of the PGS group			
Family ID	**FA**	**MA**	**Indication**
C7	41	42	AMA
C8	41	35	RIF/AMA
C9	36	40	RIF
C10	37	38	RIF
Average	38.8 (±2.6)	38.8 (±3)	

Table 3.14: Families of the PGS group used in the recombination analysis. *Female and male ages and the indication(s) for PGS for each couple are shown. (AMA: Advanced maternal age, RIF: Repeated IVF failure).*

Any possible effects of aberrant recombination in the morphology and chromosomal status of each embryo were also examined. Chromosomal classification post aCGH for these embryos was performed as described in the Materials and Methods section 2.5.2.7. Since only meiotic errors were of interest in this chapter, embryos were characterised as euploid when no change above or below the threshold for whole chromosomal gain or loss was observed for any of the chromosomes, aneuploid when one or two chromosomes showed an abnormality and complex aneuploidy when three or more chromosomes were involved in an abnormality. Out of the 53 embryos in the PGD group (table 3.15), 18 (34%) showed developmental arrest, 20 (38%) had reached the morula stage and 15 (28%) were blastocysts at day five or six. The chromosomal status was normal in 23 (44%) embryos, 16 (31%) were aneuploid, carrying one or two aneuploid chromosomes and 13 (25%) were complex aneuploid, carrying three or more aneuploid chromosomes. aCGH failed to produce a result in one embryo from this group (embryo C1.5).

In the PGS group, 24 embryos were analysed, in which six (25%) were arrested, ten (42%) were morulae and eight (33%) had reached the blastocyst stage. Of the 24 embryos, two had no interpretable aCGH result (embryos C7.6 and C9.5), five (23%) were euploid, 11 (50%) were aneuploid, with one or two aneuploid chromosomes and 6 (17%) were complex aneuploid with three or more chromosomes with an aneuploidy. Data for the embryos in the PGS group are found in table 3.16.

Table 3.15: Data of embryos in the PGD group				
Couple ID	**Embryo ID**	**PN**	**Morphology**	**Chromosome status**
C1	C1.1	2	Blastocyst	Euploid
	C1.2	2	Morula	Aneuploid
	C1.3	2	Blastocyst	Complex aneuploid
	C1.4	2	Blastocyst	Aneuploid
	C1.5	2	Arrested	No result
	C1.6	2	Arrested	Aneuploid
	C1.7	2	Blastocyst	Euploid
	C1.8	0	Arrested	Aneuploid
C2	C2.1	2	Morula	Euploid
	C2.2	2	Morula	Euploid
	C2.3	2	Morula	Aneuploid
	C2.4	2	Morula	Euploid
	C2.5	2	Morula	Euploid
	C2.6	2	Arrested	Complex aneuploid
	C2.7	0	Morula	Euploid
	C2.8	0	Morula	Aneuploid
C3	C3.1	2	Blastocyst	Euploid
	C3.2	2	Morula	Euploid
	C3.3	2	Morula	Complex aneuploid
	C3.4	2	Blastocyst	Euploid
	C3.5	2	Morula	Aneuploid
	C3.6	2	Morula	Euploid
	C3.7	2	Arrested	Complex aneuploid
	C3.8	2	Blastocyst	Euploid
	C3.9	2	Arrested	Complex aneuploid
	C3.10	2	Blastocyst	Aneuploid
	C3.11	2	Morula	Euploid
C4	C4.1	2	Morula	Aneuploid
	C4.2	2	Arrested	Complex aneuploid
C5	C5.1	2	Blastocyst	Aneuploid
	C5.2	2	Blastocyst	Euploid

Table 3.15 (cont): Data of embryos in the PGD group				
Couple ID	**Embryo ID**	**PN**	**Morphology**	**Chromosome status**
C6	C6.1	2	Arrested	Aneuploid
	C6.2	2	Blastocyst	Euploid
	C6.3	2	Arrested	Euploid
	C6.4	2	Blastocyst	Euploid
	C6.5	2	Arrested	Complex aneuploid
	C6.6	2	Blastocyst	Euploid
	C6.7	2	Morula	Complex aneuploid
	C6.8	2	Arrested	Complex aneuploid
	C6.9	2	Blastocyst	Complex aneuploid
	C6.10	0	Blastocyst	Aneuploid
	C6.11	2	Arrested	Complex aneuploid
	C6.12	2	Arrested	Aneuploid
	C6.13	2	Morula	Aneuploid
	C6.14	2	Arrested	Euploid
	C6.15	2	Morula	Aneuploid
	C6.16	2	Morula	Euploid
	C6.17	2	Morula	Complex aneuploid
	C6.18	2	Arrested	Complex aneuploid
	C6.19	2	Arrested	Euploid
	C6.20	1	Morula	Aneuploid
	C6.21	1	Arrested	Euploid
	C6.22	3	Arrested	Euploid

Table 3.15: List of the 53 embryos from PGD cycles used in the recombination analysis. *The number of pronuclei seen after fertilisation, the developmental stage at day five or six as well as the chromosomal status, as defined by the aCGH result, of each embryo are shown.*

Table 3.16: Data of embryos in the PGS group				
Couple ID	**Embryo ID**	**PN**	**Morphology**	**Chromosome status**
C7	C7.1	2	Morula	Aneuploid
	C7.2	2	Arrested	Aneuploid
	C7.3	2	Morula	Aneuploid
	C7.4	2	Arrested	Complex aneuploid
	C7.5	2	Morula	Euploid
	C7.6	1	Morula	No result
	C7.7	0	Arrested	Complex aneuploid
C8	C8.1	2	Blastocyst	Aneuploid
	C8.2	2	Blastocyst	Complex aneuploid
	C8.3	2	Blastocyst	Aneuploid
C9	C9.1	2	Morula	Euploid
	C9.2	2	Arrested	Aneuploid
	C9.3	2	Morula	Euploid
	C9.4	2	Arrested	Complex aneuploid
	C9.5	2	Arrested	No result
	C9.6	2	Blastocyst	Aneuploid
	C9.7	2	Morula	Complex aneuploid
	C9.8	2	Morula	Aneuploid
	C9.9	2	Morula	Complex aneuploid
C10	C10.1	2	Blastocyst	Aneuploid
	C10.2	2	Blastocyst	Euploid
	C10.3	2	Blastocyst	Aneuploid
	C10.4	2	Blastocyst	Euploid
	C10.5	2	Blastocyst	Aneuploid

Table 3.16: List of the 24 embryos from PGS cycles used in the recombination analysis. *The number of pronuclei seen after fertilisation, the developmental stage at day five or six and the chromosomal status, as defined by the aCGH result, of each embryo are indicated.*

3.3.2 WGA of samples using SurePlex

Whole genome amplification was performed on whole embryos of the PGD group, on day five or six of development, post diagnosis for a single gene disorder. These embryos were not selected for transfer to the uterus, or cryopreservation due to the presence of a mutation or due to developmental arrest. Embryonic DNA in families C7 and C9 of the PGS group was amplified by WGA post screening for chromosomal aneuploidy on day five or six on whole embryos that were found to be aneuploid and/or were arrested and therefore unsuitable for transfer. WGA on embryos from families C8 and C10 was performed as part of the clinical PGS procedure on trophectoderm (TE) samples biopsied from blastocysts on day five or six. Recombination analysis for these embryos was performed on the same TE WGA products.

WGA was performed using SurePlex (BlueGnome, UK). Success of the amplification was confirmed by running the amplified samples on a 2% agarose gel. A smear of brighter intensity at around 400 base pairs represented successful amplification and all amplified samples produced a smear. As mentioned in the Results section 3.3.1, one embryo from the PGD group (C1.5) and two embryos from the PGS group (C7.5 and C9.5) had no interpretable aCGH result, even though a smear was present, albeit fainter than the rest, on the agarose gel (figure 3.23 for embryos from families C1 and C7). This was probably due to experimental errors during aCGH for embryos C1.5 and C9.5, since those embryos produced good results post FPCR (performed on the same WGA product). Embryo C7.6 however produced poor result even after FPCR, as mentioned in the results section 3.3.3.2, indicating poor DNA quality of the embryo as a starting material for WGA.

Figure 3.23: Agarose gel of WGA products of embryos from families C1 and C7

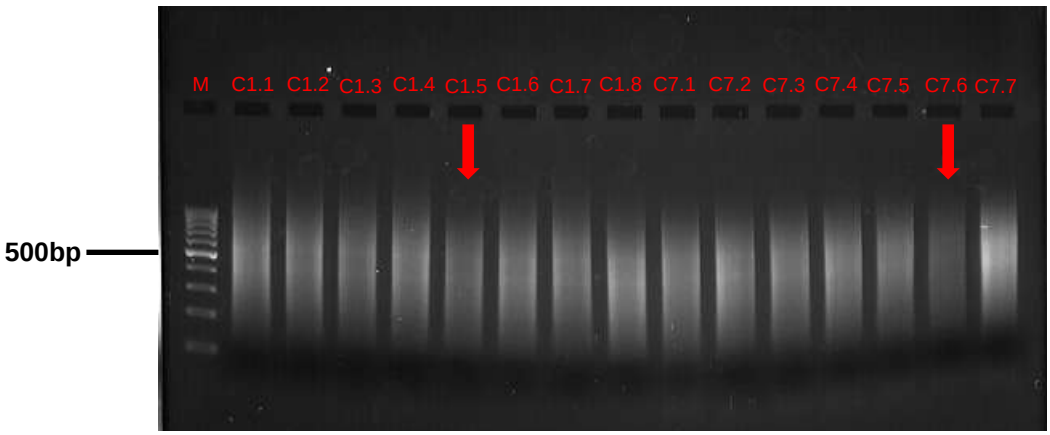


Figure 3.23: *An expected smear at around 400bp is present for all the embryos, however it is fainter for embryos C1.5 and C7.6, both of which produced no interpretable aCGH result. M: 100bp molecular weight marker.*

3.3.3 FPCR amplification of STR markers

Loci on chromosomes 1, 5, 16, 19 and 17 were examined for recombination. The first four were considered as recombination hotspots, whereas the latter as a recombination silent spot. Two STR marker loci were amplified on chromosomes one, five and 16, three on chromosome 19 and five on chromosome 17. Details of the markers are found on table 3.17. Figure 3.24 presents the exact chromosomal position of each set of markers, along with the genetic distance of each locus.

Table 3.17: STR marker information

Chromosome	Marker	Chromosomal location		Genetic distance (cM)	Type
1	D1S495	1p21.1	102,561,360-102,561,512	1.64	Dinucleotide
	D1S486	1p21.2	102,147,806-102,147,958		Dinucleotide
5	D5S1991	5p15.2	14,876,610-14,876,840	3.08	Dinucleotide
	D5S2081	5p15.2	13,476,968-13,477,164		Dinucleotide
16	D16S492	16q12.2	54,656,244-54,656,460	1.92	Tetranucleotide
	D16S3053	16q12.2	55,548,751-55,548,994		Dinucleotide
17	NF1int1	17q11.2	29,466,325-29,466,487	0	Dinucleotide
	D17S1307	17q11.2	29,473,353-29,473,561		Dinucleotide
	NF1int17	17q11.2	29,553,012-29553634		Dinucleotide
	NF1int29	17q11.2	29,569,864-29570488		Dinucleotide
	D17S1166	17q11.2	29,649,016-29,649,216		Dinucleotide
19	D19S219	19q13.32	45,993,737-45,993,910	1.13	Dinucleotide
	D19S207	19q13.32	46,303,962-46,304,113		Dinucleotide
	D19S412	19q13.32	47,010,982-47,011,111		Dinucleotide

Table 3.17: List of the markers used to amplify loci on five chromosomes to detect recombination. *The chromosomal location (Ensembl release 60, www.ensembl.org) and type of the repeat of each marker are indicated. Genetic distances were calculated using data from Kong et al, 2010.*

Figure 3.24: Chromosomal position of each set of markers used for recombination detection

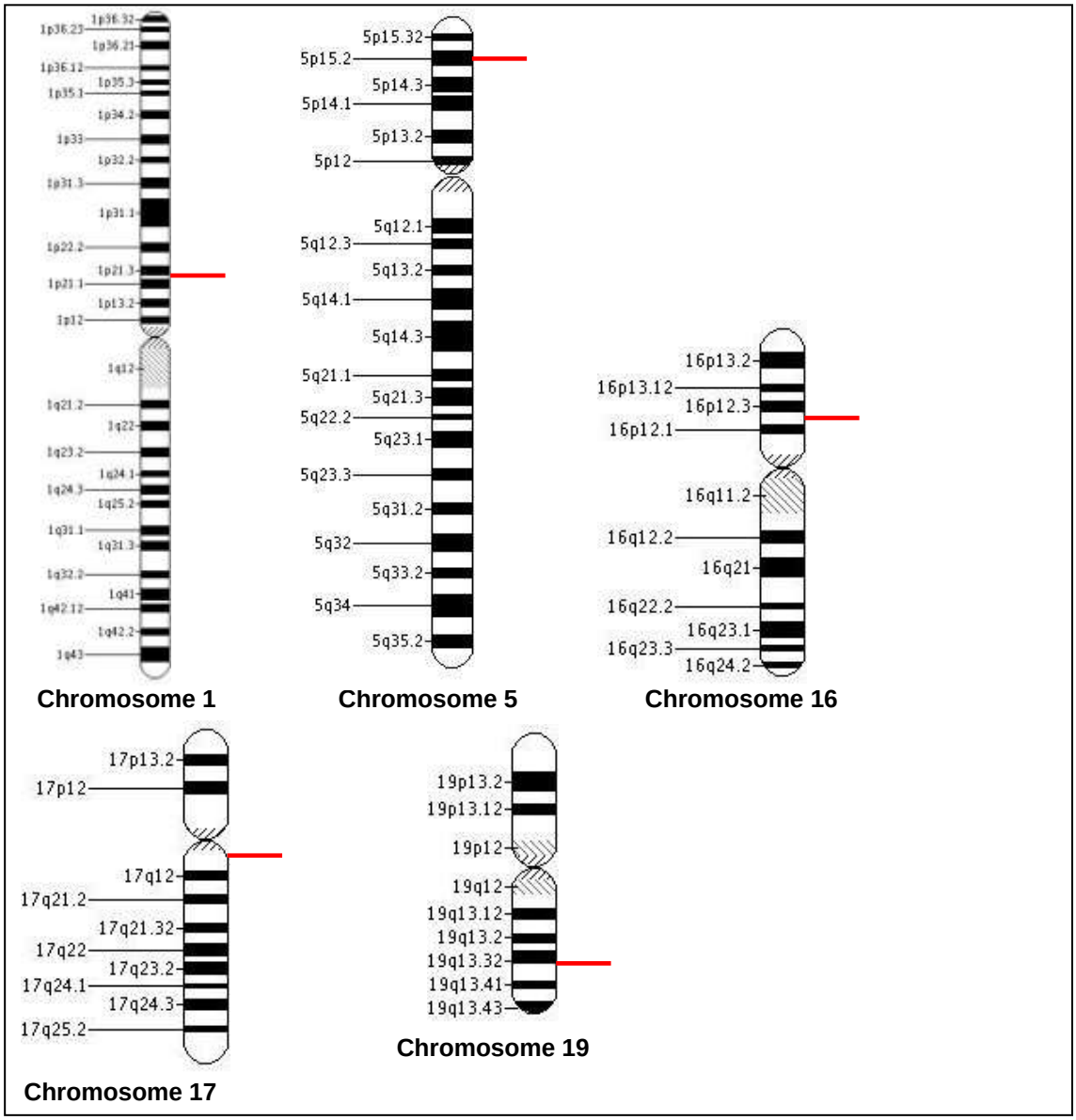


Figure 3.24: *The red line on each chromosome indicates the exact band on the chromosome where the set of markers is located.*

3.3.3.1 Conditions for amplification

Depending on the fluorescent dye of each primer set and the size of the amplified product, markers were amplified in multiplexes if possible. This was performed in order to amplify more than one locus simultaneously and therefore reduce the number of reactions.

Each primer set was first tested on all parental genomic DNAs in order to check the range of the size of the products and optimise the reaction conditions. Based on these results three multiplexes were developed. The first, a triplex, amplified both markers on chromosome 1 plus one marker on chromosome 5, the second and third, both duplex reactions, amplified loci on chromosomes 17 and 19 respectively. All multiplex reactions were performed using the Qiagen multiplex kit, as described in the Materials and Methods, section 2.4.3.4. The rest of the markers were amplified in single reactions using either the High Fidelity or *Taq* polymerase (Materials and Methods, sections 2.4.3.2 and 2.4.3.3). Details on the conditions of all the FPCR reactions are found in table 3.18.

Table 3.18: Amplification conditions for STR markers

Loci amplified	Polymerase	Annealing temperature (°C)
D1S486 D1S495 D5S1991	HotStarTaq®	60
D17S1166 D17S1307	HotStarTaq®	60
D19S412 D19S207	HotStarTaq®	60
D5S2081	High Fidelity	60
D16S492	*Taq*	60
D16S3053	*Taq*	60
NF1int1	*Taq*	60
NF1int17	*Taq*	60
NF1int29	*Taq*	60
D19S219	High Fidelity	58

Table 3.18: Conditions of multiplex and single reactions to amplify STR markers. *The HotStarTaq® DNA polymerase was a component of the Qiagen multiplex kit that was used in all multiplex reactions.*

Figure 3.25 presents an example of the result obtained by the genetic analyser for the first multiplex, amplifying markers D1S495, D5S1991 and D1S486 for the female and male partner of family C1, as well as embryo C1.6.

Figure 3.25: Example of genotyping of three STR markers

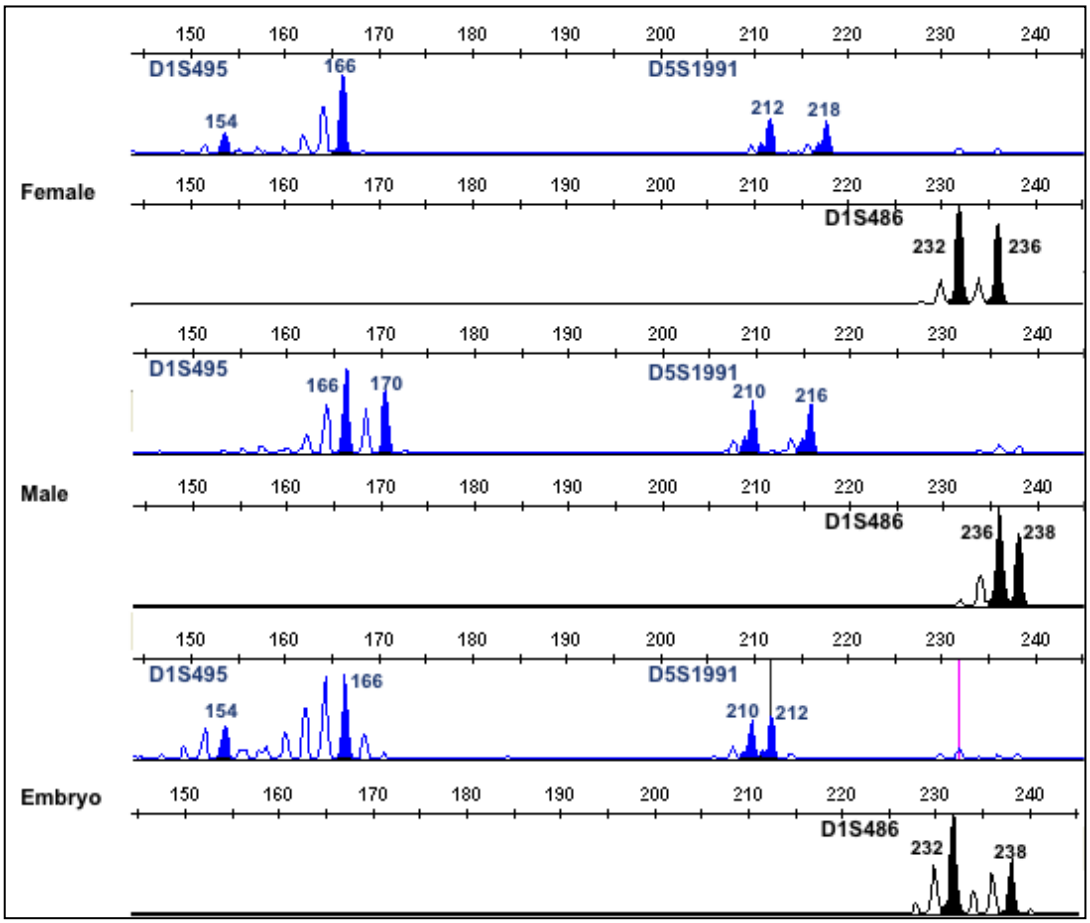


Figure 3.25: *This presents the result of the first multiplex, amplifying markers D1S495, D5S1991 and D1S486 for the two partners of family C1 and embryo C1.6.*

3.3.3.2 Marker amplification efficiency

In total 14 markers were amplified by FPCR on 20 parental genomic DNA samples and 77 embryonic DNA samples that were previously amplified by WGA. Detailed results of all the markers for each sample are found in table 8.1 of Appendix C. Amplification of all 280 loci was successful on 20 genomic DNA samples from the parents. Of the 1078 loci amplified on the embryos, amplification failure was seen in 31 loci (2.9%). Notably, 24 of the 31 cases of amplification failure were seen in embryos of the C7 family, indicating possible poor embryo quality. This was made apparent especially in embryo C7.6, which failed to produce an interpretable aCGH result and 10 out of the 14 markers failed to amplify.

Allele drop out (ADO) could also be detected for some loci in heterozygote samples. Heterozygosity was seen in 720 amplified loci and one allele failed to amplify in 38. Therefore, the ADO rate was 5.4%. These events were scored as definite ADO, in embryos that the segregation of allele markers was determined and a heterozygote result was expected. In some samples however, homozygote allele sizes may have been a result of ADO, recombination or aneuploidy. Homozygote results with a doubt to their origin were disregarded when calculating ADO and recombination frequencies.

Amplification of multiple loci could also have detected possible contamination present in the amplified embryonic samples. Paternal contamination was not expected as fertilization was performed by ICSI to prohibit contamination by sperm. Maternal contamination was possible via cumulus cells surrounding the oocyte that were not removed prior to fertilisation. If contamination from cumulus cells had occurred it should have been detected in all loci amplified in an embryo as an extra maternal allele. No embryo had evidence of maternal contamination in all the loci examined. Extra alleles present in some embryos for loci on one chromosome but not the rest indicated trisomy for that chromosome and not maternal contamination or amplification artefacts as described in the example given in figure 3.28.

3.3.3.3 Parental informativity

In order to detect recombination, the segregation of the marker alleles needed to be determined on parental DNA. This was possible only when the parents were informative for the markers tested. Parental informativity for all the markers is shown in table 8.1 of Appendix C.

For chromosomes 1, 5 and 16 two-marker sites were amplified. Heterozygosity of the parents for both markers as well as difference in the size of at least one marker allele between the parents were essential to detect recombination. For chromosome 1, out of the 20 parents tested, ten were not informative. For chromosome 5, 14 and for chromosome 16, 11 were not informative. For chromosomes 17 and 19, five and three markers were tested respectively and amplification of more than two markers increased the ability to detect recombination as parental informativity was increased. Lack of informativity on these chromosomes occurred when an individual was homozygote for all the markers or was heterozygote for only one. Eight parents were not informative for chromosome 17 and six were not informative for

chromosome 19. It is important to note that the female partner of family C6 was affected with a whole deletion of the *NF1* gene, on chromosome 17. All the markers for that chromosome were located within the gene and therefore only one allele was detected, rendering the female partner not informative for recombination detection for that locus.

3.3.4 Recombination events

Female and male recombination was detected by cross-over events occurring during meiosis in the formation of oocytes and sperm, which could be identified on informative loci only. On loci where two markers were tested a single cross-over event could be detected, whereas detection of double cross-over was possible on chromosomes 17 and 19, where five and three markers were tested respectively. When calculating the number of recombination events, double cross-over was regarded as two separate events.

Correct segregation of the marker alleles on each parental chromosome was essential to confidently identify recombination. Initially all parents were genotyped for all the markers. With the expected marker allele sizes known, genotyping of the embryos was performed. The marker alleles in phase with each other were determined by the combination of parental alleles that resulted in the smallest recombination fraction in the embryos for that family, ie with the smallest number of recombinants. Families C4 and C5 included two embryos each. Haplotyping showed evidence of recombination, however, it was not possible to determine which embryo carried a recombined chromosome and which did not.

Double recombinants could only be detected for the loci on chromosome 17 and 19 as five and three markers were tested for them respectively. For chromosome 17 only one embryo was identified as a double recombinant, of maternal origin (C3.1, Appendix C) in a family with a total of 11 embryos. Altering the segregation of the alleles to avoid the presence of an embryo with a double cross-over event would have increased the number of embryos with recombination to 10 out of 11, instead of two out of 11. As mentioned above, the haplotypes selected were those that produced the lowest number of recombinants. Four embryos showed evidence of double cross-over for chromosome 19 in families C1, C3 and C6. Both cases of the double cross-over in family C1 were paternal in origin, whereas the double events in families C3 and C6 (one each) were maternal.

Figures 3.26, 3.27, 3.28 and 3.29 show examples of different families in which cross-over events were identified on chromosomes 1, 16, 17 and 19 respectively. No recombination was detected on the chromosome 5 locus.

As seen in figure 3.26, haplotyping of the parents and their eight embryos for the chromosome 1 locus revealed female recombination on embryo C1.3. A female and male cross-over event was detected on embryo C1.5 and embryo C1.8 carried only maternal alleles. This might have been a result of paternal cross-over, monosomy or ADO on one or both paternal alleles, depending on which paternal chromosome was inherited in that embryo. Loss of chromosome 1 was not seen by aCGH on that embryo, therefore monosomy was excluded. Since the source of this result was not apparent this embryo was not included in the calculation of the recombination frequency. Any other embryo with similar doubts was also excluded.

Figure 3.26: Haplotyping of family C1 for the chromosome 1 locus

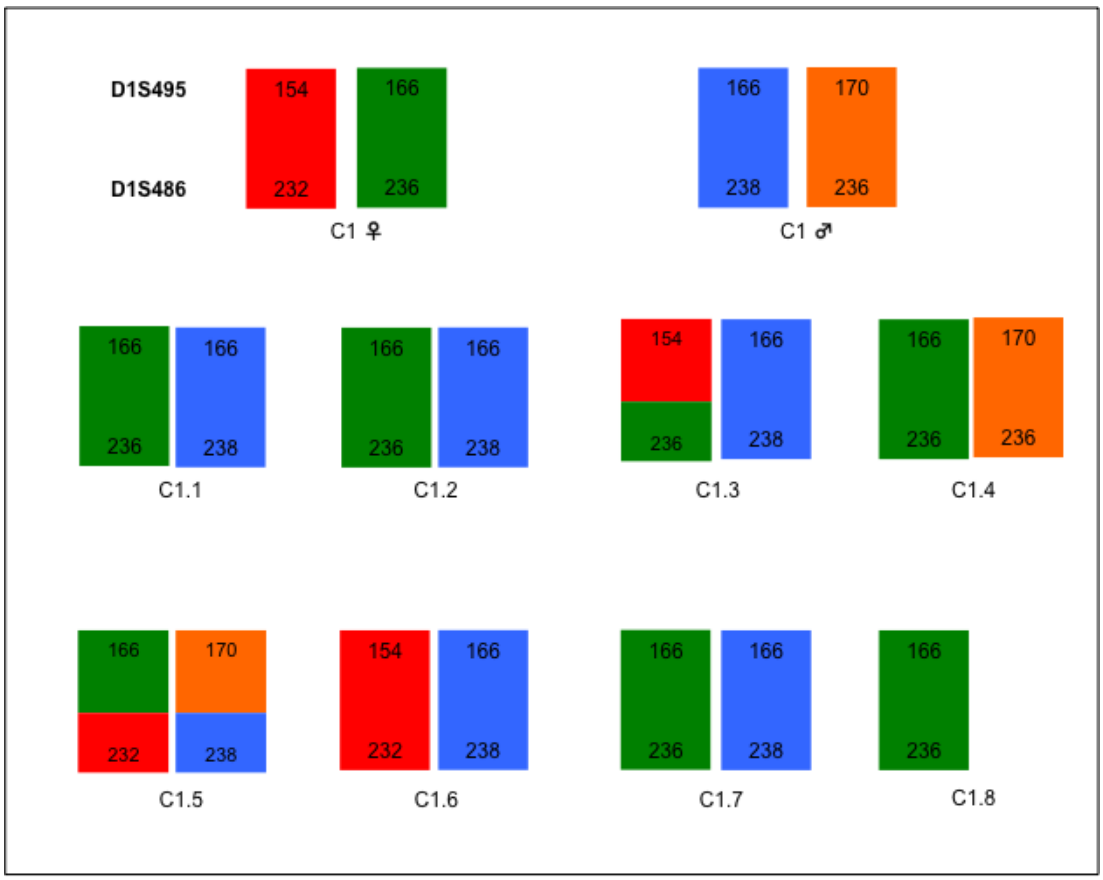


Figure 3.26: *Embryos C1.1, C1.2, C1.4, C1.5 and C1.7 established the phase of marker alleles. Embryos C1.3 and C1.5 showed deviation from the expected haplotypes, representing a female cross-over event in embryos C1.3 and C1.5 and a male event in embryo C1.5. Embryo C1.8 carried only the maternal alleles for that locus.*

Figure 3.27 shows the haplotyping results for chromosome 16 for family C4. As mentioned above, this family consisted of two embryos only. Inheritance of the expected paternal alleles was seen on both embryos. The maternal allele sizes for the two markers, however, did not segregate in the same way in both embryos, indicating recombination in one of the two. Arbitrarily, embryo C4.2 was selected as the recombinant embryo. This assumption did not affect the final calculation of the recombination frequency.

Figure 3.27: Haplotyping of family C4 for the chromosome 16 locus

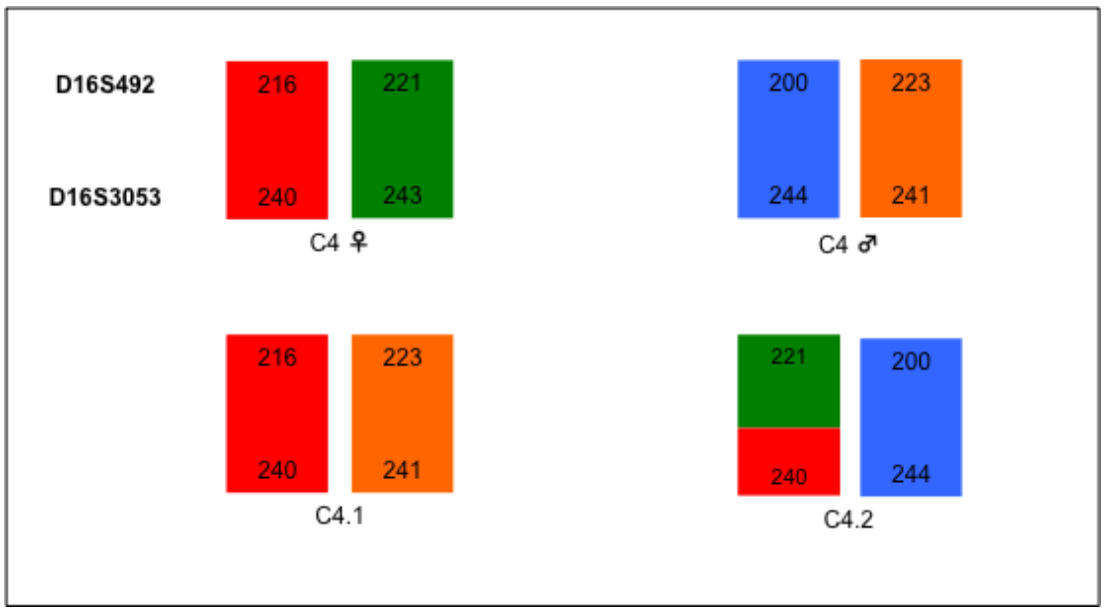


Figure 3.27: *Female recombination occurred in one of the two embryos. Due to the number of embryos, it was not possible to determine which of the two carried the cross-over. Embryo C4.2 was arbitrarily selected as the recombinant.*

The majority of cross-over events was seen on chromosome 19. An example is shown in figure 3.28. Female and male recombination events were detected in embryos C7.4 and C7.5 respectively. The site of the maternal cross-over was shown to be between the distal D19S219 and the middle D19S207 marker. The site of the paternal cross-over could not be detected, as the father was homozygote for the middle marker D19S207. Amplification failure occurred for markers in embryo C7.6, probably due to degraded embryo DNA, an indication of which was also given by a weak smear on the agarose gel and the fact that aCGH was not successful. Evidence for trisomy 19 was seen in embryo C7.7 as it carried the expected maternal alleles from both chromosomes and alleles from one paternal. However, gain of chromosome 19 was not detected by aCGH on that embryo. Maternal contamination as a source of the extra peaks was excluded since extra maternal alleles were not detected for any other chromosome with informative loci. The presence of random peaks, an artefact of the WGA, at the expected allele sizes in the analysis of the amplified products might be one explanation for these results.

Figure 3.28: Haplotyping of family C7 for the chromosome 19 locus

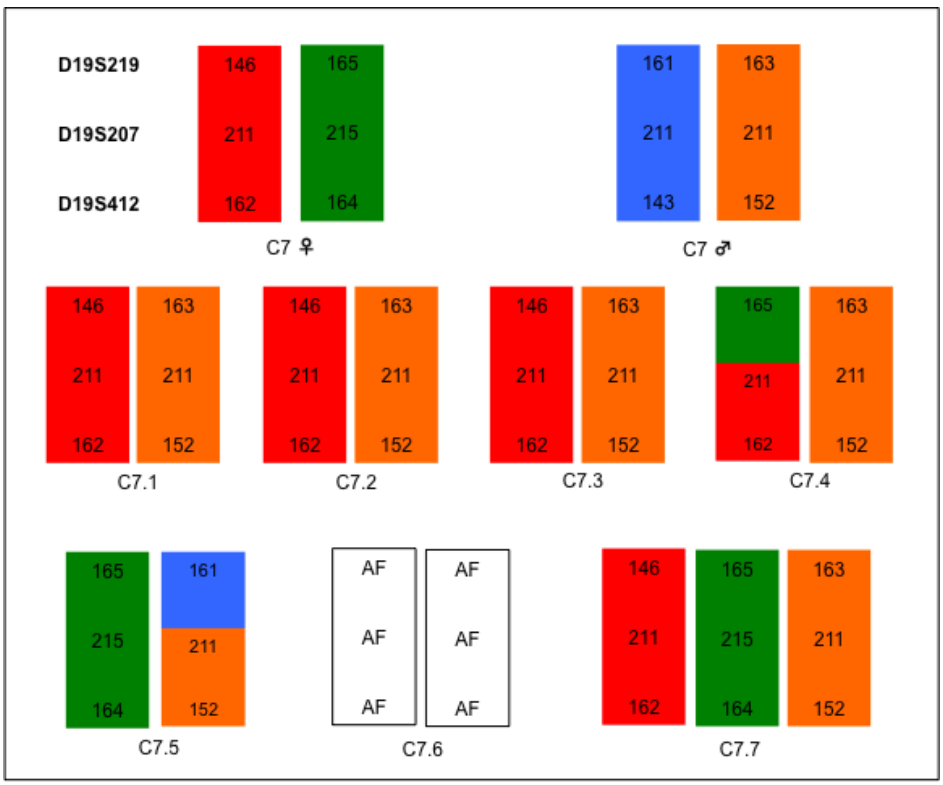


Figure 3.28: *This family consisted of seven embryos. Embryo C7.8 failed to produce a result for this locus. Female recombination between markers D19S219 and D19S207 was detected in embryo C7.4. Male recombination was seen in embryo C7.5, however the site of the cross-over event could not be detected in the father, as he was homozygote for the middle marker D19S207. Embryo C7.7 carried both maternal chromosomes and one paternal.*

Recombination in embryos was even detected for the silent spot on chromosome 17. Two embryos of family C10 showed male recombination between the markers D17S1307 and NF1int17, as presented in figure 3.29. Embryo C10.3 of the same family carried only one paternal chromosome 17, indicating monosomy for that chromosome. This aneuploidy was confirmed by loss of that chromosome in aCGH.

Figure 3.29: Haplotyping of family C10 for the chromosome 17 locus

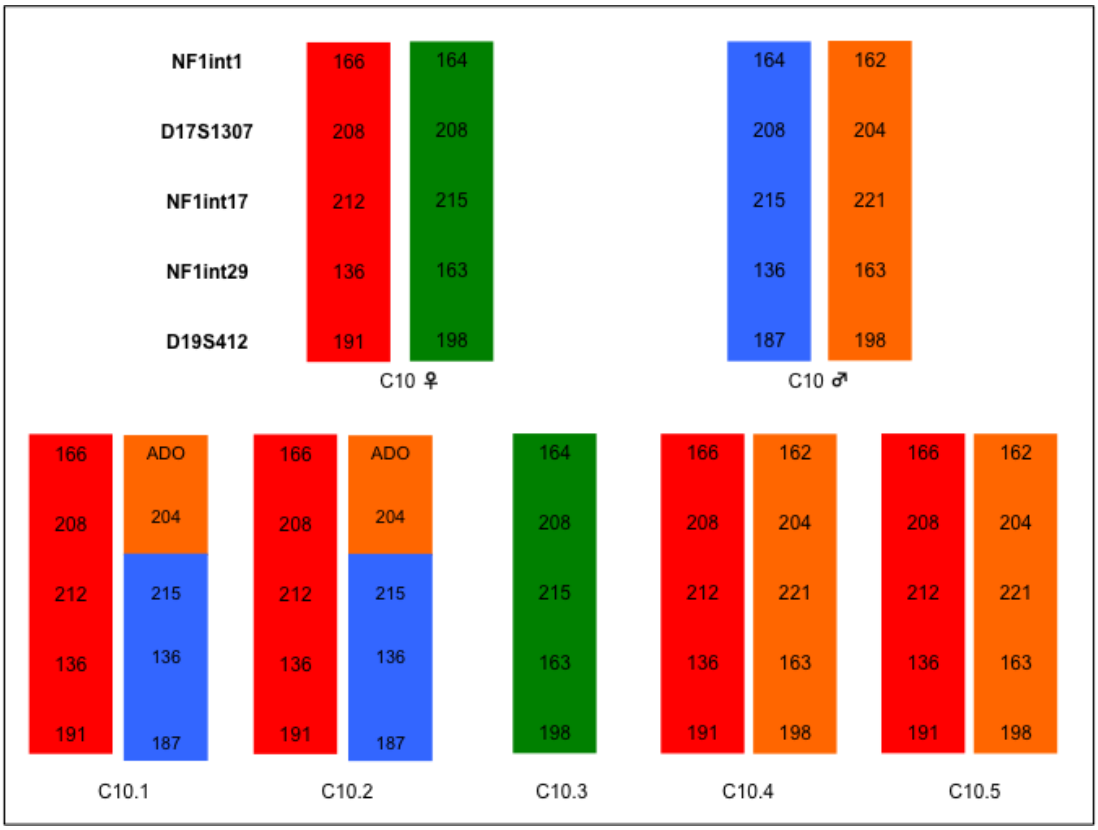


Figure 3.29: Haplotyping of family C10 for the chromosome 17 locus. *Male recombination was detected in embryos C10.1 and C10.2 between the markers D17S1307 and NF1int17. Only one paternal chromosome was detected for embryo C10.3.*

3.3.5 Recombination frequency

Tables 3.19 and 3.20 present the cumulative embryo recombination results at each locus for each family in the PGD and the PGS group respectively. Female (Rec f) and male (Rec m) recombination frequencies were calculated separately as the number of cross-over events over the number of informative female (M f) and male (M m) meioses respectively. The total recombination frequency was calculated as the total number of female and male recombination events over the total number of female and male informative meioses:

$$\text{Female recombination frequency} = \frac{\text{Rec f}}{\text{M f}}$$

$$\text{Male recombination frequency} = \frac{\text{Rec m}}{\text{M m}}$$

$$\text{Total recombination frequency} = \frac{\text{Rec f} + \text{Rec m}}{\text{M f} + \text{M m}}$$

For example, the female partner of family C1, which consisted of eight embryos, was informative for the chromosomes 1 locus, therefore in eight informative meioses there were two female recombination events detected in two embryos. The recombination frequency was calculated to be 0.25 (2/8=0.25). Similarly and as the male partner of the same family was informative for this locus and one male recombination event was seen in one embryo the male recombination frequency was 0.13 (1/8=0.13). The total recombination frequency for family C1 for chromosome one was 0.19 [(2+1)/(8+8)=0.19]. The graphs in figure 3.30 demonstrate the overall recombination frequency results per family and per chromosome.

Table 3.19: Recombination in the PGD group																				
Family ID (no of embryos)		**Chromosome 1**			**Chromosome 5**			**Chromosome 16**			**Chromosome 17**			**Chromosome 19**			**Total**		
		♀	♂	**Total**	♀	♂	**Total**	♀	♂	**Total**	♀	♂	**Total**	♀	♂	**Total**	♀	♂	**Total**
C1 (8)	Informativity	8	8	16	8	0	8	8	0	8	8	8	16	0	8	8	**32**	**24**	**56**
	Rec. events	2	1	3	0	0	0	2	0	2	0	0	0	0	6	6	**4**	**7**	**11**
	Rec. frequency	0.25	0.13	0.19	0	0	0	0.25	0	0.25	0	0	0	0	0.75	0.75	**0.13**	**0.29**	**0.20**
C2 (8)	Informativity	8	8	16	0	0	0	0	8	8	8	0	8	8	0	8	**24**	**16**	**40**
	Rec. events	0	1	1	0	0	0	0	0	0	2	0	2	1	0	1	**3**	**1**	**4**
	Rec. frequency	0	0.13	0.06	0	0	0	0	0	0	0.25	0	0.25	0.13	0	0.13	**0.13**	**0.06**	**0.10**
C3 (9)	Informativity	11	0	11	0	0	0	0	11	11	11	0	11	11	11	22	**33**	**22**	**55**
	Rec. events	0	0	0	0	0	0	0	0	0	3	0	3	2	3	5	**5**	**3**	**8**
	Rec. frequency	0	0	0	0	0	0	0	0	0	0.27	0	0.27	0.18	0.27	0.23	**0.15**	**0.15**	**0.15**
C4 (2)	Informativity	0	2	2	0	2	2	2	2	4	2	0	2	0	0	0	**4**	**6**	**10**
	Rec. events	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	**1**	**0**	**1**
	Rec. frequency	0	0	0	0	0	0	0.5	0	0.25	0	0	0	0	0	0	**0.25**	**0**	**0.1**
C5 (2)	Informativity	0	0	0	2	0	2	0	0	0	2	0	2	2	0	2	**6**	**0**	**6**
	Rec. events	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	**1**	**0**	**1**
	Rec. frequency	0	0	0	0	0	0	0	0	0	0.5	0	0.5	0	0	0	**0.17**	**0**	**0.17**
C6 (22)	Informativity	0	0	0	0	0	0	22	0	22	0	0	0	22	22	44	**44**	**22**	**66**
	Rec. events	0	0	0	0	0	0	4	0	4	0	0	0	4	0	4	**8**	**0**	**8**
	Rec. frequency	0	0	0	0	0	0	0.18	0	0.18	0	0	0	0.18	0	0.09	**0.18**	**0**	**0.12**
Total	**Informativity**	**27**	**18**	**45**	**10**	**2**	**12**	**32**	**21**	**53**	**31**	**8**	**39**	**43**	**41**	**84**	**143**	**90**	**233**
	Rec. events	**2**	**2**	**4**	**0**	**0**	**0**	**7**	**0**	**7**	**6**	**0**	**6**	**7**	**9**	**16**	**22**	**11**	**33**
	Rec. frequency	**0.07**	**0.11**	**0.09**	**0**	**0**	**0**	**0.22**	**0**	**0.13**	**0.19**	**0**	**0.15**	**0.16**	**0.22**	**0.19**	**0.15**	**0.12**	**0.14**

Table 3.19: Informativity, recombination events and frequency for the PGD group. *For each family and each locus the cumulative embryo results are presented. The number of embryos of each family is indicated in parentheses next to the family ID. The total results for all chromosomes for each family are indicated in the last column and the total results for all families for each chromosome are shown in the last row.*

Table 3.20: Recombination in the PGS group																				
Family ID (no of embryos)		**Chromosome 1**			**Chromosome 5**			**Chromosome 16**			**Chromosome 17**			**Chromosome 19**			**Total**		
		♀	♂	**Total**	♀	♂	**Total**	♀	♂	**Total**	♀	♂	**Total**	♀	♂	**Total**	♀	♂	**Total**
C7 (7)	Informativity	7	7	14	0	7	7	0	0	0	7	7	14	7	7	14	**21**	**28**	**49**
	Rec. events	1	1	2	0	0	0	0	0	0	0	0	0	1	1	2	**2**	**2**	**4**
	Rec. frequency	0.14	0.14	0.14	0	0	0	0	0	0	0	0	0	0.14	0.14	0.14	**0.10**	**0.07**	**0.08**
C8 (3)	Informativity	0	0	0	0	3	3	3	0	3	3	0	3	3	3	6	**9**	**6**	**15**
	Rec. events	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0**	**0**
	Rec. frequency	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0**	**0**
C9 (9)	Informativity	0	9	9	0	0	0	0	9	9	0	9	9	9	9	18	**9**	**36**	**45**
	Rec. events	0	0	0	0	0	0	0	0	0	0	0	0	1	1	2	**1**	**1**	**2**
	Rec. frequency	0	0	0	0	0	0	0	0	0	0	0	0	0.11	0.11	0.11	**0.11**	**0.03**	**0.04**
C10 (5)	Informativity	0	5	5	5	0	5	0	5	5	5	5	10	0	5	5	**10**	**20**	**30**
	Rec. events	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	**0**	**2**	**2**
	Rec. frequency	0	0	0	0	0	0	0	0	0	0	0.40	0.20	0	0	0	**0**	**0.1**	**0.07**
Total	**Informativity**	**7**	**21**	**28**	**5**	**10**	**15**	**3**	**14**	**17**	**15**	**21**	**36**	**19**	**24**	**43**	**49**	**90**	**139**
	Rec. events	**1**	**1**	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**2**	**2**	**2**	**4**	**3**	**5**	**8**
	Rec. frequency	**0.14**	**0.05**	**0.07**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.10**	**0.06**	**0.11**	**0.08**	**0.09**	**0.06**	**0.06**	**0.06**

Table 3.20: Informativity, recombination events and frequency for the PGS group. *For each family and each locus the cumulative embryo results are presented. The number of embryos of each family is indicated in parentheses next to the family ID. The total results for all chromosomes for each family are indicated in the last column and the total results for all families for each chromosome are shown in the last row.*

3.3.5.1 Recombination frequency per family

As seen in graph A (figure 3.30) the highest female recombination frequency was seen in family C4, whereas the male of family C1 had the highest frequency. The same family had the highest total recombination frequency (0.2). Family C8 showed no recombination and families C4, C5 and C6 showed no male recombination.

3.3.5.2 Recombination frequency per chromosome

When analysing the recombination frequency per chromosome some differences were noted between the PGD and the PGS groups. No female or male recombination were recorded on chromosome 5 for both groups, even though it was regarded a recombination hot spot. Families of the PGS group showed no female recombination on chromosomes 16 and 17. This is not surprising, especially for chromosome 16, as only three female meioses were informative for that locus in the PGS group. Female recombination frequency was higher in the PGS group for chromosome 1 and lower for chromosome 19 when compared to the PGD group (figure 3.30B).

No recombination events occurring in the sperm were detected on chromosome 16; also no male events were detected on chromosome 17 in the PGD group. Once again the informativity in that locus was very low with only eight male informative meioses. The male recombination frequencies observed for chromosomes 1 and 19 were higher for the PGD group (figure 3.30C).

When comparing the total recombination frequencies per chromosome (figure 3.30D) the highest rates for chromosomes 1, 17 and 19 were observed in the PGD group. No recombination was observed for chromosome 16 in the PGS group. The overall total recombination for all chromosomes analysed was seen in the PGD group and was 0.14, compared to 0.06 of the PGS group. The difference was not statistically significant ($p>0.05$, t test).

Figure 3.30: Recombination frequency per family and per chromosome

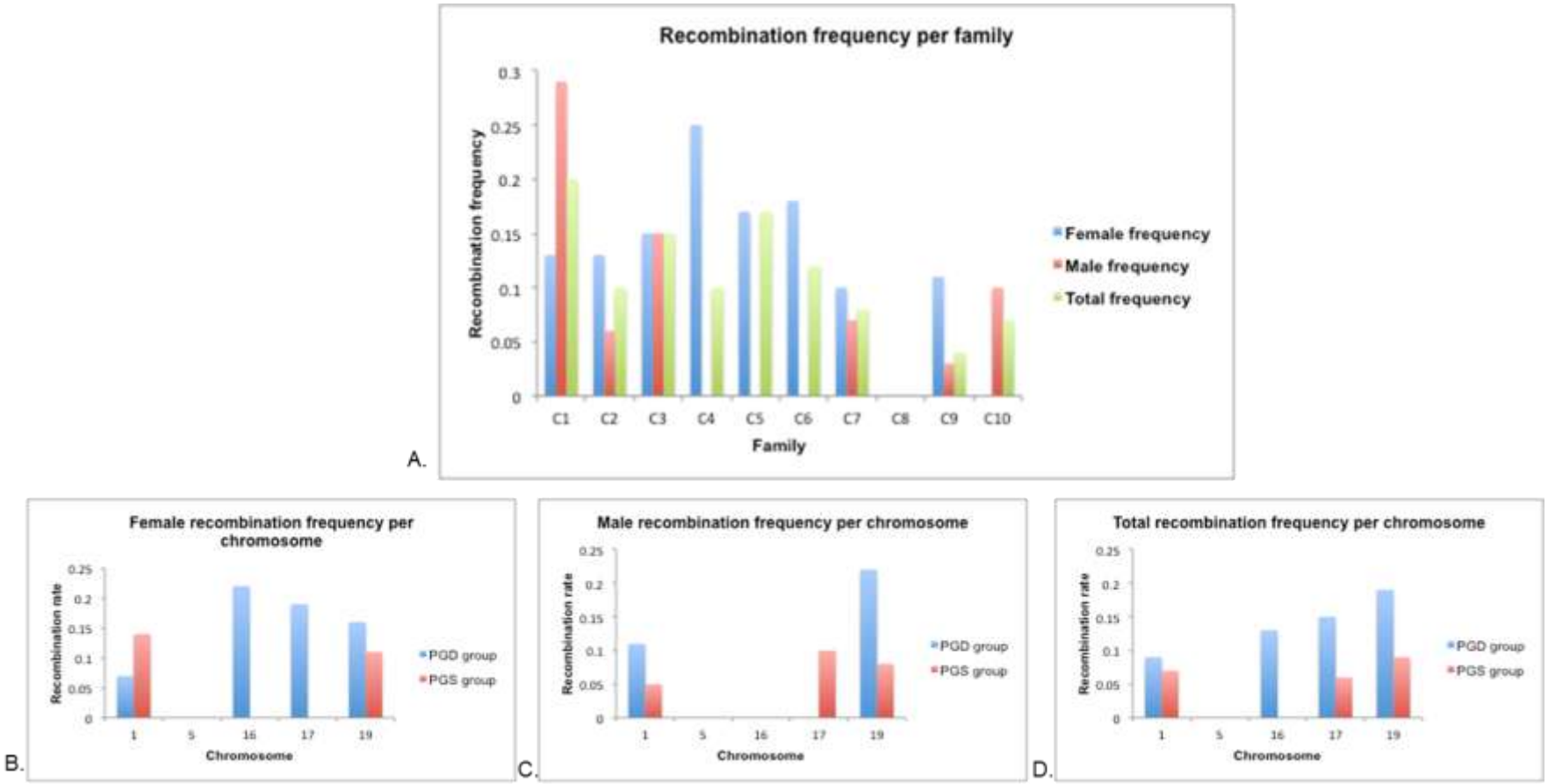


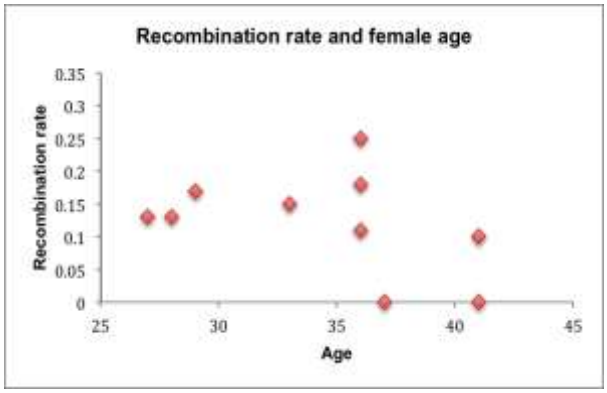
Figure 3.30: *Graph A shows the different female, male and total recombination frequencies per family. Graphs B, C and D show the female, male and total recombination frequencies as seen per chromosome.*

3.3.5.3 Sex and age effects on recombination frequency

Figure 3.31 shows how recombination frequency changed with female and male age. No trend was observed on the rate with increasing female age, however a decline on the rate was observed with increasing male age.

Figure 3.31: Effect of female and male age on recombination

A



B

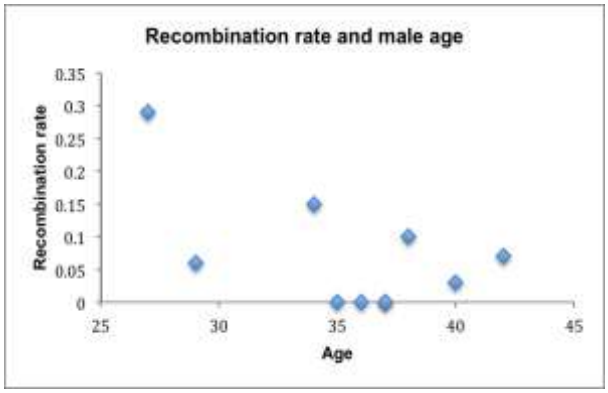


Figure 3.31: *Graph A shows the recombination frequency observed in all the females, arranged according to increasing age and graph B the rate of all the males.*

Small differences were detected in the recombination frequency between females and males. As seen in figure 3.32, female recombination frequency was higher in the PGD group. On the other hand, no differences between the sexes were detected in the PGS group. Therefore, in all families female recombination frequency was higher than the male.

Figure 3.32: Sex effect on recombination frequency

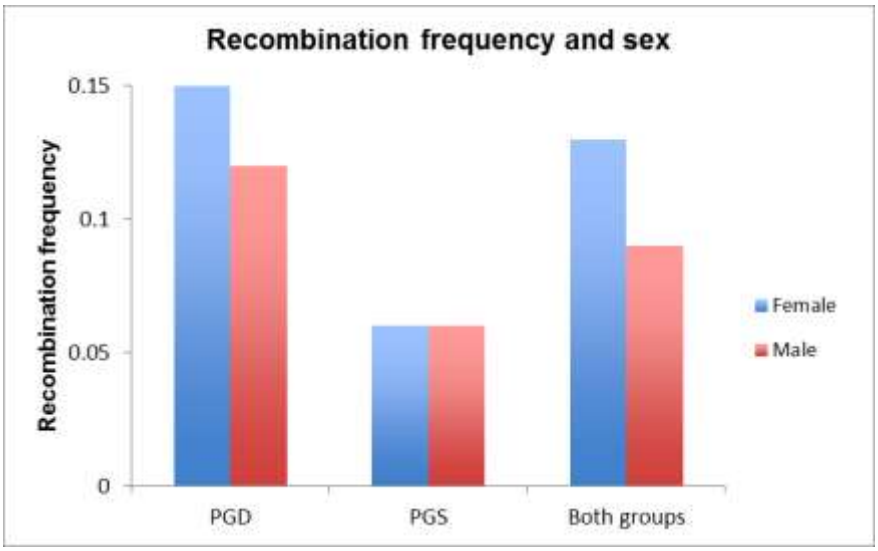


Figure 3.32: *Female and male recombination frequency for each group as well as the overall rates in both groups are indicated.*

By combining all females and males in age groups additional differences were observed. Female and male recombination frequencies were higher in younger individuals of 35 years or less. Recombination frequency decreased as age increased in males and females but not significantly ($p>0.05$, t-test), the decrease observed among males was bigger than females. The only statistical significance observed was that the frequency of older men was significantly lower than the frequency observed in women of the same age group ($p=0.04$, t-test), as shown in the graph in figure 3.33.

Figure 3.33: Differences in recombination frequencies between females and males of different age groups

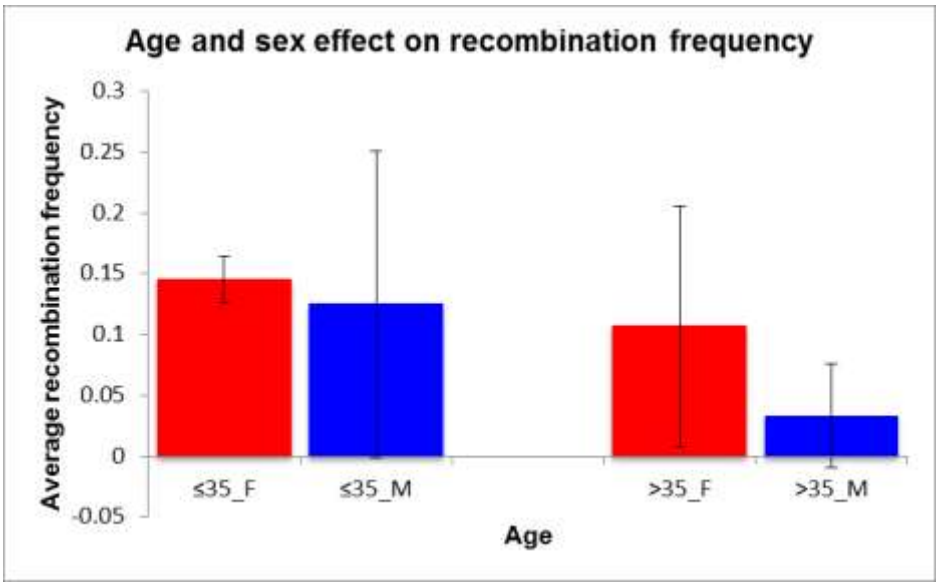


Figure 3.33: *All individuals were separated according to their age and the average recombination frequency was calculated.*

3.3.5.4 Recombination frequency, chromosomal status and morphology

Possible effects of recombination on the embryo chromosomal status as well as embryo morphology were also examined. In the total of 77 embryos analysed, cross-over events were detected in 30. Table 3.21 presents all these embryos that showed recombination, the individual embryo recombination frequencies as well as their chromosomal and development status. For each embryo the maximum female and male informative loci was five, since that was the number of loci investigated and, therefore, the maximum total number of informative loci was ten. The individual embryo recombination frequencies were calculated as the number of cross-over events seen in each embryo over the number of informative loci.

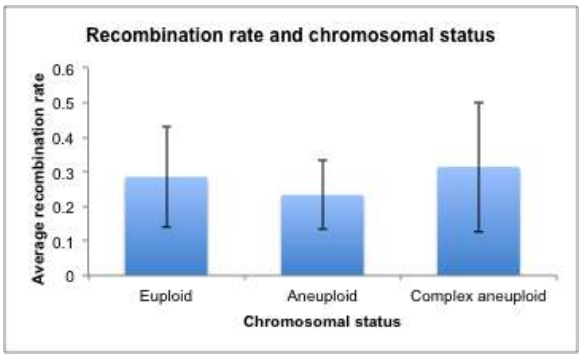
Table 3.21: Characteristics of embryos in which recombination was detected											
Embryo	**Female informative loci**	**Female recombination events**	**Female recombination frequency**	**Male informative loci**	**Male recombination events**	**Male recombination frequency**	**Total informative loci**	**Total recombination events**	**Embryo recombination frequency**	**Chromosomal status**	**Morphology**
C1.3	4	1	0.25	4	0	0.00	8	1	0.13	Complex aneuploid	Blastocyst
C1.4	4	1	0.25	4	0	0.00	8	1	0.13	Aneuploid	Blastocyst
C1.5	4	1	0.25	4	2	0.50	8	3	0.38	No result	Arrested
C1.6	4	1	0.25	4	2	0.50	8	3	0.38	Aneuploid	Arrested
C1.7	4	0	0.00	4	2	0.50	8	2	0.25	Euploid	Blastocyst
C1.8	4	0	0.00	4	1	0.25	8	1	0.13	Aneuploid	Arrested
C2.1*	3	1	0.33	2	0	0.00	5	1	0.20	Euploid	Morula
C2.5*	3	1	0.33	2	0	0.00	5	1	0.20	Euploid	Morula
C2.8	3	1	0.33	2	1	0.50	5	2	0.40	Aneuploid	Morula
C3.4*	3	2	0.67	2	0	0.00	5	2	0.40	Euploid	Blastocyst
C3.5*	3	1	0.33	2	0	0.00	5	1	0.20	Aneuploid	Morula
C3.8	3	0	0.00	2	1	0.50	5	1	0.20	Euploid	Blastocyst
C3.9	3	2	0.67	2	0	0.00	5	2	0.40	Complex aneuploid	Arrested
C3.10	3	0	0.00	2	1	0.50	5	1	0.20	Aneuploid	Blastocyst
C3.11	3	0	0.00	2	1	0.50	5	1	0.20	Euploid	Morula
C4.2	2	1	0.50	3	0	0.00	5	1	0.20	Complex aneuploid	Arrested
C5.2*	3	1	0.33	0	0	0.00	3	1	0.33	Euploid	Blastocyst
C6.2	2	1	0.50	1	0	0.00	3	1	0.33	Euploid	Blastocyst
C6.6	2	2	1.00	1	0	0.00	3	2	0.67	Euploid	Blastocyst
C6.8	2	1	0.50	1	0	0.00	3	1	0.33	Complex aneuploid	Arrested
C6.17	2	1	0.50	1	0	0.00	3	1	0.33	Complex aneuploid	Morula
C6.18	2	2	1.00	1	0	0.00	3	2	0.67	Complex aneuploid	Arrested
C6.22	2	1	0.50	1	0	0.00	3	1	0.33	Euploid	Arrested
C7.2	3	1	0.33	4	1	0.25	7	2	0.29	Aneuploid	Arrested
C7.4	3	1	0.33	4	0	0.00	7	1	0.14	Complex aneuploid	Arrested
C7.5	3	0	0.00	4	1	0.25	7	1	0.14	Euploid	Morula
C9.5	1	1	1.00	4	0	0.00	5	1	0.20	No result	Arrested
C9.6	1	0	0.00	4	1	0.25	5	1	0.20	Aneuploid	Blastocyst
C10.1*	2	0	0.00	4	1	0.25	6	1	0.17	Aneuploid	Blastocyst
C10.2*	2	0	0.00	4	1	0.25	6	1	0.17	Euploid	Blastocyst

Table 3.21: Embryos in which recombination was detected. *The embryo recombination frequency, chromosomal and developmental status are also indicated. The star (*) indicates embryos that showed recombination at the silent spot on chromosome 17.*

Figure 3.34A presents the comparison of the average recombination frequency of embryos with different chromosomal complements. Those that did not have an interpretable aCGH result were excluded from the analysis. The average frequencies of the euploid, aneuploid and complex aneuploid embryos were 0.29 (±0.15), 0.23 (±0.1) and 0.31 (±0.19) respectively. Despite the fact that the average frequency of complex aneuploid embryos was the highest none of the differences were statistically significant. The average recombination frequencies at different development stages were also compared (figure 3.34B). The lowest frequency was recorded for morulae at 0.24 (±0.09), whereas arrested embryos showed the highest recombination frequency at 0.33 (±0.14). The rate of blastocysts was 0.27 (±0.15). No statistical differences were detected within these groups.

Figure 3.34: Differences in recombination frequency of euploid, aneuploid and complex aneuploid embryos and at different developmental stages

A



B

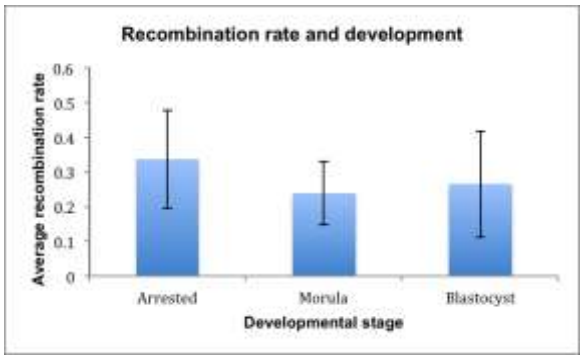


Figure 3.34: *Graph A shows the similar frequencies of diploid and aneuploid embryos. Arrested embryos showed the highest frequency, followed by blastocysts and morulae had the lowest (graph B).*

No recombination was detected in 47 embryos and the characteristics of those are found in table 3.22. Apart from investigating the effect of recombination in the chromosomes and the development within embryos that carried cross-over events, these embryos and those that did not show any recombination were also compared.

Table 3.22: Characteristics of embryos that showed no recombination		
Embryo	**Chromosomal status**	**Morphology**
C1.1	Euploid	Blastocyst
C1.2	Aneuploid	Morula
C2.2	Euploid	Morula
C2.3	Aneuploid	Morula
C2.4	Euploid	Morula
C2.6	Complex aneuploid	Arrested
C2.7	Euploid	Morula
C3.1	Euploid	Blastocyst
C3.2	Euploid	Morula
C3.3	Complex aneuploid	Morula
C3.6	Euploid	Morula
C3.7	Complex aneuploid	Arrested
C4.1	Aneuploid	Morula
C5.1	Aneuploid	Blastocyst
C6.1	Aneuploid	Arrested
C6.3	Euploid	Arrested
C6.4	Euploid	Blastocyst
C6.5	Complex aneuploid	Arrested
C6.7	Complex aneuploid	Morula
C6.9	Complex aneuploid	Blastocyst
C6.10	Aneuploid	Blastocyst
C6.11	Complex aneuploid	Arrested
C6.12	Aneuploid	Arrested
C6.13	Aneuploid	Morula
C6.14	Euploid	Arrested
C6.15	Aneuploid	Morula
C6.16	Euploid	Morula
C6.19	Euploid	Arrested
C6.20	Aneuploid	Morula
C6.21	Euploid	Arrested
C7.1	Aneuploid	Morula
C7.3	Aneuploid	Morula
C7.6	No result	Morula
C7.7	Complex aneuploid	Arrested
C8.1	Aneuploid	Blastocyst
C8.2	Complex aneuploid	Blastocyst
C8.3	Aneuploid	Blastocyst
C9.1	Euploid	Morula
C9.2	Aneuploid	Arrested
C9.3	Euploid	Morula
C9.4	Complex aneuploid	Arrested
C9.7	Complex aneuploid	Morula
C9.8	Aneuploid	Morula
C9.9	Complex aneuploid	Morula
C10.3	Aneuploid	Blastocyst
C10.4	Euploid	Blastocyst
C10.5	Aneuploid	Blastocyst

Table 3.22: List of embryos in which no cross-over events were detected. *The chromosomal status and developmental stage are also indicated.*

Figure 3.35 presents all the embryos; in graph A the embryos were grouped according to their chromosomal status. Within the euploid embryos the majority carried cross-over events, whereas the number of aneuploid and complex aneuploid embryos that showed no recombination was higher than those that did. Embryos that had reached the morula stage and did not show recombination were more than those that did and finally, arrested embryos and blastocysts with recombination were more common than those with no recombination. Comparison of these percentages did not reveal any significant differences (Fisher's exact test, $p>0.05$).

Figure 3.35: Embryos with and without recombination

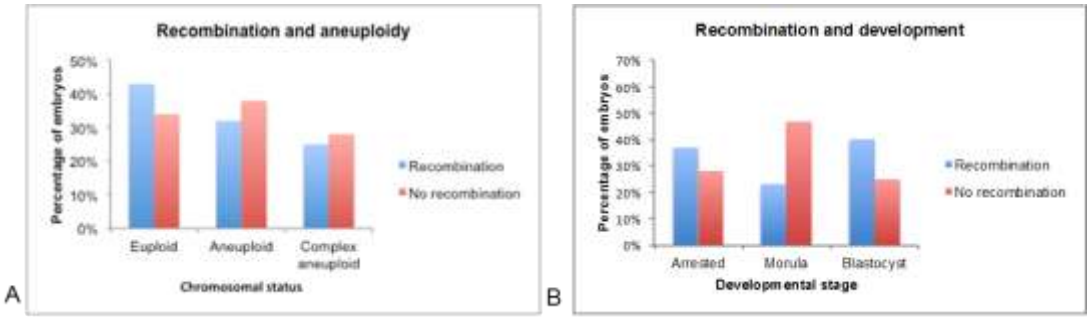


Figure 3.35: *Embryos with or without recombination grouped according to the chromosomal status (A) or their stage of development (B).*

3.3.6 Parental origin of aneuploidy

Amplifying polymorphic markers located on different chromosomes on DNA from parents and their embryos provided the ability to detect possible aneuploidies present in the embryos, as well as their parental origin. Moreover, the chromosomal status of each embryo was known through aCGH, therefore, any indication of aneuploidy seen with the STR markers could be confirmed in the aCGH result. Table 3.23 presents all the embryos with marker results that suggested aneuploidy, confirmation or not of this aneuploidy by aCGH as well as the parental origin of chromosomal abnormalities. The result of the markers on chromosome 17 for embryo C1.5, indicated monosomy 17, however no aCGH result was available for that embryo and was therefore was not included in this set of results.

Seventeen embryos showed evidence of aneuploidy by the STR analysis in one locus. Of these 17 possible aneuploidies a true gain of loss of the chromosome was confirmed by aCGH in seven. These were five monosomies and two trisomies. The most commonly involved chromosome in changes that were confirmed by aCGH

was chromosome 16, seen in four cases of aneuploidy, chromosome 17 in two and chromosome 19 was involved in one aneuploidy. Maternally derived aneuploidies were the most common, as five were detected. The remaining two were paternally inherited.

The possible aneuploidies as indicated by the STR markers that were not detected by aCGH were 10. Of these, seven showed possible monosomies and three trisomies. The indication of monosomy not confirmed by aCGH might have been a result of ADO, where expected heterozygote samples appeared as homozygote. For example, embryo C7.2 the paternal allele 212 for marker D16S429 on chromosome 16 failed to amplify and since the parents were not informative for the other marker on chromosome 16 (D16S3053, both homozygote 240), a false indication of monosomy was given (appendix C). Possible uniparental disomy (UPD) was also detected for chromosome 16 in embryo C7.7 since that embryo had two maternal alleles for marker D16S492 and no paternal. This could not be confirmed by the other marker (D16S3053) due to homozygosity of the parents with the same allele size and also by aCGH since two pairs of chromosome 16 would have been present in the embryo, thus would result to a profile identical to a euploid sample.

Table 3.23: Parental origin of aneuploidy, as detected by STR markers			
Embryo	**Aneuploidy detection by STR markers**	**Confirmation of aneuploidy by aCGH**	**Parental origin of error**
C1.8	Monosomy Chromosome 5	Not confirmed	Paternal
C2.6	Monosomy Chromosome 5	Not confirmed	Paternal
C2.7	Monosomy Chromosome 5	Not confirmed	Paternal
C4.2	**Monosomy Chromosome 17**	**Confirmed**	**Maternal**
C6.5	**Monosomy Chromosome 16**	**Confirmed**	**Maternal**
C6.9	**Trisomy Chromosome 16**	**Confirmed**	**Maternal**
C6.15	**Trisomy Chromosome 16**	**Confirmed**	**Paternal**
C6.20	Monosomy Chromosome 19	Not confirmed	Paternal
C7.2	Monosomy Chromosome 16	Not confirmed	Paternal
C7.4	**Monosomy Chromosome 16**	**Confirmed**	**Maternal**
C7.5	Monosomy Chromosome 5	Not confirmed	Maternal
C7.7	UPD Chromosome 16	Not confirmed	Maternal
	Trisomy Chromosome 19	Not confirmed	Maternal
C8.2	Trisomy Chromosome 19	Not confirmed	Paternal
C8.3	Trisomy Chromosome 19	Not confirmed	Paternal
C9.2	Monosomy Chromosome 5	Not confirmed	Maternal
C9.8	**Monosomy Chromosome 19**	**Confirmed**	**Maternal**
C10.3	**Monosomy Chromosome 17**	**Confirmed**	**Paternal**

Table 3.23: Embryos with indication of aneuploidy as detected by the STR markers. *Presence of aneuploidies was confirmed by aCGH. The parental origin of the aneuploidy could also be detected with the use of the STRs.*

3.3.7 Summary of results for section 3.3: Investigation of recombination in preimplantation embryos

- FPCR amplification was highly successful on whole genome amplified embryos by SurePlex with a low amplification failure and allele drop out rate.
- Detection of recombination in embryos using polymorphic markers was highly dependant on marker informativity.
- Recombination frequency varied between families and individuals and it was higher in females than males. Chromosome 19 showed the highest recombination frequency among all chromosomes analysed, whereas no recombination events were detected on chromosome 5. Recombination frequency decreased with age in females and males, but the decrease observed in males was higher. Recombination frequency in females over 35 is significantly higher than recombination frequency in males in the same age group.
- No significant differences were observed in the recombination frequency of euploid, aneuploid and complex aneuploid embryos and embryos at different developmental stages.
- The use of aCGH and polymorphic markers offered the ability to detect the parental origin of aneuploidy in some embryos. The majority of the aneuploidies were maternally derived.

4 Discussion

4.1 Validation of aCGH for clinical use in PGS

4.1.1 24sure array platform

The first aim of the present study was the validation of the 24sure microarray platform for the analysis of all chromosomes in preimplantation embryos. The same platform has been widely used for clinical PGS cases and research studies. Fishel *et al* published the first aCGH analysis with 24sure on polar bodies from a 41-year old woman with a history of 13 failed IVF cycles, resulting in a live birth (Fishel *et al*, 2010). Since then, 24sure has been used in PGS cycles to test blastomeres (Ata *et al*, 2012) and TE samples (Fragouli *et al*, 2011a). Despite the good clinical results in all applications, no validation on samples of known chromosomal status had been described at the start of this study.

4.1.2 Use of cell lines for the validation

Prior to clinical application, each technique needs to be validated to ensure efficiency, accuracy and, in the case of preimplantation diagnosis, speed of analysis. aCGH is a new technique for the detection of numerical chromosomal abnormalities in embryos and no clear guidelines of the steps that need to be undertaken for its validation are available. The European Society of Human Reproduction and Embryology (ESHRE) has published two sets of guidelines for PGD and PGS (Thornhill *et al*, 2005; Harton *et al*, 2011b; Harton *et al*, 2011c). These guidelines however, are restricted to PCR- and FISH-based analyses. Validation of each protocol is recommended on single cells of known genetic and/or chromosomal status. Testing in “spare” polar bodies, single blastomeres and trophectoderm cells prior to aCGH is advised (Harper and Harton, 2010) however, these are samples carrying unknown chromosomal complements. The ESHRE PGS task force has published two studies describing the technical and clinical aspects of the validation of aCGH in PGS post polar body biopsy (Geraedts *et al,* 2011, Magli *et al,* 2011). Validation using cell lines is ideal, since there is an *a priori* knowledge of their chromosomal status. Ovarian epithelial cell lines were used for the validation in this study.

The chromosomal constitution of the four cell lines used, as well as the chromosomal stability within each line, was confirmed by FISH analysis. Results revealed that these lines were indeed stable, with the expected aneuploidies,

detected in lines TOV-21G and SKOV-3 and euploidy confirmed in lines IOSE11 and IOSE 19 with acceptable rates (>90% of cells analysed for all cell lines).

4.1.3 Analysis of single cells by aCGH after cleavage stage biopsy

Several studies have been published describing the use of cell lines for the validation of aCGH at the single cell level. However, a variety of WGA techniques and array platforms were used. The use of MDA with BAC arrays was validated for the detection of aneuploidies on 12 single cells from aneuploid cell lines, as well as six cells with segmental rearrangements as small as 34Mb. All expected, whole and segmental chromosome changes, were successfully detected. Further validation of aCGH was performed on two embryos that were found to be aneuploid by FISH during PGS. Discrepancy between the aCGH and the FISH result was observed in one of the two embryos, which was attributed to mosaicism (Le Caignec *et al*, 2006). The use of GenomePlex for WGA, the method used in the present study for the validation of single cell aCGH, with BAC arrays has been described previously (Fiegler *et al*, 2007). Whole chromosome and segmental changes were detected in a small number of single cells from tumor cell lines, as well as patients with trisomy 21 and Prader-Willi syndrome. The authors suggested that there was less variability in their results when compared to the study by Le Caignec *et al,* where MDA was used. Fuhrmann *et al* described the ability to detect segmental changes as small as 4.4 and 5Mb could be detected on single cancer cells using a BAC array (Fuhrmann *et al*, 2008). Although analysis by 24sure can detect changes bigger than 10Mb, the purpose of PGS is the accurate detection of whole chromosomal changes. Finally, blind analysis on 24 single cells from aneuploid cell lines was performed as part of validation of SNP arrays, which showed 100% accuracy (Treff *et al*, 2010a).

This study describes the use of specific types of WGA (GenomePlex and SurePlex) in combination with the 24sure arrays (24sure) for which, no other validation using cell lines, has been described before. WGA by random fragmentation of the DNA and subsequent amplification, using GenomePlex was very efficient as 95% of the single cells were successfully amplified. The potential causes of WGA inefficiency or failure for the single cells include failed transfer of cells to the tubes during isolation and transfer of anucleate cells or cells with degraded DNA. Four of the amplified single cells produced a weak smear on the agarose gel. Subsequent aCGH analysis

failed for three out of these four cells. Despite the weak smears, these cells were analysed by aCGH in order to maximise the data in the validation process. During a PGS case, similar samples should still be analysed, as successful analysis is possible. This is supported by the one cell that was successfully analysed by aCGH despite the faint smear. Additionally, quality indicators provided by the software together with the aCGH result (discussed in the Discussion section 4.1.5) indicate the quality of the amplification. Blind aCGH analysis was performed on 40 single cells revealing, upon decoding, a 100% concordance in the aCGH result and the chromosomal status of the cells.

4.1.4 Analysis of mosaic models by aCGH for PGS after blastocyst biopsy

An alternative approach for chromosomal analysis in PGS is after blastocyst biopsy. Compared to cleavage stage embryo, blastocyst biopsy is thought to be more appropriate, as six to ten cells from the trophectoderm can be analysed as one sample therefore minimising problems faced during single-cell analysis. In addition, the levels of mosaicism are not as high at that stage. However, mosaicism can still be present in the blastocyst and studies have shown that the TE reflects the chromosomal constitution of the inner cell mass (Evsikov and Verlinsky, 1998). Aneuploid and euploid cells were mixed in known ratios to analyse the effect of mosaicism in aCGH. All samples were successfully amplified by SurePlex. As described above, amplification of single cells by GenomePlex was successful on 95% of cells. The 100% amplification efficiency in the mosaic samples might have been due to the increased number of cells in the starting material. These two WGA methods were based on a similar technology, including, fragmentation of the genome, library preparation and library amplification by universal primers. The library preparation is performed by an isothermal reaction during GenomePlex, whereas thermal cycling is used during SurePlex (data from www.rubicongenomics.com). Gutiérrez-Mateo *et al*, showed that GenomePlex had significantly higher rates of amplification failure when compared to SurePlex on single blastomeres. This indicated that SurePlex gives a better representation of a cell's genome, even when the DNA is not of high quality (Gutiérrez-Mateo *et al*, 2011).

Analysis of the $\log_2$ ratio for all clones on chromosome 10, showed an increasing trend of the ratio in all samples, as the proportion of aneuploid cells increased. However, variability was seen between the TE and blastocyst models as well as within the repeated samples of each model. The presence of cells with degrading DNA might affect the aCGH outcome. This might be more apparent in samples with fewer cells, as it was observed in this study. In the blastocyst model, where the total number of cells was 100, with 50% aneuploid cells, the $\log_2$ ratio was 0.4, compared to 0.28 for the TE model with the same proportion of aneuploid cells but only eight cells were in the sample in total.

Vermeesch *et al*, have described the effect on aCGH of a sample containing 20% of a cell line with trisomy 13 and a normal cell line (Vermeesch *et al*, 2005). This resulted in an increase of the ratio for all the chromosome 13 clones. In this study, a shift from normality was observed when the percentage of aneuploid cells was over 25%. A sample with 20% aneuploid cells was not analysed, but a small shift would have probably been detected. Moreover, the starting material in the experiment described by Vermeesch *et al*, was genomic DNA and not amplified material from a small number of single cells. Detection of low-level mosaicism might be easier in this type of samples.

The effect of mosaicism on SNP arrays has also been described through the use of cell lines. Northrop *et al*, chose to mix single cells from male and female cell lines in known proportions and examine the change of the X chromosome when the samples were run against a female reference DNA (Northrop *et al*, 2010). A copy number change, as monosomy X, was detected when the proportion of male cells in the sample was over 25%, the same result that was obtained when 100% of male cells were present. This comes in contrast to the gradual change in the ratio observed in this study, as the number of aneuploid cells increased in the sample.

Moreover, detection of mosaicism by aCGH when the proportion of aneuploid cells in the sample is 30% or more has been described in another study (Fragouli *et al*, 2011a). This conclusion was drawn after TE biopsy and analysis by aCGH and further reanalysis by FISH on the remainder of the embryo, to confirm the findings. The mosaic models used in this study were prepared with precise knowledge on the ratio of aneuploid cells present in the sample, thus providing a better means of analysis of the effects of mosaicism, than analysing a biopsied sample containing an unknown ratio of aneuploidy. Overall, several studies, including this, have shown

that mosaicism can be detected by aCGH and SNP arrays. However, if the biopsied sample contains mosaic cells that are a result of mitotic non-disjunction, with reciprocal abnormalities (monosomy and trisomy), no error will be detected by any type of array, as the abnormalities will result in a ratio of zero. Abnormalities from mitotic non-disjunction will still be detected if the biopsied sample contains a clear majority of one of the two cell lines.

4.1.5 Key quality indicators for aCGH

As discussed in the Discussion section 4.1.3, the agarose gel was the first indicator of a successful WGA. Samples producing a bright smear were the ideal starting material for labelling and hybridisation on the arrays and were expected to produce a good aCGH result. The software, BlueFuse, provided indicators on the quality of each aCGH experiment. These were the percentage of clones included in the analysis, the signal to background ratio and the mean spot amplitude. In our lab, 65% has been set as an acceptable value for the percentage of clones included as a threshold. Results with a percentage of clones, lower than the threshold are considered failed. The values of the clone inclusion of the mosaic TE models were compared with those of the mosaic blastocyst models. Even though the number of cells in the TE groups was eight and in the blastocyst groups 100 the average percentage of clones included in the TE groups was 81.74%, higher than in the blastocyst groups, which was 77.71%. This was surprising as it was expected that aCGH on a larger number of cells would produce better quality result. The reason for this was probably the incorporation of high volume of PBS during isolation of the 100-cell samples in the tube, which compromised the amplification of the samples.

Aside from the quality of starting material, other factors that can influence the result are the time of the labelling and hybridisation. Experiments with different timings revealed that the combination that provided the best result, with the indicators well above the desired values, was long labelling for 18 hours and short hybridisation for three hours. Acceptable values were also obtained with three hours labelling and 16 hours of hybridisation. Knowing that both these combinations will provide an interpretable result is beneficial when performing a PGS case, as experiments can be set up according to time availability.

4.1.6 Concordant results between blastocyst and TE

Four blastocysts were biopsied and both the TE and the remainder of the embryos were analysed by aCGH. The purpose of this was twofold. First, this procedure acted as a “dry-run” case before such PGS cases were performed. The efficiency of the biopsy procedure was confirmed as all samples produced a result and embryologists confirmed re-expansion of the blastocyst post biopsy. The second purpose was to examine whether the results obtained from the TE sample were representative of the whole blastocyst. Overall, concordance between the TE and the blastocyst was confirmed in four embryos. However, this was not the case in a study, where analysis was performed in three different biopsied TE samples and the ICM from each blastocyst by SNP array (Northrop *et al*, 2010). Of the 50 blastocysts analysed, 24% showed discordant results between the four samples of each blastocyst. The rest were either all euploid or all presented the same aneuploidy. Within the blastocysts with discordant results, the authors did not find any preferential segregation of the abnormalities in the TE samples and findings were attributed to mosaicism.

In this study, two of the four blastocysts were euploid and therefore confirmation could only be performed by the sex chromosomes, which was indeed achieved in both. The third pair showed loss of chromosome 21, which was present in both. Finally, the TE sample of the last pair seemed euploid, however the change of $\log_2$ ratio did not correspond to a male or a female sample. Even though there was a shift for both chromosomes, it was below the 0.3 threshold for chromosome X and around -0.4 for chromosome Y, instead of -0.8, expected for female samples. This could be accredited to poor hybridisation, which may produce bad separation of the sex chromosomes. This could not have been the case though, as all TE samples were run together and a more dynamic change was observed in the other female TE sample. Moreover, all blastocyst samples were run together in a later, separate experiment and similar $\log_2$ ratios were observed for the sex chromosomes. All the above, indicated that this was possibly a triploid embryo with a 69,XXY karyotype. aCGH cannot detect ploidy in the sample, as the abnormality present in all chromosomes will be averaged out, giving a euploid result. If, however, there is a sex mismatch, in a polyploid sample, a separation in the sex chromosomes should be expected, though not as dynamic as in diploid samples, due to the false normalisation of the autosomes from the software. This also suggests that hypo- or

hyperploid samples carrying an extra aneuploidy for one of the autosomes should still be detected by aCGH.

4.1.7 Follow-up analysis of untrasferred embryos by FISH

Previous studies of CGH, where all the cells of untransferred embryos were analysed individually by FISH, have speculated that discordant results were due to mosaicism and/or technical artefacts (Gutiérrez-Mateo *et al*, 2011). In this study, reanalysis by FISH was performed on seven embryos, which were found to be aneuploid and thus not transferred, as well as one euploid that was not transferred due to bad morphology, after PGS cycles using aCGH. Blastocyst biopsy was performed on two embryos, whereas cleavage stage biopsy was performed on the remaining six. Probes for the FISH analysis were selected according to the aneuploidies detected by aCGH and their availability in the lab.

An indication for mosaicism was detected in both blastocysts analysed by aCGH, with one (A1.1) carrying only that abnormality, whereas the other presented abnormalities affecting three chromosomes. FISH analysis for chromosome 14 that was deemed mosaic after aCGH, for embryo A1.1, revealed that 33% of the nuclei were monosomic for that chromosome. The average $\log_2$ ratio for that chromosome was -0.20, thus indicating mosaicism. From the mosaic TE models, as described in the Discussion section 4.1.4, a similar increase in the ratio was observed when the proportion of cells was between 25% and 37.5%. The mixing experiments were conducted with an aneuploid cell line carrying trisomy for chromosome 10, thus examining the effect in chromosomal gain. This cannot be extrapolated for chromosomal losses due to differences in the ratios. In a trisomic sample the theoretical ratio of the test over the reference DNA would be 3:2, whereas in a monosomic it would be 1:2. FISH analysis of all individual nuclei in the blastocyst can confidently reveal the true level of mosaicism in the remaining embryo. This was also made apparent with embryo A2.1, where chromosomes 1 and 5 had a $\log_2$ ratio of 0.24 and 0.25 respectively, but 88% and 85% of the total nuclei analysed, showed to carry that abnormality. A higher ratio was expected for both chromosomes, if the proportion of aneuploid cells was the same in the biopsied samples.

Possible experimental and biological variation may be a feature of the aCGH result. The mixing experiments revealed that there was a small variation between samples

with the same starting material. For example, as seen in table 3.2 in the Results section, within the 8-cell group, the $\log_2$ ratio of sample 6(A) was 0.28, whereas the $\log_2$ ratio of sample 6(B) was 0.37. Both of these samples contained 75% of aneuploid cells and the difference in the $\log_2$ ratio revealed experimental variation. The aCGH results of the TE biopsied samples discussed here were indicative of mosaicism, a result of biological variation. This provided the knowledge that an abnormality was present but it was not possible to accurately predict the exact proportion of aneuploid cells present in the sample.

Reanalysis of embryos found to be aneuploid after biopsy of single blastomeres, confirmed that mosaicism at that stage renders aCGH not to be representative of the chromosomal situation in the whole of the embryo. FISH analysis confirmed all the aneuploidies that were detected by aCGH, therefore aCGH did not give any false positive results. Of these five embryos, only one carried the aneuploidy detected by aCGH in all the nuclei analysed by FISH. In the rest the abnormality was seen only in a proportion of nuclei. Mitotic non-disjunction was also detected in one embryo, which carried both monosomic and trisomic cells for chromosome 15 and aCGH had shown loss for that chromosome. The one embryo that was found to be euploid by aCGH on day three, was classified as diploid/chaotic mosaic by day five FISH analysis.

4.1.8 Is PGS beneficial?

PGS was regarded as a promising technique in aiding infertile couples during the first years of its application. However, several RCTs revealed that it was not beneficial and even resulted in reduced pregnancy rates in patients with AMA (Mastenbroek *et al*, 2011). This was attributed to technical and biological limitations. FISH analysis does not allow the enumeration of all chromosomes and therefore a lot of aneuploidies remained undetected. Moreover, the majority of PGS cases were performed at cleavage stage biopsy on single blastomeres, which due to mosaicism might not be representative of the chromosomal status of the remainder of the embryo. The complete enumeration of chromosomes by aCGH together with biopsy at stages when mosaicism may be less of a concern, polar body and TE, is an alternative that can overcome the above-mentioned limitations.

Potential and true benefit of PGS performed with these procedures can only be uncovered through properly conducted RCTs. Indeed the ESHRE PGS task force

has performed a pilot study as the first step towards a multicentre RCT, examining the outcome of polar body testing by aCGH using the 24sure platform (Geraedts *et al*, 2011). Encouraging results were obtained after analysis of TE samples in a randomised pilot study, on good prognosis patients. Using the same platform, as the one used in this study, 24sure, it was revealed that the clinical and on-going pregnancy rates were significantly higher in couples that received PGS than those that did not (Yang *et al,* 2012). The choice of the patient cohort for randomisation in the study by Yang *et al* was couples with maternal age less than 35 years, with no previous IVF treatment, seeking IVF due to tubal or male factor. The reproductive potential in this group of couples is not comparable to those that normally choose to go through PGS, who are considered infertile. On average, 7.7 blastocysts were analysed per couple, a high number that is rarely achieved in couples that undergo PGS. The stage of transfer is another issue with blastocyst biopsy. aCGH analysis is possible within 24 hours, thus transfer may be performed on day six. However, data have suggested that transfer on day six may be close to the limit of the implantation window and dyssynchrony of the endometrium and the embryo may result on poor pregnancy rates (Van Voorhis and Dokras, 2008). Moreover, transfer of frozen/thawed embryos in a natural or mildly stimulated cycle may provide benefits in the receptivity of the endometrium, early implantation and the development of the placenta, since it reflects more the natural process (Pinborg, 2012).

Very recently, the validation of a new, rapid technique to detect imbalances in all chromosomes has been described (Treff *et al,* 2012). Quantitative real-time PCR (qPCR) was performed on samples containing five cells from cell lines of known karyotypes and TE samples biopsied from blastocysts. The chromosomal status of the blastocysts was previously determined by SNP arrays on TE biopsied samples from each embryo. The authors suggested that, with this selection of embryos, there was control over the chromosome abnormalities and the risks of mosaicism were minimised. Results were obtained in four hours, showing 97.6% reliability in the diagnosis post analysis of the cell lines and 98.6% consistency for the 24-chromosome analysis in the blastocysts. Although, this seems a promising technique, especially when opting for a fresh transfer, further assessment is needed with RCTs.

A lot of progress has also been observed in the field of whole genome sequencing of small amounts of DNA. Tests have been performed on biopsied samples from

blastocysts to detect the suitable method of amplification that will result in a template of sufficient quality for the sequencing (Peters *et al*, 2011). The introduction of the long fragment read (LFR) technology, describes the ability to perform whole genome sequencing and haplotyping on the DNA from 10 to 20 single cells, following amplification by a modified MDA, the protocol found best suited for sequencing analysis in blastocysts (Peters *et al*, 2011). This type of sequencing provides information on the diploid genome with a low error rate, more sensitive than that provided by aCGH or SNP array analysis (Peters *et al*, 2012).

Even if technology advances, the problems in the diagnosis generated by mosaicism will still be present. Mosaicism at the cleavage stage may result in the biopsy of cells not representative of the whole embryo. At the blastocyst stage, mosaicism is an issue and despite the fact that this study showed that it could be detected by aCGH in TE samples, its true level on the remainder of the embryo will never be known. The only stage when mosaicism does not pose any risks is the oocyte. Therefore, analysis of both polar bodies in couples that are at high risk of maternal errors, like those with AMA, by aCGH may be the only stage in which PGS will actually be of benefit. However, even at that stage some biological limitations should be taken into consideration. Depending on the segregation of the sister chromatids in meiosis II, following premature separation of the chromatids in meiosis I, the zygote will be euploid or aneuploid. Identification of reciprocal errors in both polar bodies indicates a euploid zygote (Scott et al, 2012a). However, anaphase lag may cause chromosomal loss in one of the polar bodies, with no reciprocal changes in the other polar body or the zygote (Handyside *et al*, 2012). This observation, together with the fact that errors that are paternal in origin cannot be detected, highlight the fact that polar body biopsy will never be 100% accurate of the chromosomal status of the zygote.

Other approaches in IVF may aid reproductively challenged couples to become pregnant that do not involve invasive techniques. For example, one study has shown that single blastocyst transfer in women older than 35 has resulted in a high pregnancy rate of 51.1% (Davis *et al,* 2008). Moreover, the use of time-lapse imaging of *in vitro* embryo development can provide a tool for predicting blastocyst formation from as early as day two of development (Wong *et al*, 2010). Time-lapse imaging does not affect embryo quality, blastocyst formation and pregnancy rates, as described in a study analysing embryos from female donor cycles (Cruz *et al*, 2011). Preliminary data of a study investigating the length of early divisions in

diploid and aneuploid murine preimplantation embryos, showed that diploid embryos divided faster (Elaimi *et al*, in preparation).

4.2 Aneuploidy in embryos from couples undergoing PGD

The second aim of this thesis was to determine the aneuploidy level in embryos from couples undergoing PGD for single gene disorders. In the majority of cases the only indication for IVF is for the purpose of PGD. Embryos are generated *in vitro* for biopsy, diagnosis and ultimately transfer of unaffected embryos. These couples do not necessarily face fertility problems, since some of them may already have a child or have gone through the termination of an affected pregnancy. Others choose not to get pregnant to avoid the risk of an affected pregnancy, but their young age and fertility checks before IVF can suggest their good fertility status. Aneuploidy in the embryos of this cohort of patients may be the reason for a lack of pregnancy, in spite the transfer of high quality, free of mutation, embryos. In some cases, aneuploidy may be the source of error during PGD (Delhanty *et al*, 1997). For example, monosomy of the chromosome carrying the disease-causing gene will result in a false negative result in the analysis of an autosomal dominant mutation. The detection of both parental genomes through the incorporation of linked to the disease-causing gene, polymorphic markers in the PGD protocol is a way to detect aneuploidy. The absence of one or the presence of one extra parental allele will result in the deviation from the expected haplotype in heterozygote samples.

A small number of studies have presented data on the chromosomal constitution of embryos from young and presumably fertile patients. Two of them (Munné *et al*, 2006 and Reis Soares *et al*, 2003) performed analysis on embryos from cycles with oocyte donors. In both, the data used were clinical results performed by FISH on single blastomeres biopsied from cleavage stage embryos with no follow-up analysis on the remainder of untransferred embryos. Munné *et al* compared the results with embryos from patients undergoing PGS, which were divided into subgroups according to maternal age. In the donor group (average maternal age: 25.6), the rate of chromosomally abnormal embryos was high (57%), however, significantly less than the rate of chromosomally abnormal embryos in all the PGS groups. The second study by Reis Soares *et al*, compared the results of the donor group, with that of a group of couples undergoing PGD for X-linked diseases. The same set of probes was used for both groups. Despite the fact that the maternal age in the donor group was lower than in the PGD group (27 and 31 years), the

frequency of abnormalities in embryos of the donor group was significantly higher than the PGD group (56.5% and 37.3%). Both studies attributed the high rate of chromosomal abnormalities in the stimulation used in donor cycles, which was aggressive in order to produce a large number of follicles. This hypothesis was contradicted after the analysis of embryos from IVF cycles with no ovarian stimulation. Aneuploidy was still present in these embryos at a rate of 36.4%, despite the fact that the maternal age was low (mean 31.4 years) (Verpoest *et al*, 2008).

4.2.1 Chromosomal analysis by FISH

In total, 86 embryos were analysed by FISH and successful analysis was possible in 82.5%. FISH failure could be attributed to fault in the fixation of the embryos on the microscope slide, but also to degraded DNA material. Failure of FISH reanalysis of day five embryos at an even higher rate of 23% has been previously described and was attributed to degeneration of the embryos (Baart *et al,* 2006). A five-probe set was used, analysing chromosomes 13, 18, 21, X and Y in two consecutive FISH rounds. This choice of probes is preferred by other groups for analysis, as it covers chromosomes that are at high risk of involvement in aneuploidy. Diploidy, aneuploidy and mosaicism differed at each developmental stage.

Uniform diploidy and aneuploidy were only seen in arrested embryos and morulae. Similar to this study, analysis of 50 embryos by FISH, revealed diploid embryos at only the cleavage and morula stages. The authors suggested that the fourth mitotic division of an embryo is the one generating most blastomeres with an aneuploidy (Gonzalez-Merino *et al*, 2003). Even though the embryonic genome is activated during the cleavage stage, maximum expression for the majority of embryonic genes occurs at the blastocyst stage (Wells *et* al, 2005). This can explain the fact that errors in mitosis can occur in later cell divisions. Moreover, it can provide an explanation for the presence of diploid embryos in only the cleavage and morula stages. The presence of maternal transcripts could have aided in the normal progression of embryos during the first stages of preimplantation development. However, failure of expression of genes, post EGA, responsible for correct chromosome segregation, cell cycle control and apoptosis might have led to developmental arrest.

Mosaicism was detected at all stages, however only diploid mosaic morulae and blastocysts were seen. The rate of mosaicism increased from 67% at the morula stage to 100% at the blastocyst. The chaotic embryos observed were all arrested. The overall rate of 50% diploid mosaic embryos is in line with other studies examining aneuploidy at all developmental stages (Bielanska *et al*, 2002). FISH analysis allowed the distinction between errors in meiosis and mitosis. In total, 13 meiotic and 48 mitotic errors were detected. Among the mitotic errors, chromosome loss (CL) was more frequent, followed by chromosome gain (CG) and mitotic non-disjunction. The frequency in the type of mitotic errors varies among studies. Some have reported CL as the most frequent and others CG (Fragouli *et al*, 2011a). Monosomy was more frequent within the meiotic errors, an observation made by other groups as well (Gonzalez-Merino et al, 2003). On the other hand, monosomy rates might be overestimated by FISH due to technical issues. Hybridisation failure of one of the two chromosomes in a diploid sample and overlapping signals may occur during FISH (Ruangvutilert *et al*, 2000b) and may result in the false scoring of a diploid cell as monosomic.

All 15 blastocysts analysed by FISH were found to be diploid mosaic and all of them consisted of 50% to 90% of diploid cells. Mosaicism in the blastocyst has been described in many studies, in which FISH was used. Similar findings, with no aneuploid and only diploid or diploid mosaic blastocysts from routine IVF patients, have been described after the use of the same set of probes (Ruangvutilert *et* al, 2000a). In a study by Fragouli *et* al, aneuploid and diploid mosaic blastocysts were identified 32.7% of 52 blastocysts analysed and only around 6% of all blastocysts contained more than 50% of diploid cells (Fragouli *et al*, 2011a). This substantial difference in the proportion of diploid cells seen in the present study could be due to various reasons. First, the average maternal age in the study by Fragouli *et al*, was 36, compared to the younger 32.5 in this study and second, nine probes were used compared to only five in this study. Consequently, fewer errors were detected in our cohort of blastocysts due to younger maternal age and possible inability to detect some errors due to the small number of probes. Similar findings in blastocysts were described by Baart *et al*, 2006, where the proportion of mosaic blastocysts was 45% in a cohort of couples of maternal age similar to this study (average age 33.1 years). Again the number of probes was higher than this study, as ten chromosomes were tested. Analysis of blastocysts by Bielanska *et al*, revealed, a proportion of diploid mosaic blastocysts similar to the one described in this study, at 90.9%, when only three chromosomes were tested (Bielanska *et al,* 2002). The average number of

diploid cells within the blastocysts was 78.2%, slightly higher than the number seen in this study, 71.6%, probably due to the two additional probes used here.

4.2.2 Chromosomal analysis by aCGH

aCGH allows the complete enumeration of the chromosomes, as well as the detection of aneuploidies on chromosomes not analysed by FISH, resulting in different chromosomal classification of embryos. The ideal way to examine aneuploidy in embryos is through complete disaggregation and analysis of all individual cells by CGH, which, however, is difficult to perform in advanced embryos (morulae and blastocysts) due to compaction of cells. For this reason disaggregation studies are limited to cleavage stage embryos. Uniformly euploid, mosaic and chaotic embryos have been described after disaggregation and analysis of all cells by mCGH (Voullaire *et al,* 2000, Wells and Delhanty, 2000) and aCGH (Mertzanidou *et al*, 2012). In this study, it was not possible to determine whether the aneuploidies seen by aCGH were meiotic or mitotic in origin, as analysis was performed in whole embryos. Embryos were scored as euploid; aneuploid when one or two chromosomes were affected and as complex aneuploid when three chromosomes or more were affected. This type of scoring has been previously described (Voullaire *et al,* 2007). Embryos carrying abnormalities affecting two chromosomes can lead to a pregnancy, as described in the analysis of spontaneous abortions (Guerneri *et al,* 1987). In this study, aneuploid embryos were divided in the two groups, aneuploid and complex aneuploid, in order to differentiate between those that could lead to implantation and pregnancy and those that would not.

Within the 52 embryos analysed successfully by aCGH, the level of euploid and aneuploid embryos was almost equal (38% and 37% respectively), whereas 25% of embryos were found to be complex aneuploid. Of the 123 errors detected in all embryos, 55 were chromosome losses and 68 were gains. The wide scale studies of embryos using FISH have revealed that monosomy occurs more often than trisomy (Munné *et al*, 2004). However, samples scored as monosomic after FISH may be a result of hybridisation failure. The results in this study support that, as trisomy occurred more often than monosomy.

Studies on blastocysts using either mCGH or aCGH have revealed that errors affecting all chromosomes may lead to aneuploidy. Frequencies of errors within the chromosomes may vary. In this study, chromosome 22 was the most commonly affected with five errors. The rest of the chromosomes showed one or two errors,

apart from chromosomes 2, 5, 6, X and Y that showed no change. This comes in contrast with the findings by Fragouli *et al*, 2010, who reported that the sex chromosomes were most frequently involved in errors (Fragouli *et al*, 2010). Abnormalities affecting chromosome 22 has been seen as the predominant error in some studies (Fragouli *et al*, 2011a), whereas other studies have reported chromosome 16 to show most frequent abnormalities (Rius *et al*, 2011).

4.2.2.1 Segmental chromosomal changes

Eight out of 52 embryos analysed by aCGH (15%), two blastocysts, three morulae and three arrested at the cleavage stage, showed evidence of segmental changes. Seven embryos carried one change and one carried three. Chromosomal material was lost in all, except one. Five embryos had additional aneuploidies. In three embryos, the sole abnormality detected, was the segmental change that involved whole chromosome arms, the termini, or changes within one arm. The smallest change detected was 17Mb, whereas the largest was 82Mb, which involved the loss of the q arm of chromosome 6. Smaller segmental changes were not scored, as they may have been artefacts of the amplification.

Similar to whole chromosome changes, segmental aberration can be derived from errors in meiosis or mitosis (Voet *et al*, 2011) as previously described in studies using comprehensive chromosome analysis to examine cleavage stage embryos (Voullaire *et al,* 2000, Wells and Delhanty, 2000). Different studies have reported various rates of segmental aneuploidies using different array platforms, different cohorts of patients and embryos of different developmental stages. Voullaire *et al* reported an 8% incidence of segmental changes by mCGH in single blastomeres of patients with RIF (Voullaire *et al*, 2002). A higher rate of 30% has been reported elsewhere (Daphnis *et al*, 2008). This was, however, the result of analysing single blastomeres from day three embryos, where segmental aneuploidy as whole chromosome aneuploidy is detected more often. A study of untransferred day five embryos from AMA cases (average age 42.4 years) revealed segmental changes in 31.8% of embryos, a higher percentage to that reported here (Rius *et al,* 2011). a recent study examining only one chromosome in PGD cycles of carriers with structural chromosomal abnormalities showed that chromosome breakage is independent of maternal age (Xanthopoulou *et al*, 2012). Vanneste *et al* reported segmental abnormalities in embryos from patients similar to our cohort (couples undergoing PGD, with no known fertility issues), analysed by SNP arrays. Analysis

of all blastomeres from disaggregated day three or four embryos showed a segmental aneuploidy at a rate of 70% of all embryos analysed (Vanneste *et al,* 2009). The high rate of changes observed could be due to the fact that the resolution of SNP arrays is much higher than that of mCGH or aCGH. In the present study evidence of segmental aneuploidies was seen in 15% of embryos. However, the sample size is small, the patients had a variety of different indications and results were drawn from embryos at different developmental stages, so no comparison with the above-mentioned studies was performed.

Fragile sites are specific loci in the genome that cause chromosome instability and result in chromosome breakage. Over 100 fragile sites of variable frequencies have been identified in the general population. Several of the rare fragile sites are associated with disease, whereas common fragile sites can be detected in all individuals (Durkin and Glover, 2007). A link between the segmental errors observed here and fragile sites can be made as each fragile site occurs at a known chromosome band (Debacker and Kooy, 2007). aCGH can detect the site of chromosome breakage, however, due to low resolution, it might not be the exact corresponding band where the break occurred. Of all the segmental changes observed, those occurring on the q arm of chromosomes 2, 6, 10 and 18 were adjacent or close to a common fragile site. The remaining abnormalities did not correspond to a common or a rare site. In conclusion, the presence of a fragile site can be the explanation of chromosome breakage, resulting in segmental errors.

4.2.3 Parental origin of chromosomal errors

Polymorphic markers used for investigating recombination, as discussed in section 4.3, provided the ability to detect aneuploidy for the chromosomes on which the markers where located, and in some cases the parental origin of the aneuploidy. Since aneuploidy in these samples was also examined with aCGH, possible aneuploidies detected by the markers were confirmed in the aCGH result. In total, seven cases of aneuploidy were detected by both techniques in seven embryos. Of these, five were maternal errors, two were paternal, one incidence of monosomy for chromosome 17 and one trisomy for chromosome 16. The maternally derived aneuploidies included two cases of monosomy for chromosome 16, one monosomy 17, one trisomy 16 and finally one monosomy 19. The overall results agree with the literature, in that maternal errors are more common than paternal.

Information on the parental origin of aneuploidies is usually derived from families of affected liveborns or aborted fetuses. Therefore, only those aneuploidies that can be present in an established pregnancy can be analysed. Trisomy 16, an abnormality that has been examined extensively, among those detected, and has been found to originate in a 100% of cases from errors in maternal meiosis (Hassold *et al,* 1995). Interestingly, in this study, a second case of trisomy 16 that was paternally derived was detected, something which does not agree with findings in established pregnancies. Four of the five cases of monosomy that were detected were maternally derived. Since 45,XO condition is the only type of monosomy not causing early miscarriage; no other data on the origin of autosomal monosomies are available from pregnancies. Similarly, no information is available regarding abnormalities on chromosome 17, which in this study was caused by an error that was paternal in origin.

Analysis of the parental origin of aneuploidy is possible by SNP arrays through the use of informative SNPs and has been presented upon analysis of single blastomeres from cleavage stage embryos (Johnson *et al,* 2010; Rabinowitz *et al*, 2012). In the study by Rabinowitz *et al*, all cases of meiotic trisomies were maternal, whereas paternal and maternal monosomies occurred in similar frequencies. Results were obtained from single blastomeres biopsied from embryos of PGS cycles and healthy egg donors. Johnson *et al*, identified maternal trisomies in significantly higher rate than paternal trisomies and again maternal and paternal monosomies occurred with the same frequency. Analysis was performed on 26 disaggregated embryos. In the present study, the parental origin could only be detected in a small number of embryos, due to lack of informativity and the small number of STR markers analysed. It can be confidently concluded that all trisomies detected were due to errors occurring in meiosis, since two alleles from one parent together with one allele from the other were observed. The stage of error in monosomies, could not be determined with confidence as the loss of one parental chromosome could have occurred in mitosis.

One case of UPD was detected for chromosome 16. Both maternal alleles were detected and no paternal for that chromosome. UPD could not be detected by aCGH as the end result was two chromosomes, similar to a euploid sample with bi-parental inheritance. UPD detection is possible by SNP arrays and was described by Rabinowitz *et al*, in 0.16% of all chromosomes analysed. It was observed that UPD occurred in blastomeres with a large number of aneuploid chromosomes

(Rabinowitz *et al,* 2012). In this case, the embryo presenting UPD was characterised as complex aneuploid and was arrested at the cleavage stage. The chromosomal classification could have been a result of a high number of mitotic errors. If trisomy 16 was present in the embryo, resulting from an error in maternal meiosis, then loss of the paternal chromosome during the mitotic divisions could have resulted in maternal UPD.

4.2.4 Fate of chromosomally abnormal embryos

FISH analysis revealed that only diploid mosaic embryos with a proportion of diploid cells higher than 50% reached the blastocyst stage. Arrested embryos were characterised diploid, haploid, aneuploid, aneuploid mosaic or chaotic. All of the abnormal constitutions could be the aetiology of developmental arrest. It was surprising that all diploid embryos detected by FISH were arrested. Interestingly, when dividing the couples according to age, the only statistical significance was observed in the distribution of diploid embryos, which was higher in the older age group. The most reasonable explanation is that errors in chromosomes not investigated with the probe set used, have led to embryo arrest. This was also observed in another study using the same number of probes (Gonzalez-Merino *et al*, 2003). The vast majority of arrested embryos were either haploid, aneuploid, aneuploid mosaic and chaotic. A very small proportion of arrested embryos were diploid. This confirms the fact that haploid embryos and embryos with extreme mosaicism do not have the ability to develop normally.

Barbash-Hazan *et al* analysed 83 embryos on day three by FISH. All embryos were found to be aneuploid. These were reanalysed on day five, to examine the embryos' ability to self-correct. Of all the aneuploid embryos, 9.7% showed self-correction in 100% of cells and 13.2% had more than 50% of diploid cells. This study also presented a linear correlation between self-correction and developmental potential (Barbash-Hazan *et al*, 2009). The reduction of the mosaicism level prior to blastocyst formation through self-correction or developmental arrest has also been suggested by other groups (Evsikov and Verlinsky 1998, Bielanska *et al*, 2002). It has been suggested than diploid mosaic embryos with low level of aneuploid cells, still have the ability to form a viable fetus (Voullaire *et al*, 2000). All blastocysts in this study were diploid mosaic with more than 50% of diploid cells. There is evidence that apoptosis occurs during preimplantation embryo development, probably in order to eliminate cells with chromosomal abnormalities (Hardy, 1999).

Therefore, normally developing embryos with a high proportion of diploid cells may have the ability to destroy aneuploid cells through apoptosis. Within the embryos analysed by aCGH, the rate of complex aneuploid embryos decreased with development. Within blastocysts, euploid embryos were significantly more than complex aneuploid. Since mosaicism cannot be accurately detected by aCGH it can be assumed that a small proportion of aneuploid cells could have been present in those that were scored as euploid.

Aneuploidy in the preimplantation embryo may result in confined placental mosaicism (CPM). Trisomies affecting the autosomes are the predominant type of aneuploidy detected in the cases of CPM (Lestou and Kalousek, 1998). CPM abnormalities may be a result of meiotic or post zygotic error and if they are confined in extra-embryonic tissue they can result in normal prenatal development and live birth (Lebedev, 2011). On the other hand, embryos with autosomal monosomy most probably cannot achieve implantation, which was concluded after determining an extremely low rate of monosomies, at 0.75% in samples from spontaneous abortions (Guerneri *et al*, 1987). Among the blastocysts analysed by aCGH four were found to be aneuploid. All of them carried a loss of at least one chromosome and therefore transfer of these could have probably led to implantation failure.

4.2.5 Comparison of aneuploidy levels in embryos from PGD and PGS

The results of the aneuploidy analysis in embryos from couples undergoing PGD was directly compared with results from PGS cycles, performed at the CRGH. This was advantageous as, couples treated in the same clinical setting, with similar stimulation protocols, embryo culture and biopsy methods were compared, thus, eliminating any bias that can arise through these factors. Embryos analysed by FISH were compared with already published follow up data from PGS cycles (Mantzouratou *et al*, 2007). Comparison of embryos analysed by aCGH was performed between embryos from PGS after blastocyst biopsy at the CRGH and the blastocysts from the PGD group. These samples were selected for the comparison as no aCGH follow up data were available and results from PGS cycles after cleavage stage biopsy may not be representative of the chromosomal constitution in the remainder of the embryo. Comparison was not performed with the arrested

embryos and the morulae of the PGD group, as they were at different developmental stages.

It was observed that, among embryos analysed by FISH, the distribution of diploid mosaic embryos was significantly higher in the PGD than the PGS group. It is worth noting that, in the PGS group, only one embryo consisted of more than 50% diploid cells, whereas all, apart from two, diploid mosaic embryos in the PGD group carried more than 50% diploid cells. On the contrary, chaotic embryos were significantly more in the PGS group. Evidence has shown that the cases of mitotic non-disjunction are more frequent in embryos from couples of advanced maternal age. It has been suggested that since the embryonic genome is not active during the first mitotic division, and mRNA and proteins are similar to those found in the oocyte, errors that occur frequently in meiosis, like non-disjunction in oocytes of advanced maternal age, will continue to occur in mitosis (Munné *et al*, 2002). This cannot be the case here, though, as the rate of mitotic non-disjunction in embryos of the PGD group was 29%, slightly higher than the 24% rate observed by Mantzouratou *et al*. The number of chromosomes examined by Mantzouratou *et al* was six, whereas five chromosomes were examined in this study. It is possible that the aneuploidy level would have increased if an additional chromosome was examined. The differences of the distribution of diploid mosaic (50% in PGD versus 14% in PGS) and chaotic embryos (9% in PGD versus 59.1% in PGS) were large and would probably still remain similar if one more chromosome was analysed. The presence of significantly more aneuploid and mosaic embryos than euploid in embryos from PGS cycles has been observed before (Rubio *et al*, 2007). Here, the proportion of diploid mosaic embryos, in the PGD group, was higher than the sum of aneuploid, aneuploid mosaic, haploid and chaotic embryos and very close to reaching significance ($p=0.0510$, Fisher's exact test).

When comparing embryos analysed by aCGH, no significant differences were observed. However, the proportion of euploid embryos was higher in the PGD group, whereas the number of complex aneuploid embryos was higher in the PGS group. The average maternal age in the PGS groups was 38.5 years and in the PGD groups 32 years. The proportion of aneuploid embryos, analysed by FISH and aCGH, was higher in the PGS groups than in the PGD groups, confirming the well-accepted fact that aneuploidy increases with maternal age.

4.2.6 Would diagnosis of a disease-causing mutation together with aneuploidy screening be beneficial?

All of the above findings suggest that aneuploidy is present in embryos of couples undergoing PGD for monogenic disorders. However, the type of errors may not be as severe as those seen in embryos from PGS couples. Distinct differences, made apparent after FISH analysis, include fewer incidences of meiotic errors in embryos from PGD cycles. Embryos that seem to develop normally contain a high proportion of diploid cells, suggesting that low-level aneuploidy may not affect implantation or a healthy pregnancy. According to the latest ESHRE data collection of the PGD consortium, the pregnancy rate in PGD cycles for single gene disorder per embryo transfer is 29% (Goossens *et al,* 2012). This can be attributed to chromosomal errors that remain undetected, as chromosomal screening is not the primary purpose of PGD. Therefore, genetic analysis of the disease, combined with chromosomal analysis will aid to the selection of embryos free of the mutation as well as euploid.

Direct PCR on biopsied samples, using primers on chromosomes 13, 16, 18, 21, 22 and X, together with primers to detect a genetic disorder has already been described. Studies report a high pregnancy rate of 54%, even in patients of advanced maternal age (Rechitsky *et al*, 2006, Verlinsky *et al*, 2006). With recent technological advances, it is now possible to perform mutation detection and aneuploidy analysis more effectively. Following WGA, the same product can be used for chromosomal analysis by aCGH, as well as analysis of the mutation by PCR amplification of suitable loci. Moreover, this can be performed simultaneously with the use of SNP arrays. However, there are many issues that need to be taken under consideration.

As in PGS, the timing of biopsy is critical. Polar body biopsy provides the ability to detect chromosomal errors and genetic mutations that are present in the oocyte. In certain countries embryo biopsy is prohibited by law and therefore polar body biopsy is the only option. Analysis of the first polar body has been described for autosomal recessive and X-linked dominant disorders (Griesinger *et al*, 2009). In cases of the diagnosis of autosomal recessive diseases following embryo biopsy, 75% of the embryos will be normal or carriers and therefore suitable for transfer. However, if polar body biopsy is performed and only the maternal genome is analysed, 50% of embryos will theoretically be available for transfer, those that are normal and those

that are carriers of the paternal mutation. Moreover, polar body biopsy cannot be performed for cases where the male partner carries an autosomal dominant mutation. Cleavage stage biopsy, which is the most common stage of biopsy in PGD, is hampered by mosaicism. Blastocyst biopsy might be the ideal choice, but mosaicism can still cause a misinterpretation of both the chromosomal and genetic status of the embryo.

Another point to consider is that the number of embryos available for transfer will be markedly reduced. As seen by the FISH results, the vast majority, 82% of embryos analysed, had at least one aneuploid cell. This is most important in cases of autosomal dominant disorders, where theoretically only 50% of embryos will be free of the mutation. Reduction in the number of embryos available for transfer was observed after simultaneous diagnosis of structural chromosomal rearrangements and numerical abnormalities by aCGH (Alfarawati *et al*, 2011). Of the embryos analysed, after biopsy at different developmental stages, 22.3% were balanced for a translocation as well as euploid and therefore available for transfer. However, a further 28.9% were balanced for a translocation but carried aneuploidies and were not transferred. In a different study, analysis of chromosomes on single blastomeres from cleavage stage embryos in cases of structural chromosomal rearrangements showed that 16% of embryos were normal or balanced for every chromosome, whereas 27.3% were normal or balanced for the chromosomal rearrangement but carried aneuploidies in other chromosomes (Fiorentino *et al*, 2011). The pregnancy rate in Alfarawati *et al* did not increase when compared to cases when FISH was used for the detection of the rearrangements. However, the authors noted that the risk of miscarriage was reduced. Fiorentino *et al* reported a high pregnancy rate, at 70.6% per embryo transfer, which was significantly higher than that reported by the PGD consortium on cases where FISH was used.

The recent RCT on young couples using aCGH on trophectoderm biopsied samples, described in section 4.1.8 of the Discussion, showed significantly higher pregnancy rates in the cycles with chromosomal screening than those without screening. The availability of a test that screens for chromosome aneuploidies, together with the genetic diagnosis of a disease, could be potentially offered to all PGD patients. As in PGS, positive outcome can only be determined by RCTs. However, the additional limitation in embryo transfer imposed by the presence of a mutation needs to be considered.

4.3 Investigation of recombination in preimplantation embryos

The third aim of this thesis was the investigation of recombination in preimplantation embryos. This was performed as a pilot study. The chromosomal status of all embryos was also determined by aCGH, in order to detect any links between recombination and aneuploidy. Until very recently no other studies on embryo recombination had been published. Taylan and Altiok presented a study on recombination within the major histocompatibility complex (MHC) on preimplantation embryos (Taylan and Altiok, 2012). As recombination is essential for proper segregation of the chromosomes in meiosis, it was hypothesized that recombination in embryos showing developmental arrest or aneuploid embryos could be different to developing, euploid embryos. Recombination was investigated in a different way compared to the majority of published studies. Instead of analysing recombination frequency using a large number of markers across the genome and across generations, in this study, recombination was analysed using few markers within generations in families with a large number of embryos. The embryos analysed here may not reflect the general population. The vast majority of them would not have resulted in a live birth. Therefore, investigating recombination in this set of samples could provide an important insight in the differences between the general population and embryos that arrest in development.

4.3.1 FPCR amplification on WGA products

WGA on single cells is possible, however, the efficiency of the technique is compromised by the small DNA amount of the starting material resulting in high ADO rates. The quality of the product is also dependent on the type of the cell and the type of lysis used (Glentis *et al*, 2009). SurePlex was selected for WGA in these samples. The main reason for this choice was the need to perform both haplotype and aneuploidy analysis in the same sample. The efficiency of the amplification was determined in the first part of this thesis through the validation of aCGH. Amplification efficiencies of 95% on single cells and 100% in samples containing eight and 100 cells were observed. All the samples, analysed here, were either whole embryos at different developmental stages, or TE biopsied samples. As expected, all samples produced a smear on a 2% agarose gel, representing successful amplification.

In total 14 polymorphic marker loci were amplified by fluorescence PCR (FPCR) on the 77 embryonic samples, corresponding to 1078 loci. The rates of amplification failure and allele drop out (ADO) observed, were 2.9% and 5.4% respectively. Overall, the ADO rate reported here is considerably lower than that reported in other studies, where single cells were analysed (Spits *et al*, 2006; Renwick *et al*, 2007), due to the fact that the number of cells in the majority of the embryos was over 10, therefore, reducing the possibility of ADO.

4.3.2 Ability to detect recombination in embryos

Although STR markers are more informative than SNPs, the lack of multiple STRs close to the selected regions limited the detection of recombination in embryos. Moreover, marker informativity was essential in order to identify recombination. At the sites where two markers were tested, in chromosomes 1, 5 and 16 apart from heterozygosity, difference in the size of the parental alleles was also essential. In chromosomes 17 and 19, where five and three markers were tested respectively, cross-over events could be detected if the individual was heterozygote for at least two of three markers, on chromosome 19, and two or three markers on chromosome 17. However, difference in the size of the marker alleles between the parents was still essential. Five loci were analysed for 20 parents, a total of 100 possible informative meioses. It was not possible to detect recombination in 48% of all loci analysed, in all parents, due to lack of informativity. Therefore, only parents that were informative for recombination detection markers were considered to have informative meioses. In chromosomes 1, 5 and 16, where two markers were tested, detection of single recombination events was possible, whereas in chromosomes 17 and 19, where more markers were investigated, double recombination events could have been detected and these were considered as two separate events.

The recombination frequency for females, males, as well as the total frequency for each family and chromosome was calculated, considering the number of events and the number of informative meioses. This introduced bias in the analysis, as limited informative meioses that showed recombination, could have resulted in a high recombination frequency, possibly not representative of the true recombination frequency. However, as informativity was a matter of equal chance between the individuals, comparisons between frequencies were still performed in an attempt to retrieve information.

4.3.3 Recombination frequency and families, chromosomes, sex and age

Differences in the total recombination frequency between families were observed. The range of the total frequency was zero (family C8) to 0.20 (family C1). The only family with no recombination detected consisted of just three embryos. All the families in the PGS group showed lower recombination frequencies than the PGD group. The total recombination frequency for each family was calculated considering both female and male recombination. It is well known that recombination varies between individuals (Cheung *et al*, 2007) and this was confirmed here.

Several observations could be made, when looking at the total recombination frequency of each chromosome. Despite being a recombination hotspot, no recombination was detected on chromosome 5 for any of the families and no recombination was seen on chromosome 16 in the PGS group. Another contradictory result to population analysis was identified for the locus on chromosome 17, which was a recombination silent spot, as recombination events were detected in both groups. All markers on that locus were located within the *NF1* gene. Population studies have shown that recombination occurs preferentially outside genes (McVean *et al* 2004). The recombination frequency of all families in both groups was found to increase as the length of the chromosome decreased. Again, this is a well-established feature of recombination (Kong *et al,* 2002). Finally, the highest rate was observed on chromosome 19, which is in line with previous studies (Dib *et al*, 1996).

Differences were observed between males and females. It has been shown that a sequence variation within the *RNF212* gene is a source of differences in the recombination frequency between the sexes. The haplotypes of two SNPs within the gene are associated with low recombination frequency in females and the highest in males (Kong *et al*, 2008). Overall, recombination frequency was higher in females than males. The recombination frequency around the telomeres of the chromosomes is higher in males, whereas in females, higher recombination is observed close to the centromeres in the general population (Broman *et al*, 2002). In this study, the most telomeric set of markers was on chromosome 19. Indeed the male recombination frequency of all families was higher than female only for that chromosome. Variation among individuals, as well as between the sexes, was also observed in the study Taylan and Altiok. Recombination was detected on single

blastomeres from embryos of PGD cases for HLA (human leukocyte antigen) compatibility (Taylan and Altion, 2012). Higher recombination was observed in maternal chromosomes than paternal, the same finding with this study.

When families were divided in groups according to age it was observed that the recombination frequency decreased for both sexes over the age of 35. The decrease was bigger in males than females. The only statistical significance was observed between the average recombination frequency in women and men over 35 years and it was higher in women. The effect of age in recombination is not clear. The ”production line” hypothesis, in maternal gametogenesis of mice, states that there is a higher degree of recombination in the oocytes formed first and that oocytes are ovulated in the same order as they were formed (Henderson and Edwards 1968). This would mean that oocytes from young women would have higher recombination than oocytes from older women. This is in line with what observed in this study. However, a large study on 70000 individuals showed that recombination frequency increased with maternal age (Kong *et al*, 2004). The authors hypothesized that, through a selection process, there is a higher chance of an oocyte with more recombination events to lead to a normal pregnancy. The study by Kong *et al*, was performed in already established families. The recombination frequency increased with maternal age because these women had a high recombination count. This contradicts the findings of this study and emphasises the fact that there are differences in preimplantation embryos that in the vast majority will not lead to a successful pregnancy and delivery, compared to what happens in the general population.

4.3.4 Recombination frequency in embryos. Effects on chromosomal status and morphology

Recombination frequency was examined within the families and also from the embryo perspective, in order to detect links between recombination with aneuploidy and development. A recombination frequency was calculated for those embryos in which cross-over events were detected. This was achieved by considering the number of loci tested and the number of events seen. Embryo recombination frequency was determined as the number of recombination events detected in each embryo over the number of informative meioses per embryo, which was maximum five, equal to the number of loci investigated. The chromosomal status was examined by aCGH and embryos were classified as described in section 4.2.2.

No significant differences were noted in the average recombination frequency of euploid, aneuploid and complex aneuploid embryos and of embryos at different developmental stages. A higher rate was observed in arrested embryos, followed by the rate of blastocysts and morulae, but of no significance. Chromosomal and developmental status was also compared between those embryos that showed recombination and those in which recombination was not detected at all. Recombination was observed in 30 of the 77 embryos analysed (39%), whereas no recombination events were observed in 47 embryos. Embryos with no recombination were also observed in the study by Taylan and Altiok. Again no statistical significant differences were observed, however the majority of euploid embryos, showed recombination, whereas the majority of aneuploid and complex aneuploid embryos did not. When comparing the two groups of embryos according to morphology, it was observed that embryos that showed recombination were more among the arrested and blastocyst groups, whereas the proportion of morulae that did not show recombination was higher than those that did. The recombination machinery is closely linked to the correct segregation of the chromosomes during meiosis. No recombination was detected in the majority of aneuploid and complex aneuploid embryos, indicating that in those embryos that possibly carried meiotic errors, errors in recombination were also present.

4.3.5 Analysis of recombination in embryos

Cross-over events were detected through the use of polymorphic markers, however this was limited by marker uninformativity and several events might have been missed. Moreover, the use of polymorphic markers was only possible when the correct haplotypes of parents and embryos were generated. This can only be performed with confidence when a large number of progeny exists. A newly developed technique, direct determining phasing (DDP) allows molecular haplotyping of the whole genome from single cells (Fan *et al*, 2011). Chromosomes are released from a metaphase cell and amplified by MDA. Direct amplification of each homologous chromosome does not require the need of parental genomes in order to prepare the haplotypes. Recombination events can even be detected with only one offspring. The ability to perform this type of technology on a single cell is fascinating, especially for the world of preimplantation genetics. Its main drawback, however, is the fact that metaphase cells are needed as a starting material, which are not easily obtained from embryos.

Double recombination events were observed in five embryos. Four of these events occurred on the chromosome 19 locus and one on chromosome 17. Two of the events on chromosome 19 were observed in the same family (C1), which also showed the highest recombination frequency among all the families investigated. While the chance of a double recombination event occurring is much less likely than a single event, without other families from previous generations, the correct phasing of alleles could not be confirmed. This was a limitation of the present study and the rational determining the allelic phase from the lowest recombination frequency for any family was used because the largest genetic distance between any of the markers was only 3.08cM. Double cross-over in preimplantation embryos, within a region of 3.81cM, has been reported elsewhere during PGD for HLA typing (Fiorentino *et al*, 2005).

No significant differences in recombination frequency were observed between the embryos of the PGD and PGS groups. This may be due to the limitations of this study imposed by the use of microsatellite markers and the small number of embryos analysed. The most suitable technique to identify recombination in embryos, that is now becoming available, is SNP arrays. Genotyping of parents and embryos with SNP arrays and subsequent identification of recombination as well as chromosomal abnormalities has been described through the use of "Karyomapping" (Handyside *et* al, 2010). This would provide a far more detailed insight on recombination. However, other investigations may unveil differences in these groups. It has been suggested that variation in the PRDM9 protein, that controls recombination hotspots, may be present in women with recurrent miscarriages, as well as infertility and therefore produce a high rate of aneuploid embryos (Cheung *et al*, 2010). Analysis of the protein and its coding gene, *PRDM9*, in these women and fertile women could confirm this assumption.

4.4 Conclusion

The main topic of this thesis was aneuploidy in preimplantation embryos. Detection of aneuploidy by aCGH, the level of aneuploidy in embryos from PGD cycles, as well as recombination in preimplantation embryos were investigated. Validation of aCGH using the 24sure platform was described. Blind analysis of single cells from epithelial cell lines revealed 100% concordance between the aCGH result and the chromosomal status of the cells. This indicated that 24sure analysis, could confidently detect aneuploidies in single blastomeres biopsied from cleavage stage embryos. Validation of 24sure following blastocyst biopsy was performed by

examining the effect of mosaicism on the aCGH result. Mosaicism could be detected by aCGH and the result could also indicate the number of aneuploid cells present in the sample. Concordant results between biopsied TE samples and the remainder of the blastocysts further validated aCGH for PGS following blastocyst biopsy. However, aCGH may be hampered by biological limitation, as observed by FISH follow-up on PGS cases where aCGH was used. The aCGH result did not always represent the chromosomal status of the embryo, especially following biopsy at cleavage stage. Several RCTs are underway in order to determine the clinical efficacy of aCGH in PGS.

Very few studies on the aneuploidy level of embryos from couples undergoing PGD for single gene disorders have been published. FISH and aCGH were used to determine that level. Diploid mosaic embryos were the predominant type of embryos, whereas chaotic and haploid embryos were the least common, among embryos analysed by FISH. aCGH revealed that the distribution of euploid and embryos with aneuploidies affecting one or two chromosomes was almost equal whereas embryos with three or more aneuploidies were the minority. Embryo aneuploidy was found to be reduced in more advanced developmental stages. FISH analysis of blastocysts revealed that they were all diploid mosaic with more than 50% of cells being diploid, whereas aCGH showed that the majority of blastocysts were euploid. Important differences between the two techniques were highlighted. The stage of chromosomal error could be determined by FISH, with mitotic errors being more common than meiotic. This was not possible by aCGH, however with this technique it was revealed that all chromosomes were affected by an error, whereas only five chromosomes were examined by FISH. Analysis of the parental origin of aneuploidies in embryos analysed by aCGH revealed that maternal errors are more common, however a paternal meiotic error not in agreement with studies on established pregnancies was also identified. Aneuploidy occurred in a lower frequency in embryos from PGD cycles when compared to data from PGS cycles.

Recombination was investigated in embryos of known chromosomal status, from PGD and PGS cycles, with the use of STR markers on loci of high and low recombination. Findings that were in line with data from population studies were observed. These included, individual and chromosome variability in recombination frequency, a higher recombination frequency in females than in males, higher recombination in telomeric regions in males than females and the fact that the highest recombination frequency was detected on chromosome 19. However, no

recombination was detected on the recombination hot spot on chromosome 5 and recombination was detected on the recombination silent spot on chromosome 17, which was not in agreement with population studies. Recombination was detected in the majority of euploid embryos, whereas no recombination was seen in the majority of aneuploid and complex aneuploid embryos. This indicated that mechanisms leading to meiotic recombination may relate to mechanisms that lead to meiotic aneuploidy.

All the three aims were covered using different techniques. FISH and aCGH were used for the detection of aneuploidy and recombination was investigated by PCR analysis. SNP arrays, an emerging technique in preimplantation genetics, could have covered all the technical needs of this thesis. Chromosomal copy number and genotyping analysis through haplotyping, which are both possible with SNP arrays, could have provided data on aneuploidy and recombination respectively. As with aCGH, the clinical effectiveness of SNP arrays in screening for chromosomal aneuploidy in preimplantation embryos still needs to be confirmed by RCTs. However, their use in a research setting is already proving highly valuable as a lot of information can be acquired through one experiment. Study of recombination in the whole genome of embryos, with the high-resolution analysis that SNP arrays provide, will examine whether the findings presented here by the pilot study are indeed representative of the recombination status in human embryos.

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6 Appendix A

Table 6.1: Appendix A - Embryos analysed for chromosomes 13, 18, 21, X and Y by FISH						
Embryo	**No of pronuclei**	**Morphology**	**Number of nuclei 1st/2nd round**	**Number of diploid cells (%)**		**Embryo classification**
				13/21	**X/Y/18**	
B1.1.1	2	Arrested	3/3	0	0	Chaotic
B2.1.1	2	Morula	26/24	23 (88%)	17 (71%)	Diploid/aneuploid/chaotic mosaic
B2.1.2	2	Arrested	2/2	0	0	Aneuploid/chaotic mosaic
B2.1.3	2	Blastocyst	93/98	78 (84%)	86 (88%)	Diploid/chaotic mosaic
B2.1.4	2	Morula	65/50	46 (71%)	33 (66%)	Diploid/chaotic mosaic
B2.1.5	2	Morula	44/44	37 (84%)	38 (86%)	Diploid/chaotic mosaic
B2.2.1	2	Blastocyst	38/38	31 (82%)	26 (68%)	Diploid/chaotic mosaic
B2.2.2	2	Morula	9/11	0	0	Aneuploid/chaotic mosaic
B2.2.3	2	Morula	16/16	13 (81%)	14 (88%)	Diploid/chaotic mosaic
B2.2.4	2	Morula	6/6	3 (50%)	3 (50%)	Diploid/chaotic mosaic
B3.1.1	2	Arrested	4/4	0	1 (25%)	Aneuploid/chaotic mosaic
B3.1.2	2	Arrested	No signals	-	-	No result
B3.1.3	0	Arrested	No signals	-	-	No result
B3.1.4	0	Arrested	4/4	0	0	Aneuploid/chaotic mosaic
B4.1.1	0	Arrested	1/3	0	1 (33%)	Chaotic
B4.1.2	0	Arrested	2/2	0	0	Haploid
B5.1.1	3	Arrested	4/4	4 (100%)	4 (100%)	Diploid
B6.1.1	2	Arrested	1/3	0	1 (33%)	Chaotic
B6.1.2	2	Arrested	6/2	0	0	Haploid
B6.1.3	2	Arrested	6/3	0	0	Haploid
B7.1.1	3	Morula	No signals	-	-	No result
B7.2.1	2	Morula	13/14	9 (69%)	10 (71%)	Diploid/chaotic mosaic
B7.2.2	2	Morula	17/9	9 (53%)	9 (100%)	Diploid/chaotic mosaic
B7.3.1	2	Arrested	2/2	2 (100%)	2 (100%	Diploid
B7.3.2	2	Blastocyst	27/24	25 (93%)	21 (88%)	Diploid/chaotic mosaic

Table 6.1: Appendix A (cont.) - Embryos analysed for chromosomes 13, 18, 21, X and Y by FISH						
Embryo	**No of pronuclei**	**Morphology**	**Number of nuclei 1st/2nd round**	**Number of diploid cells (%)**		**Embryo classification**
				13/21	**X/Y/18**	
B8.1.1	2	Blastocyst	42/42	33 (79%)	28 (67%)	Diploid/aneuploid/chaotic mosaic
B8.1.2	2	Arrested	3/3	0	0	Aneuploid/chaotic mosaic
B8.1.3	2	Arrested	No signals	-	-	No result
B8.1.4	2	Arrested	2/2	2 (100%)	2 (100%)	Diploid
B8.1.5	2	Arrested	No signals	-	-	No result
B8.1.6	0	Arrested	1/3	0	0	Chaotic
B9.1.1	2	Morula	12/11	2 (17%)	6 (55%)	Diploid/chaotic mosaic*
B9.2.1	2	Arrested	4/4	0	2 (50%)	Aneuploid/chaotic mosaic
B9.2.2	2	Arrested	2/1	0	1 (100%)	Aneuploid
B10.1.1	2	Morula	No signals	-	-	No result
B10.1.2	2	Blastocyst	No signals	-	-	No result
B10.1.3	2	Morula	No signals	-	-	No result
B10.1.4	2	Morula	15/15	8 (53%)	12 (80%)	Aneuploid/chaotic mosaic
B10.1.5	0	Arrested	No signals	-	-	No result
B11.1.1	2	Arrested	7/7	1 (14%)	3 (43%)	Chaotic
B11.1.2	2	Morula	3/3	0	2 (67%)	Aneuploid
B11.1.3	2	Blastocyst	24/17	22 (92%)	10 (59%)	Diploid/aneuploid/chaotic mosaic
B11.1.4	2	Blastocyst	5/5	3 (60%)	3 (60%)	Diploid/chaotic mosaic
B11.1.5	2	Arrested	2/1	2 (100%)	1 (100%)	Diploid
B11.1.6	2	Blastocyst	21/10	12 (57%)	9 (90%)	Diploid/chaotic mosaic
B12.1.1	2	Morula	2/2	2 (100%)	2 (100%)	Diploid
B13.1.1	2	Morula	No signals	-	-	No result
B13.1.2	2	Arrested	No signals	-	-	No result
B13.1.3	2	Blastocyst	No signals	-	-	No result
B13.1.4	2	Arrested	No signals	-	-	No result

Table 6.1: Appendix A (cont.) - Embryos analysed for chromosomes 13, 18, 21, X and Y by FISH						
Embryo	**No of pronuclei**	**Morphology**	**Number of nuclei $1^{st}/2^{nd}$ round**	**Number of diploid cells (%)**		**Embryo classification**
				13/21	**X/Y/18**	
B13.1.5	2	Blastocyst	39/36	26 (67%)	24 (67%)	Diploid/chaotic mosaic
B13.1.6	2	Blastocyst	No signals	-	-	No result
B13.2.1	2	Morula	17/17	12 (71%)	10 (59%)	Diploid/chaotic mosaic
B13.2.2	2	Blastocyst	65/39	41 (63%)	19 (49%)	Diploid/aneuploid/chaotic mosaic
B13.2.3	2	Morula	39/38	32 (82%)	31 (82%)	Diploid/chaotic mosaic
B13.2.4	0	Morula	33/34	28 (85%)	27 (79%)	Diploid/chaotic mosaic
B13.2.5	0	Blastocyst	37/35	32 (86%)	32 (91%)	Diploid/chaotic mosaic
B14.1.1	2	Morula	11/11	7 (64%)	8 (73%)	Diploid/chaotic mosaic
B14.1.2	2	Arrested	2/2	2 (100%)	2 (100%)	Diploid
B14.1.3	2	Morula	9/9	6 (67%)	8 (89%)	Diploid/chaotic mosaic
B14.1.4	2	Arrested	3/3	0	3	Aneuploid
B15.1.1	2	Arrested	2/2	0	0	Chaotic
B15.1.2	2	Arrested	No signals	-	-	No result
B15.1.3	2	Arrested	No signals	-	-	No result
B16.1.1	2	Blastocyst	86/73	79 (92%)	56 (77%)	Diploid/aneuploid/chaotic mosaic

Table 6.1: Appendix A (cont.) - Embryos analysed for chromosomes 13, 18, 21, X and Y by FISH						
Embryo	**No of pronuclei**	**Morphology**	**Number of nuclei 1st/2nd round**	**Number of diploid cells (%)**		**Embryo classification**
				13/21	**X/Y/18**	
B17.1.1	2	Morula	12/12	12 (100%)	11 (92%)	Diploid
B17.1.2	2	Morula	8/7	8 (100%)	7 (100%)	Diploid
B17.1.3	2	Morula	5/5	5 (100%)	5 (100%)	Diploid
B18.1.1	2	Morula	20/18	12 (60%)	15 (83%)	Diploid/chaotic mosaic
B18.1.2	2	Arrested	10/10	1 (90%)	9 (91%)	Aneuploid
B18.1.3	2	Arrested	5/5	5 (100%)	5 (100%)	Diploid
B18.1.4	2	Blastocyst	38/35	24 (63%)	26 (74%)	Diploid/chaotic mosaic
B18.1.5	2	Arrested	17/15	15 (88%)	14 (93%)	Diploid
B18.1.6	2	Morula	15/15	10 (67%)	13 (87%)	Diploid/chaotic mosaic
B18.1.7	2	Blastocyst	53/51	43 (81%)	45 (88%)	Diploid/chaotic mosaic
B18.1.8	2	Morula	28/26	25 (89%)	20 (77%)	Diploid/chaotic mosaic
B18.1.9	2	Arrested	No signals	-	-	No result
B19.1.1	2	Morula	18/18	12 (67%)	16 (89%)	Diploid/aneuploid/chaotic mosaic
B19.1.2	2	Morula	10/9	0	0	Aneuploid/chaotic mosaic
B19.1.3	0	Blastocyst	6/6	4 (67%)	7 (78%)	Diploid/chaotic mosaic
B19.1.4	3	Morula	No signals	-	-	No result
B19.1.5	2	Morula	12/12	4 (33%)	5 (42%)	Diploid/chaotic mosaic*
B19.1.6	2	Morula	14/13	13 (93%)	13 (100%)	Diploid
B19.1.7	2	Arrested	No signals	-	-	No result
B19.1.8	2	Arrested	No signals	-	-	No result
B19.1.9	3	Morula	12/11	8 (67%)	10 (91%)	Diploid/chaotic mosaic

Table 6.1: Appendix A – Detailed results of embryos analysed by FISH to detect aneuploidy. *Embryo IDs are given as the couple number, followed by the cycle number and the embryo number in each cycle. The numbers are separated by a dot. Probes for chromosomes 13, 18, 21, X and Y were used in two consecutive FISH rounds. The number of pronuclei and developmental stage of each embryo are indicated. Also the number of analysable nuclei in each round and the number of diploid cells detected in each FISH round are shown. The chromosomal classification of each embryo is presented in the last column. *Diploid/chaotic mosaic embryos with <50% of diploid cells.*

7 Appendix B

Table 7.1: Appendix B - Embryos from PGD cycles analysed by aCGH				
Embryo	**No of pronuclei**	**Morphology**	**Classification**	**Chromosomal complement**
B20.1.1	2	Blastocyst	Euploid	46,XY
B20.1.2	2	Morula	Aneuploid	45,XX,-1
B20.1.3	2	Blastocyst	Complex aneuploid	60,XY,+3,+7,+8,+9,-10q22.3-qter +11,+12,+13,+15,+17,+18,+19,+20, +21,+22
B20.1.4	2	Blastocyst	Aneuploid	44,XX,-7,-22
B20.1.5	2	Arrested	No result	-
B20.1.6	2	Arrested	Aneuploid	45,XX,-12
B20.1.7	2	Blastocyst	Euploid	46,XX
B20.1.8	0	Arrested	Aneuploid	47,XX,+22
B21.1.1	2	Morula	Aneuploid	46,XY,-6q14.1-qter
B21.1.2	2	Morula	Aneuploid	46,XY,-8pter-p22
B21.1.3	2	Morula	Aneuploid	45,XX,-4
B21.1.4	2	Morula	Euploid	46,XX
B21.1.5	2	Morula	Euploid	46,XX
B21.1.6	2	Arrested	Complex aneuploid	41,XX,-2,-3,+5q23.1-qter,-6q14.3-qter,+7,+9,-11,-12pter-12q12.2,+13,-16,-17,-18,-19,-20
B21.1.7	0	Morula	Euploid	46,XX
B21.1.8	0	Morula	Aneuploid	47,+4
B22.1.1	2	Blastocyst	Euploid	46,XY
B22.1.2	2	Morula	Euploid	46,XX
B22.1.3	2	Morula	Complex aneuploid	53,XY,+3,+4,+9,+11,+15,+16,+18
B22.2.1	2	Blastocyst	Euploid	46,XX
B22.2.2	2	Morula	Aneuploid	47,XX,+20
B22.2.3	2	Morula	Euploid	46,XX
B22.3.1	2	Arrested	Complex aneuploid	43,XX,-2,-12,-18
B22.3.2	2	Blastocyst	Euploid	46,XY
B22.3.3	2	Arrested	Complex aneuploid	45,XX,-13,+16,+17,-18q11-q13.2,-19
B22.3.4	2	Blastocyst	Aneuploid	45,XX,-18
B22.3.5	2	Morula	Aneuploid	46,-5q33.3-qter
B23.1.1	2	Morula	Aneuploid	45,XY,-2
B23.1.2	2	Arrested	Complex aneuploid	50,XX,+5,+8,-9,+13,+14,-15,-17,+18,+19,+21
B24.1.1	2	Blastocyst	Aneuploid	45,XX,-22
B24.1.2	2	Blastocyst	Euploid	46,XY
B25.1.1	2	Arrested	Aneuploid	47,XXY
B25.1.2	2	Blastocyst	Euploid	46,XY
B25.1.3	2	Arrested	Euploid	46,XX
B25.1.4	2	Blastocyst	Euploid	46,XY
B25.1.5	2	Arrested	Complex aneuploid	43,XY,-6,-10p15.2-q11.21,-13,-16,+18,-19
B25.1.6	2	Blastocyst	Euploid	46,XY
B25.1.7	2	Morula	Complex aneuploid	48,XXY,-6,+7,-10,+11,+15
B25.1.8	2	Arrested	Complex aneuploid	42,XX,-1,+5,-7,-9,+10,-12,-19,-21
B25.1.9	2	Blastocyst	Complex aneuploid	44,XY,-1,-2q,-4,-9,+10,-11,-13,+14, -15,+16,+17,+19,+20,-21,-22
B25.1.10	1	Blastocyst	Aneuploid	46,XX,+4,-16

Table 7.1: Appendix B (cont.) - Embryos from PGD cycles analysed by aCGH				
Embryo	**No of pronuclei**	**Morphology**	**Classification**	**Chromosomal complement**
B25.2.1	2	Arrested	Complex aneuploid	44,XX,+1,-2,+6,-7,-8,+14,-15,+17,+18,-21,-22
B25.2.2	2	Arrested	Aneuploid	47,XX,+17
B25.2.3	2	Morula	Aneuploid	45,XX,-22
B25.2.4	2	Arrested	Euploid	46,XY
B25.2.5	2	Morula	Aneuploid	47,XX,+16
B25.2.6	2	Morula	Euploid	46,XX
B25.2.7	2	Morula	Complex aneuploid	54,XY,+5,+7,+14,+16,+17,+18,+19,+21
B25.2.8	2	Arrested	Complex aneuploid	43,XY,+2,-4,-20,-21
B25.2.9	2	Arrested	Euploid	46,XX
B25.2.10	1	Morula	Aneuploid	47,XX,+16
B25.2.11	1	Arrested	Euploid	46,XX
B25.2.11	3	Arrested	Euploid	46,XY

Table 7.2: Appendix B (cont.) - Embryos from PGS cycles analysed by aCGH			
Embryo	**No of pronuclei**	**Classification**	**Chromosomal complement**
B26.1.1	2	Aneuploid	45,XY,-14
B26.2.1	2	Euploid	46,XY
B26.2.2	2	Aneuploid	45,XY,-17
B26.2.3	2	Euploid	46,XY
B26.2.4	2	Aneuploid	47,XX,+22
B27.1.1	2	Aneuploid	47,XX,+16
B28.1.1	2	Aneuploid	47,XX,-10
B28.1.2	2	Complex aneuploid	50,XY,+16,+17,+21,+22
B28.1.3	2	Aneuploid	49,XY,+4,+5
B29.1.1	2	Euploid	46,XY
B30.1.1	2	Aneuploid	48,XX,+4,+5
B30.1.2	2	Complex aneuploid	43,XO,-11,-19,-22
B31.1.1	2	Aneuploid	44,XY,-13,-22
B31.2.1	2	Aneuploid	45,XY,-13
B31.3.1	2	Complex aneuploid	47,XY,+9,+15,-18
B31.3.2	2	Complex aneuploid	46,XXY,-4,+15,-16
B31.3.3	2	Complex aneuploid	47,XX,+7,-20,+21
B32.1.1	2	Aneuploid	44,XY,-13,-21
B32.1.2	2	Aneuploid	45,XY,-18
B32.1.3	2	Aneuploid	45,XX,-22
B32.1.4	2	Euploid	46,XY
B32.1.5	2	Euploid	46,XX
B32.1.6	2	Aneuploid	46,XX,-18,+19
B32.1.7	2	Aneuploid	47,XX,+9
B33.1.1	2	Euploid	46,XX
B33.1.2	2	Aneuploid	47,XX,+2
B33.1.3	2	Euploid	46,XY
B34.1.1	2	Complex aneuploid	47,XY,+1,-7,+8,+19
B34.1.2	2	Complex aneuploid	45,XY,-8,+13,-16
B34.1.3	2	Complex aneuploid	47,XY,+16,-18,+22
B34.1.4	2	Aneuploid	46,XX,-4q32.2-qter
B35.1.1	2	Euploid	46,XX
B35.1.2	2	Euploid	46,XX
B35.1.3	2	Aneuploid	45,XY,-9
B35.1.4	2	Euploid	46,XX
B35.1.5	2	Aneuploid	48,XX,+7,+20
B35.1.6	2	Aneuploid	45,XY,-19

Tables 7.1 and 7.2: Appendix B – Details of embryos from PGD and PGS cycles analysed by aCGH. *Embryo IDs are given as the couple number, followed by the cycle number and the embryo number in each cycle. Each number is separated by a dot. The number of pronuclei, as scored by the embryologists, embryo morphology (for embryos from PGD cycles), the chromosomal classification and complement of each embryo are provided. All embryos from PGS cycles were blastocysts part of the clinical PGS program, where TE biopsy was used. The aCGH result is the clinical result for each embryo.*

8 Appendix C

Table 8.1: Appendix C - Polymorphic marker results of all parents and embryos analysed for recombination. *The results for all the loci for each parent and embryo are shown. Also information on the informativity of the parents is provided. In heterozygote samples each allele is separated with a "/" and in the embryos the maternal allele is given first followed by the parental.*

Key*:*

Yellow: Loci where recombination was detected

Orange: Loci where double recombination was detected

Pink: Loci that showed evidence of possible aneuploidies

Green: Heterozygote samples that showed definite allele drop out (ADO)

Purple: Loci that had either recombination or ADO. These samples caused doubt of their status and were not included in the calculation of the recombination frequency.

Blue: Sites that showed amplification failure

Table 8.1: Appendix C - Polymorphic marker results for recombination detection									
	Chromosome 1		Chromosome 5		Chromosome 16		Chromosome 19		
Sample	D1S495	D1S486	D5S1991	D5S2081	D16S492	D16S3053	D19S219	D19S207	D19S412
C1 ♀	154/166	232/236	212/218	218/216	198/220	242/244	165	213	151/153
	Informative		Informative		Informative		Not informative		
C1 ♂	166/170	238/236	210/216	218	220	242/244	163/165	215/211	156/166
	Informative		Not informative		Not informative		Informative		
C1.1	166	236/238	212/210	218	220	244	165/163	213/ADO	151/156
C1.2	166	236/238	212/210	218	220	244	165	213/211	151/166
C1.3	154/166	236/238	218/210	216/218	220	244	165	213/211	153/166
C1.4	166/170	236	218/216	216/218	220	242/244	165	213/211	153/166
C1.5	166/170	232/238	218/210	AF	220	244	165	213/211	151/156
C1.6	154/166	232/238	212/210	218	220	242/244	165/163	213/211	151/156
C1.7	166	236/238	212/216	218	220	244	165/163	213/211	153/156
C1.8	166	236/ADO?	218	216	198/220	242/244	165	213/211	151/156

Table 8.1: Appendix C (cont.) - Polymorphic marker results for recombination detection									
	Chromosome 1		Chromosome 5		Chromosome 16		Chromosome 19		
Sample	D1S495	D1S486	D5S1991	D5S2081	D16S492	D16S3053	D19S219	D19S207	D19S412
C2 ♀	170/164	236/234	210/216	217	217/224	242	171/175	215/217	143/152
	Informative		Not informative		Not informative		Informative		
C2 ♂	168/160	236/232	210/216	215	217/228	242/246	161	211/215	164
	Informative		Not informative		Informative		Not informative		
C2.1	164/168	234/236	216/210	217/215	224/228	242/246	175/161	217/211	152/164
C2.2	170/168	236	216/210	217/215	224/217	242	171/161	215	143/164
C2.3	164/160	234/232	216/210	217/215	224/217	242	171/161	215	143/164
C2.4	170/168	236	216/210	217/215	224/228	242/246	175/161	217/211	152/164
C2.5	164/160	234/232	216/210	217/215	224/228	242/246	175/161	217/211	152/164
C2.6	164/160	234/232	216	217	224/217	242	175/161	ADO/211	152/ADO
C2.7	164/168	234/236	216	217	217/228	242/ADO?	171/161	215/211	143/ADO
C2.8	164/160	234/236	ADO/210	217/215	217	242	175/161	217/215	143/164
C3 ♀	170/166	236/238	210/216	217	220	205/224	152/163	198/214	164/156
	Informative		Not informative		Not informative		Informative		
C3 ♂	164	236	216	215/225	242/244	240/242	161/165	211/215	166/164
	Not informative		Not informative		Informative		Informative		
C3.1	166/164	238/236	216	217/215	220/205	244/240	152/161	198/211	164/166
C3.2	166/164	238/236	210/216	217/225	220/205	242/240	152/161	198/211	164/166
C3.3	170/164	236	216	217/215	220/205	242/240	152/161	198/211	164/166
C3.4	166/164	238/236	216	217/215	220/224	244/242	163/165	214/215	156/164
C3.5	166/164	238/236	216	217/215	220/205	242/240	152/161	198/211	164/ADO?
C3.6	166/164	238/236	216	217/215	220/205	242/240	152/ADO	198/211	164/ADO?
C3.7	166/164	238/ADO	210/216	217/225	220/205	244/240	152/161	198/211	164/166
C3.8	170/164	236	216	217/225	220/205	242/240	163/165	214/215	156/166
C3.9	170/164	236	210/ADO	217/225	220/205	244/240	163/165	198/215	156/164
C3.10	170/164	236	216	217/225	220/205	244/240	163/165	214/215	156/166
C3.11	166/164	238/236	216	217/225	220/205	244/240	163/165	214/215	156/166

Table 8.1: Appendix C (cont.) - Polymorphic marker results for recombination detection									
	Chromosome 1		Chromosome 5		Chromosome 16		Chromosome 19		
Sample	D1S495	D1S486	D5S1991	D5S2081	D16S492	D16S3053	D19S219	D19S207	D19S412
C4 ♀	**162**	**236**	**210/216**	**218**	**216/221**	**240/243**	**146/165**	**213/215**	**152/164**
	Not informative		**Not informative**		**Informative**		**Not informative**		
C4 ♂	**151/162**	**234/236**	**212/216**	**216/220**	**200/223**	**244/241**	**146/165**	**213/215**	**154**
	Informative		**Informative**		**Informative**		**Not informative**		
C4.1	162/151	236/234	210/216	218/220	216/223	240/241	ADO/165	213/215	152/154
C4.2	162	236	216	218/220	221/200	240/244	146/165	213/215	164/154
C5 ♀	**168/170**	**236**	**210/216**	**215/225**	**209/221**	**242**	**161/165**	**213/211**	**162**
	Not informative		**Informative**		**Not informative**		**Informative**		
C5 ♂	**164/166**	**236**	**210/216**	**217**	**221**	**242**	**161**	**213**	**164/166**
	Not informative		**Not informative**		**Not informative**		**Not informative**		
C5.1	170/164	236	210	215/217	209/221	242	165/161	211/213	162/166
C5.2	168/164	236	210	215/217	221	242	165/161	211/213	162/166

Table 8.1: Appendix C (cont.) - Polymorphic marker results for recombination detection									
	Chromosome 1		Chromosome 5		Chromosome 16		Chromosome 19		
Sample	D1S495	D1S486	D5S1991	D5S2081	D16S492	D16S3053	D19S219	D19S207	D19S412
C6 ♀	152/163	236	209	214/216	216/212	236/242	171/163	198/213	154/152
	Not informative		Not informative		Informative		Informative		
C6 ♂	161/165	236	208/214	216	216	240/242	153/163	198/213	164/156
	Not informative		Not informative		Not informative		Informative		
C6.1	163/165	236	209/208	214/216	216	236/240	171/153	198	154/164
C6.2	163/165	236	209/214	216	216	242/240	171/153	198	154/164
C6.3	163/161	236	209/214	214/216	212/216	242/240	163/153	213/198	152/164
C6.4	152/161	236	209/208	214/216	216	236/240	163/153	ADO/198	152/164
C6.5	163/161	236	209/214	216	216	240	ADO?/163	198/213	154/156
C6.6	163/161	236	209/208	214/216	212/216	236/242	171/153	198	152/164
C6.7	163/161	236	209/214	214/216	212/216	242/240	171/163	198/213	154/ADO
C6.8	163/165	236	209/214	216	212/216	236/242	163/153	213/198	152/164
C6.9	163/165	236	209/208	214/216	212/216	242+236/240	163/153	213/198	152/164
C6.10	163/165	236	209/ADO	216	212/216	242/240	163	213	152/156
C6.11	163/165	236	209/208	214/216	216	236/242	163	213	152/156
C6.12	163/ADO	236	209/214	214/ADO	216	236/240	171/153	198	154/164
C6.13	152/165	236	209/214	216	216	236/242	171/163	198/213	154/156
C6.14	152/165	236	209/214	216	216	236/242	163	213	152/156
C6.15	152/161	236	209/208	214/216	216	236/240+242	171/163	198/213	154/156
C6.16	152/165	236	209/208	216	216	236/240	163	213	152/156
C6.17	163/161	236	209/214	216	212/216	242	171/163	198/213	152/156
C6.18	ADO/161	236	ADO/214	216	216	ADO/240	163	198/213	152/156
C6.19	152/161	236	209/208	214/216	212/216	242/240	163/153	ADO/198	152/164
C6.20	ADO/161	236	209/208	216	216	AF	163	213	152
C6.21	152/165	236	209/208	214/ADO	216	242/240	171/163	198/ADO	154/156
C6.22	ADO/161	236	209/208	214/ADO	212/216	236/240	163/153	213/198	152/164

Table 8.1: Appendix C (cont.) - Polymorphic marker results for recombination detection									
	Chromosome 1		Chromosome 5		Chromosome 16		Chromosome 19		
Sample	D1S495	D1S486	D5S1991	D5S2081	D16S492	D16S3053	D19S219	D19S207	D19S412
C7 ♀	150/168	238/236	208	217/215	197/216	240	146/165	211/215	162/164
	Informative		Not informative		Not informative		Informative		
C7 ♂	152/164	236/238	211/208	217/215	212	240	161/163	211	143/152
	Informative		Informative		Not informative		Informative		
C7.1	168/164	236/238	AF	AF	197/212	AF	146/163	211	162/152
C7.2	150/164	236/238	208/211	217	216	240	146/163	211	162/152
C7.3	168/164	ADO?/238	208	217/ADO?	216/212	AF	146/163	211	162/152
C7.4	150/164	238	AF	217	212	240	165/163	211	162/152
C7.5	150/164	AF	ADO/211	217	197/212	AF	165/161	215/211	164/152
C7.6	ADO/164	AF	AF	AF	AF	240	AF	AF	AF
C7.7	150/ADO	238	ADO/211	AF	197/216	240	146+165/163	211	162/+164/152
C8 ♀	165	235/232	209	214/219	197/217	240/242	153/165	198/217	156
	Not informative		Not informative		Informative		Informative		
C8 ♂	165/171	237	207/211	217/215	197/205	240	165/161	213/215	143/152
	Not informative		Informative		Not informative		Informative		
C8.1	165	235/237	209/211	214/215	217/205	242/240	153/165	198/213	156/143
C8.2	165/171	235/237	209/211	214/215	217/205	242/240	165/161	217/215	156/152
C8.3	165	235/237	209/211	214/215	197/205	240	153/161	198/215	156/152

Table 8.1: Appendix C (cont.) - Polymorphic marker results for recombination detection									
	Chromosome 1		Chromosome 5		Chromosome 16		Chromosome 19		
Sample	**D1S495**	**D1S486**	**D5S1991**	**D5S2081**	**D16S492**	**D16S3053**	**D19S219**	**D19S207**	**D19S412**
C9 ♀	**159**	**236**	**209**	**217**	**221**	**235/241**	**175/165**	**215/213**	**162/151**
	Not informative		**Not informative**		**Not informative**		**Informative**		
C9 ♂	**157/163**	**238/236**	**209**	**217/215**	**205/217**	**243/241**	**163/167**	**213**	**166/164**
	Informative		**Not informative**		**Informative**		**Informative**		
C9.1	159/163	236	209	ADO/215	221/205	235/243	175/163	215/213	162/166
C9.2	159/157	236/238	209	ADO/215	221/217	235/241	175/167	215/213	162/164
C9.3	159/157	236/238	209	217/215	221/217	235/241	175/167	215/213	162/164
C9.4	159/157	236/ADO?	209	217	221/217	241	175/167	215/213	162/164
C9.5	159/163	236	209	217/215	221/217	235/241	165/163	213	162/166
C9.6	159/163	236	209	217	221/205	241/243	165/167	213	151/166
C9.7	159/163	236	209	217/215	221/205	235/243	165/163	213	151/166
C9.8	159/157	236/238	209	217/215	221/217	235/241	163	213	166
C9.9	159/157	236/238	209	217	221/205	235/243	165/167	213	151/166
C10 ♀	**159/163**	**236**	**208/214**	**215/217**	**200/217**	**239**	**146**	**213**	**162/166**
	Not informative		**Informative**		**Not informative**		**Not informative**		
C10 ♂	**152/168**	**238/236**	**210**	**213/217**	**220/228**	**241/236**	**162/165**	**213/217**	**154/166**
	Informative		**Not informative**		**Informative**		**Informative**		
C10.1	159/152	236/238	208/210	215/213	200/228	239/236	146/165	213/217	162/166
C10.2	163/168	236	208/210	215/217	217/220	239/241	146/162	213	162/154
C10.3	163/168	236	208/210	215/213	200/220	239/241	146/162	213	166/154
C10.4	163/168	236	214/210	217/213	200/220	239/241	146/162	213	166/154
C10.5	159/168	236	208/210	215/217	200/228	239/236	146/162	213	162/154

Table 8.1: Appendix C (cont.) - Polymorphic marker results for recombination detection					
	Chromosome 17				
Sample	**NF1int1**	**D17S1307**	**NF1int17**	**NF1int29**	**D17S1166**
C1 ♀	**164/166**	**208**	**212/216**	**136**	**189/191**
	Informative				
C1 ♂	**164**	**208**	**208/212**	**136**	**191/189**
	Informative				
C1.1	164	208	212/208	136	189/191
C1.2	166/164	208	216/212	136	191/189
C1.3	166/164	208	216/212	136	191/189
C1.4	ADO?/164	208	212	136	191/189
C1.5	AF	208	212/ADO	136	AF
C1.6	164	208	212/208	136	189/191
C1.7	164	208	212/208	136	189/191
C1.8	164	208	212	136	189
C2 ♀	**162/160**	**205**	**221/229**	**165/161**	**195**
	Informative				
C2 ♂	**164**	**208**	**216**	**165/167**	**199**
	Not informative				
C2.1	160/164	205/208	221/216	165/167	195/199
C2.2	160/ADO	205/208	229/216	161/165	195/199
C2.3	162/164	205/208	221/216	165	ADO/199
C2.4	162/164	205/208	221/216	165	195/199
C2.5	160/164	205/208	221/216	165	195/199
C2.6	AF	205/208	221/ADO	165	195/199
C2.7	162/164	205/208	221/216	165	195/199
C2.8	162/164	205/208	221/216	165	195/199
C3 ♀	**160/164**	**200/208**	**223/212**	**157/136**	**195/189**
	Informative				
C3 ♂	**164**	**208**	**208/210**	**136**	**191**
	Not informative				
C3.1	160/164	200/208	223/210	157/136	195/191
C3.2	160/164	200/208	223/208	157/136	195/191
C3.3	160/164	200/208	223/208	157/136	195/191
C3.4	ADO/164	200/208	212/210	136	195/191
C3.5	160/164	ADO/208	223/210	157/136	189/191
C3.6	164	208	212/210	136	189/191
C3.7	160/ADO	200/208	223/210	157/136	195/191
C3.8	164	208	212/210	136	189/191
C3.9	160/164	200/208	223/210	157/136	195/191
C3.10	164	208	212/210	136	189/191
C3.11	160/164	200/208	223/210	157/136	195/191
C4 ♀	**164**	**208**	**215/213**	**163/136**	**198/199**
	Informative				
C4 ♂	**164**	**208**	**212**	**138**	**189/198**
	Not informative				
C4.1	164	208	215/212	163/138	198
C4.2	164	208	212	138	189
C5 ♀	**160/164**	**200/208**	**227/219**	**157/136**	**195/191**
	Informative				
C5 ♂	**164**	**208**	**212**	**136**	**189/191**
	Not informative				
C5.1	160/164	200/208	227/212	157/136	195/191
C5.2	164	208	219/212	136	195/189

Table 8.1: Appendix C (cont.) - Polymorphic marker results for recombination detection					
	Chromosome 17				
Sample	**NF1int1**	**D17S1307**	**NF1int17**	**NF1int29**	**D17S1166**
C6 ♀	**164**	**208**	**212**	**138**	**188**
	Not informative				
C6 ♂	**164**	**208**	**210/212**	**136**	**188**
	Not informative				
C6.1	164	208	212	136	188
C6.2	164	208	210	136	188
C6.3	164	208	212/210	138/136	188
C6.4	164	208	212	138/136	188
C6.5	164	208	212	138/136	188
C6.6	164	208	212/210	138/136	188
C6.7	164	208	212	136	188
C6.8	164	208	212	136	188
C6.9	164	208	210	136	188
C6.10	164	208	210	136	188
C6.11	164	208	212	138/136	188
C6.12	164	208	210	136	188
C6.13	164	208	212	136	188
C6.14	164	208	212	136	188
C6.15	164	208	212	138/136	188
C6.16	164	208	212/210	138/136	188
C6.17	164	208	212/210	138/136	188
C6.18	164	208	212/210	138/136	188
C6.19	164	208	212/210	138/136	188
C6.20	164	208	212	138	188
C6.21	164	208	212	138	188
C6.22	164	208	210	136	188
C7 ♀	**158/166**	**208/204**	**216/221**	**169/163**	**198**
	Informative				
C7 ♂	**162/164**	**204/208**	**212/239**	**138/161**	**189/198**
	Informative				
C7.1	AF	204/208	216/239	169/161	198
C7.2	AF	208	216/212	169/138	198
C7.3	AF	204/208	216/239	169/161	198
C7.4	AF	204/208	216/239	169/161	198
C7.5	AF	204/208	216/239	169/161	198
C7.6	AF	204/208	AF	163/161	AF
C7.7	AF	208	216/ADO	169/161	198
C8 ♀	**164**	**204/208**	**211**	**136/138**	**191/189**
	Informative				
C8 ♂	**164**	**208**	**211**	**136**	**189**
	Not informative				
C8.1	164	204/208	211	136	191/189
C8.2	164	204/208	211	136	191/189
C8.3	164	204/208	211	136	191/189

Table 8.1: Appendix C (cont.) - Polymorphic marker results for recombination detection					
	Chromosome 17				
Sample	**NF1int1**	**D17S1307**	**NF1int17**	**NF1int29**	**D17S1166**
C9 ♀	**164**	**208**	**212/214**	**136**	**191**
	Not informative				
C9 ♂	**162/164**	**204/208**	**217/229**	**161/163**	**210/198**
	Informative				
C9.1	AF	208	212/229	136/163	191/198
C9.2	164	208	214/229	136/163	191/198
C9.3	162/164	208/204	214/217	136/161	191/210
C9.4	164/162	208/204	212/217	136/161	191/210
C9.5	164/162	208/204	214/217	136/161	191/210
C9.6	164/162	208/204	214/217	136/161	191/210
C9.7	164	208	214/229	136/163	191/198
C9.8	164/162	208/204	214/217	136/161	191/210
C9.9	AF	208/204	212/217	136/161	191/210
C10 ♀	**166/164**	**208**	**212/215**	**136/163**	**191/198**
	Informative				
C10 ♂	**164/162**	**208/204**	**215/221**	**136/163**	**187/198**
	Informative				
C10.1	166/ADO	208/204	212/215	136	191/187
C10.2	166/ADO	208/204	212/215	136	191/187
C10.3	164	208	215	163	198
C10.4	166/162	208/204	212/221	136/163	191/198
C10.5	166/162	208/204	212/221	136/163	191/198

9 Publications from this thesis

9.1 Published paper

- **Mamas T**, Gordon A, Brown A, Harper J, Sengupta S. 2012. Detection of aneuploidy by array comparative genomic hybridization using cell lines to mimic a mosaic trophectoderm biopsy. *Fertil Steril.* 97(4): 943-947.

9.2 Abstract presentations from this thesis

Oral presentations

- **Mamas T**, Kakourou G, Dhanjal S, Cawood S, Doshi A, Serhal P, Xanthopoulou L, Mantzouratou A, Delhanty J, Harper JC, SenGupta S. 2009. Detection of chromosomal aneuploidy in embryos from preimplantation genetic diagnosis cases for monogenic disorders. *Reprod Biomed Online*. 18 (Supplement 3): S11. (Preimplantation Genetic Diagnosis International Society conference, Miami, USA).
- **Mamas T**, SenGupta S, Dafou D, Gordon T, Harper JC. 2009. Detection of chromosomal abnormalities in single cells from epithelial cell lines by array comparative genomic hybridisation. *J. Med Genet*. 46 (Supplement 1): S19. (British Society of Human Genetics conference, Warwick University, UK).
- **Mamas T**, Craig A, Garcia-Bernando H, SenGupta S, Gordon T, Brown A, Harper JC. 2010. Validation of single cell array comparative genomic hybridisation using aneuploidy and normal cells from epithelial cell lines. (Association of Clinical Embryologists meeting, Bristol, UK).
- **Mamas T**, Gordon T, Brown A, Doshi A, Serhal P, SenGupta S, Harper JC. 2011. Validation of array comparative genomic hybridisation for preimplantation genetic screening. (UCL Institute for Women's Health 6th annual meeting, London, UK).
- **Mamas T**, Xanthopoulou L, Heath C, Doshi A, Serhal P, SenGupta S. 2012. Incidence of aneuploidy in embryos from fertile couples. (UCL Institute for Women's Health 7th annual meeting, London, UK).

9.3 Poster presentations

- **Mamas T**, Dhanjal S, Jaroudi S, Kakourou G, SenGupta S. 2007. Preimplantation Genetic Diagnosis for inherited cancer predisposition – Different strategies. *J Med Genet*. 44 (Supplement 1): S69. (British Society of Human Genetics conference, York, UK).
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