Errors in Chromosome Segregation during Oogenesis and early Embryogenesis

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Abstract

Errors in chromosome segregation occurring during human oogenesis and early embryogenesis are very common. Meiotic chromosome development during oogenesis is subdivided into three distinct phases. The crucial events including meiotic chromosome pairing and recombination takes place from around 11 weeks until birth. Oogenesis is then arrested until ovulation, when the first meiotic division takes place with the second meiotic division not completed until after fertilization. It is generally accepted that most aneuploid fetal conditions, such as for example trisomy 21 Down syndrome, is due to maternal chromosome segregation errors. The underlying reasons are not yet fully understood. It is also clear that superimposed on the maternal meiotic chromosome segregation errors there is a large amount of mitotic errors taking place post-zygotically during the first few cell divisions in the embryo. In this chapter we summarize current knowledge of errors in chromosome segregation during oogenesis and early embryogenesis, with special reference to the clinical implications for successful assisted reproduction.

Keywords

Oogenesis, embryogenesis, chromosome segregation, non-disjunction, meiotic recombination, MLH1, crossing-over, chiasma, aneuploidy

1. Introduction

1.1 Meiosis

Meiosis, derived from the Greek for diminution, means to halve. The process of meiosis halves the chromosome number of a diploid cell during gametogenesis, producing haploid gametes. This involves two cell divisions (meiosis I and meiosis II; Fig.1, Table 1) without an intermediate DNA replication step. The first of these meiotic cell divisions is reductional, i.e. the chromosome complement of a diploid oogonial cell/primary oocyte is halved leading to haploid secondary oocytes. During the second meiotic division, chromatids separate in the same way as in mitosis, thus the chromosome number is not changed (Table 1). Fusion of a haploid sperm cell and a haploid oocyte at fertilization restores the somatic diploid chromosome number.

The process of meiosis is evolutionarily conserved from yeast to man, though with marked differences between sexes of the same species and between different species.

1.2 Differences in the meiotic process between the human male and female

There are drastic differences in the meiotic process between human males and females (Table 2). In particular the timing of events, the numbers of gametes produced and the level of chromosome abnormalities in sperm compared to oocytes are very different.

1.2.1 Timing of meiotic events

In the human male, testicular pre-meiotic spermatogonia start dividing mitotically at puberty. The meiotic process, including the reductional division at meiosis I, the equational division at meiosis II followed by maturation of spermatids to spermatozoa is ongoing in the testes throughout life. In the human female, however, meiosis is subdivided into three separate stages. The first part of meiosis I, i.e. prophase I (Table 1), takes place in fetal ovaries and continues until birth, at which time meiosis is arrested, and is not continued until ovulation. The second part of the reductional division of meiosis I takes place just before ovulation, and the process continues into the second meiotic division. Meiosis is then arrested again, at the metaphase II (MII) stage (Table 1). The anaphase and telophase stages of the second meiotic division are not completed until after the oocyte has been penetrated by the spermatozoon at fertilisation.

The study of the meiotic chromosome behaviour in human males is much more advanced than that in females as the difference in timing of meiosis between the sexes means it is much easier to obtain tissue material from males. Testicular biopsies of post-pubertal men have yielded large amounts of data. However, in females, biopsies of fetal ovaries, individual oocytes at the metaphase I (MI) stage (prior to ovulation) and individual oocytes arrested at the MII stage (post-ovulation and pre-fertilisation) are scarce and difficult to obtain for study. Accordingly, a large body of knowledge has accumulated over the last 40 years or so, of the meiotic chromosome behaviour of normally fertile

men and those with reproductive problems (see Martin, Chapter 00). The situation is very different in the human female; it is only within the last decade that technology has been developed to allow the relevant investigations of chromosome behavior in fetal ovaries and the meiotic divisional stages occurring at ovulation and following fertilization. Meiotic investigations of chromosome behavior in human fetal ovaries have been performed by few research groups, whilst chromosome analysis of the meiosis divisional stages has primarily been undertaken as research projects within a number of Assisted Conception Units.

1.2.2 Frequency of gametes produced

With respect to availability of material for study, it should also be noted that there are incredible differences in gamete production between sexes. There are normally at least 300 million spermatozoa produced daily in post-pubertal human males, which stands in sharp contrast to the meager production of around 300-400 oocytes in the lifetime of human females.

1.2.3 Incidence of meiotic chromosome errors

For reasons not yet fully understood, errors in meiotic chromosome behavior giving rise to aneuploidy in offspring are more common in the human female than in the male. Remarkably, these errors are much more common in the human female than in any other species investigated to date. The reason for this species difference also remains unknown and requires further study.

1.3 Content of this chapter

In this chapter we will summarize current knowledge of errors in chromosome segregation during oogenesis and early embryogenesis, with special reference to the clinical implications for successful assisted reproduction. The main problems occurring during oogenesis are (1) errors in meiotic chromosome pairing and recombination at the meiosis I prophase; (2) insufficient elimination of chromosomally abnormal oocytes during the meiotic chromosome pairing stage; and (3) abnormalities in segregation of chromosomes at the anaphase I (AI) and anaphase II (AII) stages. The first two of these problems may be investigated in fetal ovaries, whilst the segregation errors can only be analysed on isolated oocytes at the MI stage captured at ovulation and when arrested at the following MII stage. Crucial information may also be obtained by investigation of the results of AI chromosome segregation, i.e. the secondary oocyte and the 1st polar body (PB). As evidenced from the analysis of chromosome constitution in early embryos, there is also a high frequency of post-zygotic mitotic chromosome segregation errors.

2. Investigation of meiotic chromosome behavior in fetal ovaries

Primordial germ cells are the gametic precursors. Around the fifth week of pregnancy they migrate to the area of the genital ridge. Subsequently, sexual differentiation occurs and, in females, ovarian development begins. Oogonia, the precursors of oocytes, increase in number following cellular

division to become oocytes. The proliferation of cells is then arrested and the oocytes begin the first stages of meiosis at around 10-11 weeks of gestation, which continues until birth (Fig. 2; [1]). It is at the prophase of the first meiotic cell division (Table 1) during this early foetal developmental stage, that the crucial events of crossing-over, chiasma formation and recombination of parental alleles take place. As human foetal ovarian material is difficult to obtain, less knowledge is available from direct microscopy analysis of crossing-over, chiasma formation and recombination in the human female than in the male. Nevertheless, both direct investigation of foetal ovarian oocytes and indirect studies of recombination by family linkage analysis have demonstrated that meiotic recombination errors, leading to aneuploid oocytes, are common in the human female.

It is important to note that the meiotic recombination process in oocytes can be studied in three very different ways, depending on their stage of maturation. First, crossover frequency and distribution along the lengths of individual chromosomes can be identified in fetal oocytes by analysis of the meiotic chromosome pairing structure, the Synaptonemal Complex (SC) using immunofluorescence against recombination proteins[2-6]. Second, there is the potential to identify crossover frequency and distribution in the form of chiasmata at the MI stage ([7])**.** Third, it is also possible to estimate crossover frequency and distribution indirectly by family linkage analysis ([8-10]).

2.1 Analysis of meiotic crossovers in fetal oocytes

Meiosis in fetal oocytes starts with pairing of the maternal and paternal homologous chromosomes along their entire length. This pairing is mediated by a meiosis-specific protein structure, the Synaptonemal Complex (SC; Fig 3). Meiotic crossover points may be directly visualized by immuno-fluorescence microscopy analysis of the SC in fetal oocytes from around 12 to 24 weeks' gestational age. The visualization of the crossover/chiasma/recombination points has been made possible by the use of (commercially available) antibodies against the recombination proteins MLH1 and MLH3 (Fig. 4).

2.2 Oocyte crossover patterns

Meiotic crossover patterns in human foetal oocytes can vary significantly ([2-6]). In a study of 95 oocytes from one fetus, a mean of 70.3 (range 48-102) recombination events per oocyte were detected. Chromosomes lacking a recombination focus altogether were also described ([4]). A more detailed analysis indicated that foetal oocytes with defects in the formation of the SCs, such as incomplete pairing or breakage, were predisposed to recombination deficiency ([**4**]). A study of 131 oocytes from nine cases ([3]) also described large variation between individual cases (range 10-107). It was concluded that if such variability persisted throughout development and into adulthood, that as many as 30% of human oocytes would be predisposed to aneuploidy i.e. an abnormal chromosome number ([3]). This is because the bivalents would lack a crossover site between the maternal and paternal homologues leading to the absence of a chiasma, which would normally hold them physically

together at MI. Thus the maternal and paternal chromosomes would not be able to orient stably at the metaphase plate in relation to the spindles, an obligatory mechanical requirement for the normal reductional chromosome segregation at AI (Fig 5a). Instead they would be expected to segregate randomly at AI (Fig. 5b). By chance, 50% of the two daughter cells, i.e. the secondary oocyte and the 1st polar body (PB)) would then contain both maternal and paternal chromosomes, while the remaining 50% would contain neither. Thus, if the original bivalent had been 'achiasmatic' the secondary oocyte and the corresponding 1st PB would be expected to be aneuploid in 50% of cases. There is also the possibility that the half chromosomes (chromatids) of an unpaired chromosome (univalent) would segregate in the same way as at mitosis, leading to an extra chromatid in either the secondary oocyte or the 1st PB (Fig 5c).

2.3 Natural elimination of abnormalities

The majority of fetal oocytes are eliminated during fetal and postnatal life, with only a small minority reaching ovulation and thus meiotic MI - AI stages (Fig. 2). There is evidence to suggest that natural selection against oocytes containing abnormalities in homologous chromosome pairing and crossing-over takes place during fetal development, leading to delay in their maturation or deletion by atresia and apoptosis ([4]). This type of selection may also continue after birth until the onset of ovulation in adulthood. There is a paucity of data describing patterns of recombination in immature oocytes, comprising the ovarian reserve in adult women. These data could be obtained by analysis of

chiasmata at the MI stage, as has been performed in spermatocytes (Fig 6a). However, the scarcity of oocyte material available for study, combined with the limited clarity of chiasmata in oocytes compared to spermatocytes at this stage (Fig. 6b; [7]) still presents challenges for obtaining this information. Chiasma analysis at the MI stage would also allow evaluation of the extent of delay and elimination by atresia and apoptosis oocytes exhibiting crossover errors during oocyte maturation from foetal life to adulthood.

3. Meiotic crossover errors detected by family linkage analysis

Meiotic crossover errors in the human female have also been investigated indirectly by linkage analysis, using DNA samples from both parents and children ([8,9]). Patterns of allelic recombination are then determined by using DNA markers along the length of individual chromosomes, including the chromosomes for which aneuploidy is common: 21 (Down syndrome), 18 (Edwards syndrome), 13 (Patau syndrome) and the sex chromosomes X and Y (e.g. Klinefelter and Turner syndromes). These studies have demonstrated that the outstanding majority of autosomal trisomies originate from recombination errors taking place during maternal oogenesis. However, errors in parental oogenesis as well as spermatogenesis give rise to the Klinefelter and XXX syndromes.

3.1 Linkage analysis of trisomy 21

It has been concluded from linkage analysis of families with a case of Down syndrome that lack of recombination during maternal oogenesis account for approximately half of all cases associated with chromosome non-disjunction at AI. Aberrant positioning of chiasmata (more distal or proximal than normal) on the long arm of chromosome 21 has also been detected and identified as a putative causal factor ([8,9]). Much effort has gone into explaining why such positioning would increase the risk for non-disjunction at AI ([8-10]). The mechanism underlying this process remains to be elucidated, however it should be noted that the same distal chiasma site on chromosome 21 is prevalent in human males and is not associated with a high rate of non-disjunction.

It was initially thought that the well-known maternal age effect in risk of having a child with Down syndrome (Fig. 7) was primarily due to changes in patterns of maternal meiotic recombination. However, it has been clearly demonstrated that this is not the case, and a so-called 'two-hit' hypothesis has been suggested to explain the maternal age affect. This postulates that bivalents with the vulnerable chiasma positions are less readily handled by the AI machinery as women grow older. It has been suggested that one explanation could be age-related impairment of the binding of homologous grandparental chromosomes by the protein cohesion, with an increased risk for primary non-disjunction of the two chromosomes 21 at AI (Fig. 5 b,c; [10]**)**. Most recently it has been suggested that the maternal age effect is a multifactorial trait, and may be influenced by environmental factors ([8,9]). Another intriguing possibility it that normal women may be trisomy 21 ovarian mosaics ([11-13])

with the potential for oocytes containing three chromosomes 21 accumulating during maturation and when reaching MI undergoing obligatory secondary non-disjunction at AI.

3.2 Linkage analyses of trisomy 13 and 18

There are few data concerning trisomy 13 (Patau syndrome) and the genetic errors that can cause it. However, a recent study of 78 cases ([14]) indicated that over 90% of cases are due to maternal meiotic errors, with approximately one third of these occurring at AII. This demonstrates some similarity to trisomy 18 in that a large proportion of these cases appear to arise predominantly because of errors at AII. Though, as the authors describe, the only similarity between chromosomes 13 and 18 is that they are gene poor, and any specific, potentially causative, genomic features have yet to be identified; if indeed there is a common cause of both trisomies ([14]).

4. Direct evaluation of oocyte aneuploidy

To date (as far as the authors are aware) there have been no detailed studies evaluating exact oocyte aneuploidy rates in MI oocytes. However, numerous investigations have been performed on MII oocytes ([15-17]), obtained as immature MII oocytes or oocytes that have failed to fertilize. These studies have demonstrated that segregation errors involving both whole chromosomes and chromatids are common (reviewed in [18]). The material investigated consists almost exclusively of MII oocytes obtained at infertility treatment clinics, where spare oocytes, spontaneously arrested at MII, have been

donated for research. However, the various stimulation regimes necessary for *in vitro* fertilization (IVF) do not appear to affect the chromosome constitution of the oocyte, as oocytes obtained from natural cycles show similar frequency of anomalies ([19,20]). Furthermore, the eggs that remain unfertilised after exposure to sperm are not cytogenetically different from those that are fertilized ([21]).

Thousands of human oocytes at the MII stage have been investigated to obtain information on the efficacy of AI segregation as well as the occurrence of structural chromosome aberrations. Traditionally, two methods have been used for analysis of oocytes, following cell fixation on a microscope slide. Chromosome analysis has been performed by karyotyping and fluorescence *in situ* hybridization (FISH) for selected chromosomes. Recently, however, a DNA based method, comparative genomic hybridization (CGH) has also been used to investigate oocyte aneuploidy.

4.1 Analysis of oocyte aneuploidy by karyotyping

4.1.1 Methodology

Karyotyping involves spreading and fixing a single cell on a microscope slide, followed by staining and microscopy analysis. However, use of a single cell risks the loss of one or more chromosomes, and their contracted nature does not allow for the use of conventional G-banding techniques for exact identification of individual chromosomes. Indeed, a large scale study of unfertilized human oocytes only resulted in the successful analysis of 45.9% of samples ([22]).

4.1.2 Results

In the most recent, large scale karyotyping study of 1397 oocytes from 792 patients (mean age 33.7± 4.7 years), numerical abnormalities were detected in 20.1% of samples ([22]). The majority of these were due to extra or missing whole chromosomes or chromatids, whereas structural abnormalities (breaks, deletions and acentric fragments) were rarer, only detected in 2.1% of cells. Numerical abnormalities caused by extra or missing chromatids were more common than aneuploidy of whole chromosomes, confirming the hypothesis that AI segregation errors of chromatids are common in human oocytes obtained through IVF studies ([23]; Fig. 5c). Although aneuploidy was detected in all chromosomes, the distribution was skewed towards a significantly higher frequency in the physically smallest autosomes (chromosomes 13-22); a result that has been confirmed in further studies ([15,30]), irrespective of the method used.

A strong positive correlation between maternal age and rate of aneuploidy, was also detected ([22]). The same effect has been observed in a sample of fresh oocytes (not stimulated by hormone injections as part of IVF treatment) investigated using spectral chromosomal analysis ([25]).

4.2 Analysis of oocyte aneuploidy using FISH and CGH

The difficulties described above for analysis of oocyte aneuploidy have led to the development and application of molecular cytogenetic methods: initially, multiprobe fluorescence *in situ* hybridisation (FISH) analysis and more recently CGH.

4.2.1 Methodology

Similar to karyotyping, a set of FISH probes designed to hybridise to specific chromosomal regions are incubated with a cell, spread on a microscope slide. The probes are labeled e.g. with a red or green fluorophore, and after incubation, the slides are washed and the results observed and recorded with the use of a fluorescent microscope. The application of FISH with chromosome-specific DNA probes to MII oocyte preparations allows the identification of specific chromosomes; an additional advantage of using FISH is that precise information can also be obtained from the chromatin of the 1st PB.

4.2.2 Results

FISH analysis of the 1st PB with probes specific for chromosomes X and 18 has been demonstrated to be able to predict the chromosome constitution of the oocyte under the assumption that the loss and gain will be reciprocal ([16]). This approach has since been developed for diagnostic purposes, with the aim of avoiding the use of oocytes that have been predicted to be aneuploid based upon 1st PB analysis of five autosomes by FISH ([26]). Micromanipulation of the PBs followed by FISH with 3–5

chromosome-specific probes is now a technique used in preimplantation genetic diagnosis (PGD). The largest study to date examined 6733 oocytes from women above the age of 35 (average 38.5 years), and a large proportion of oocytes were aneuploid with respect to the chromosomes tested (13, 15, 18, 21 and 22;[27]). In total, 41.7% of oocytes were considered to be aneuploid because of AI malsegregation, the majority involving chromatids rather than whole chromosomes. Loss of a chromatid from the 1st PB was the most common anomaly scored but it should be noted that technical FISH error could also produce this result.

Several researchers have used multiprobe FISH to analyse MII oocytes and the associated 1st PBs ([15,17,28]). A number of interesting features have emerged from these studies. In agreement with karyotyping studies, anomalies were not randomly distributed between the chromosomes. The smaller chromosomes, 13, 16, 18, 21 and X were most frequently aneuploid, with no anomalies detected for chromosomes 1, 9, or 12 (Fig 8). In addition, analysis of an MII oocyte and its corresponding 1st PB revealed a previously not well recognized mechanism leading to aneuploid gametes. In two cases an extra chromosome was detected in the 1st PB with no accompanying loss in the MII oocyte; and in a third case, extra chromatids were present in both the MII oocyte and the 1st PB ([17]). All three of these mature eggs must have been derived from cells that were trisomic for the chromosomes involved (13 and 21) when entering meiosis during fetal life. Without further investigation it is not possible to determine whether this pre-existing aneuploidy arose during the pre-

meiotic divisions (germinal mosaicism) or if it was present in the embryonic gonad (gonadal mosaicism). The first instance of gonadal mosaicism cytogenetically proven this way was reported in a woman who had had three previous conceptions with trisomy 21 and for whom PGD was performed. FISH analysis of unfertilized MII oocytes and corresponding first PBs proved that ovarian mosaicism was the cause of the abnormal conceptions ([29]; see also Table 2 in Hultén et al[12]).

4.3 Analysis of oocyte aneuploidy using CGH

4.3.1 Methodology

To obtain an accurate estimate of aneuploidy in human oocytes requires a method that does not involve the spreading of chromosomes on a slide. A DNA based method such as comparative genomic hybridisation (CGH) is appropriate, but for single cell analysis the DNA must first be amplified from the 6-10 picograms initially present to the 200 nanograms required. DNA from a small number of cells from a chromosomally normal control source is similarly amplified, test and control DNAs are then labelled with red or green fluorochromes respectively and co-hybridised to prepared slides of normal male metaphase chromosomes, which act as indicators (Fig 9). The two DNAs compete for hybridisation sites allowing differences in the number of copies of any of the chromosomes to be detected by analysis of the green:red ratio along each indicator chromosome on the slide (Fig. 10). The approach of using CGH analysis to acquire data on aneuploidy in human oocytes has been validated by several recent studies ([24,30-34]).

4.3.2 Results

As the 1st PB and the MII oocyte are reciprocal products of the first meiotic division, analysis of one of these cells should predict the chromosome constitution of the other, although there are exceptions as in cases of germinal or gonadal mosaicism, where there is pre-existing aneuploidy in the oocyte. The 1st PB GH approach has been applied in both clinical practice and research projects, providing an indirect means of assessing oocytes for aneuploidy involving any chromosome. As regards IVF treatment the aim is the identification and transfer of embryos derived from chromosomally normal oocytes, which are likely to have the greatest chance of forming a viable pregnancy. However, the application of this technology in a clinical context to either PBs or embryonic blastomeres (i.e. Preimplantation Genetic Screening - PGS) is not without problems, one of which is the length of time the method requires ([34]). Standard CGH requires 3-4 days and the analysis is labor intensive, preventing analysis of large numbers of cells. Blastocyst transfer, usually on day-5 post-fertilization, provides sufficient time for PB CGH analysis to be completed; however, this is not appropriate for all patients undergoing IVF. An alternative is to cryopreserve the zygotes immediately after biopsy of the 1st and 2nd PBs or after blastomere biopsy on day 3 of development. The success of improved freezing (vitrification) protocols in routine IVF makes this a more attractive option.

The use of lymphocyte metaphases for CGH analysis has recently been replaced by the use of genomic microarrays for pre- and postnatal genetic analysis, but for single cell analysis this approach is not yet sufficiently robust for application in clinical practice.

4.4 The aneuploidy rate in human oocytes

Recent karyotyping data that includes chromatid anomalies and the more specific FISH analyses indicated that the overall rate of chromosome and chromatid imbalance in human oocytes is about 11% for women of maternal age 32-34 years ([15,17,22,35]). However, several independent studies of aneuploidy in MII oocytes and corresponding 1st PBs have used CGH to obtain exact information on all chromosomes. There are now data on at least 221 MII oocyte-PB complexes donated from 82 patients ([24,30,31] and unpublished data). The results confirm that CGH is able to detect chromatid losses and gains, as well as whole chromosome changes, and partial aneuploidies thought to be due to chromosome breakage.

Current data suggest an aneuploidy rate of 20.8% ([24,31] and unpublished data), double the rate found by karyotyping ([22]). Chromosome loss and gain occurred almost equally in the oocyte and 1st PB in the abnormal samples, in accordance with the theoretical expectations of the outcome of primary meiotic non-disjunction of the two homologs. These data provide no support for the frequent occurrence of anaphase lag. As evidence gained by analysis of preimplantation embryos shows that

those with autosomal monosomies and with autosomal trisomies of the largest autosomes rarely progress beyond initial implantation, over half of the abnormal oocytes will lead to IVF failure. The mechanism of gonadal or germinal mosaicism involving secondary meiotic non-disjunction, leading to aneuploid oocytes was confirmed by the observation of oocytes or PBs with an additional or missing chromosome, without the reciprocal anomaly being scored in the corresponding PB or oocyte ([24,31-33]).

Abnormalities affected all chromosomes except 7 and 14 but most frequently the X and then chromosomes 21, 22, followed by 8, 12 and 20 ([24,30] and unpublished data). The mechanisms involved whole chromosome non-disjunction, chromatid imbalance due to precocious chromatid separation, chromosome breakage and, rarely, gonadal/germinal mosaicism. It is of interest that the larger autosomes, numbers 1 to 12, were affected solely by whole chromosome non-disjunction and unaffected by chromatid anomalies. This is thought to reflect the increased cohesion of larger bivalents as well as the role of crossing over in holding paired homologues together, as larger chromosomes have greater numbers of chiasmata. It is further suggested that smaller chromosomes with few or no crossovers are more likely to separate early, in turn predisposing them to random disjunction as well as to chromatid anomalies. However, the X chromosome stands out as a special case; eight of sixteen X chromosome anomalies were seen in just three patients (age range 18-42). At least half of these were chromatid anomalies.

From both the X chromosome and the autosome data it is clear that age-independent mechanisms are operating in the younger women, and there may be a causal link to their infertility. These mechanisms would include germinal or gonadal mosaicism as well as a predisposition to the production of aneuploid gametes due to reduced recombination in meiosis I.

4.5 Aneuploidy in early human embryos

4.5.1 Constitutional aneuploidy

Parental meiotic chromosome segregation errors will lead to constitutional aneuploidy, thereby affecting all cells of the embryo. Almost all recent information on the chromosomal status of the early human embryo has been derived from PGD and follow-up studies of non-transferred embryos. However, these are rarely complete enough to allow determination of parental meiotic chromosome segregation errors. Reliable conventional karyotyping of single embryonic cells is virtually impossible; but the advent of interphase FISH analysis, using fluorescently labeled chromosome-specific probes, has made it possible to determine the copy number of individual chromosomes in the nuclei of single cells obtained from preimplantation embryos. The data obtained from PGD follow-up studies, in particular those carried out for aneuploidy screening, complement those obtained from research studies on human oocytes. Munné et al. ([36]) reported on the diagnostic analysis of single cells from over 2000 embryos using FISH probes for up to 14 chromosomes. Cells were tested for a

minimum of four chromosomes from: 1, 4, 6, 7, 13, 14, 15, 16, 17, 18, 21, 22, X and Y. Those most frequently involved in aneuploidy were chromosomes 15, 16, 21 and 22; those least involved were 6, 14, X and Y. Because follow-up analyses were not carried out on the majority of non-transferred embryos, in most cases it was not possible to distinguish chromosome segregation errors that occurred during parental meiosis from those that arose post-zygotically in the embryo.

As a follow-up to aneuploidy screening for couples who were having difficulties conceiving via IVF due to repeated implantation failure (RIF) or advanced maternal age (AMA) or who were experiencing repeated miscarriage (RM), mainly after natural pregnancies, studies have been carried out to determine the mechanisms leading to aneuploidy and implantation or pregnancy failure. Embryos from 75 couples undergoing preimplantation genetic screening (PGS) have been investigated for abnormalities of chromosomes 13, 15, 16, 18, 21 and 22 using FISH in two rounds of hybridization ([37] and unpublished data). This included the screening of single blastomeres on day 3 and full follow-up analysis on day 5/6 of all the cells of non-transferred embryos. In total, ninety four PGS cycles were included in the study, and 847 embryos biopsied, with results obtained for 91% of these. Approximately one-fifth (19%) were normal disomic for the chromosomes tested at diagnosis on day 3 whilst 81% showed an abnormal result. Despite the low normality rate, the pregnancy rate per cycle that progressed to embryo biopsy was 29.5%; 32.9% per cycle in which embryos were suitable to be transferred. Satisfactory follow-up was obtained from 536 embryos. All those

diagnosed as chromosomally abnormal were confirmed as abnormal on follow-up, of which 94% were mosaics with mixed cell types and only 5.3% were uniformly abnormal. Although mosaicism in general is common in human embryos generated by IVF and increases with the number of days spent in culture, this almost universal aneuploid mosaicism is beyond what is normally seen in embryos from routine IVF patients ([38-42]).

Parental meiotic chromosome segregation errors, detected because all embryonic cells were affected with the same aneuploidy, were identified overall in only 14.8% of embryos, most frequently for chromosomes 18, 21, and 22. Errors in post-zygotic mitotic segregation were detected mostly for chromosomes 13, 15 and 16. There was a significant difference in the distribution of embryos that were uniformly abnormal ($p<0.005$) and those caused by parental meiotic segregation errors ($p<0.005$) between the referral groups. The rates for parental meiotic errors were 24% for the RM group, 20% for the AMA group and only 8.9% for RIF patients. There were similarities in the abnormalities affecting embryos from the couples with RM and AMA, whereas couples with RIF appeared to be different due to the low frequency of identifiable abnormalities in their embryos caused by parental meiotic chromosome segregation errors. For this patient group, post-zygotic abnormalities appear to be the main factor leading to implantation failure. The RM patients resemble those with advanced maternal age, although the average age (36 years) in the RM group is much lower than in the AMA group (42 years). It would appear from these data that even for the RM and

AMA groups, aneuploidy caused by parental meiotic chromosome segregation errors is far less of a risk than the mosaic form caused by post-zygotic mitotic errors.

4.5.2 Mosaic aneuploidy

In general, the embryos of women undergoing IVF who are younger than 37 years of age are chiefly at risk of mosaic aneuploidy caused by post-zygotic segregation errors rather than the full constitutional type with all embryo cells being aneuploid, this type originating from parental meiotic segregation errors. Interphase FISH detection of the X and Y chromosomes for the purposes of embryo sexing first indicated that mosaicism is a common feature of human preimplantation embryos ([38]). Subsequent studies using autosomal probes for testing between three and nine chromosomes detected mosaic aneuploidy in more than half of the embryos investigated ([39,41,42]). The development of single cell CGH analysis, allowing the copy number of every chromosome to be determined, and its application to analyse every cell from a total of 24 good quality three day old embryos demonstrated that 62% were mosaic ([43,44]). The extent of mosaic abnormality varied between the presence of a single abnormal cell to every cell being abnormal, but with the chromosomal constitution varying randomly from cell to cell (a feature termed 'chaotic'). Importantly, the CGH analysis also showed that a quarter of the embryos were totally euploid, with no chromosome imbalance by day 3 of development. Clearly, it is these euploid embryos that have the greatest potential to implant and develop normally thereafter.

5. Preimplantation genetic diagnosis for chromosomal disorders

Preimplantation genetic diagnosis (PGD) for chromosomal disorders involves analysis of a single cell, either the 1st PB or a blastomere taken on day 3 of development. As it is not possible to visualize the whole chromosome set with accuracy, PGD almost always involves FISH with chromosome-specific DNA probes. This enables the number of copies of each chromosome to be determined. The highest efficiency and accuracy is achieved by using probes for three pairs of chromosomes for each hybridization experiment. Probe mixes to detect five chromosomes are available commercially, but these involve some compromise on efficiency and accuracy.

5.1 Specific PGD for carriers of chromosomal rearrangements

One of the most common reasons for requesting specific PGD is where one parent is a carrier of a balanced chromosomal rearrangement (such as a translocation or an inversion) and this has led to infertility or frequent miscarriage. For each couple an individual protocol must be developed to detect embryos that have an unbalanced form of the rearrangement. A three probe strategy has been designed for reciprocal translocations, such that two of the probes are located either side of the breakpoint on one of the involved chromosomes and the third probe maps to any position on the second involved chromosome ([45]; Fig. 11). The main risk for these couples is due to segregation problems of the rearranged chromosomes at the first meiotic division, leading to the production of a

high proportion of unbalanced gametes ([46,47]). However, from the data gathered on the outcome of this type of specific PGD, it is apparent that the level of abnormality detected far exceeds that expected due to parental meiotic segregation errors alone. Much of the abnormality is chaotic in nature (affecting multiple chromosomes) and clearly post-zygotic in origin ([46-49]). The frequency of abnormality in the embryos from these cycles approaches 75%, adding weight to the suggestion that these particular couples are afflicted by two pathologies, the increased risk of parental meiotic segregation errors, compounded by additional factors that result in above normal rates of post-zygotic anomalies. It is possibly these additional factors that lead to the couple being referred for PGD. It should be noted, however, that it is nevertheless possible for most of the parents that carry a translocation or inversion to achieve a normal pregnancy by this type of advanced specific PGD, with the option available for confirmation of a normal pregnancy by prenatal diagnosis using fetal cells obtained by Chorionic Villus Biopsy (CVS) at 12-13 weeks of pregnancy or amniocentesis at around 16 weeks.

5.2 Preimplantation genetic screening for aneuploidy

Preimplantation genetic screening (PGS) was developed to improve the outcome for couples that were unable to achieve an ongoing pregnancy due to AMA, RIF or RM. In all these situations the parents themselves have normal somatic karyotypes. The aim is to provide a general screen for the most common aneuploidies affecting implantation and spontaneous abortion. Probe sets used invariably

contain those specific for chromosomes 13, 18 and 21; and frequently also include probes for chromosomes 16 and 22. The X and Y chromosomes may also be selected.

5.3 First polar body or blastomere for the diagnosis of aneuploidy?

Up to 20% of embryos overall will have an error that is due to meiotic chromosome segregation errors during the production of gametes in either parent, of these, 90% will be maternal. Initially it might seem that analysis of the 1st PB would be the most effective procedure for the identification of aneuploid embryos. However, as previously stated, at least 50% of embryos created by IVF are chromosomally mosaic, with a mixture of normal and abnormal cells ([42,50]) and this proportion increases in couples with RIF ([37,51]). This type of aneuploidy is due to errors in post-zygotic mitotic divisions.

5.4 Problems with 1st PB analysis

There are several problems with analysis limited to the 1st PB. Meiotic segregation of the primary oocyte will lead to two haploid cell nuclei, each normally expected to contain 23 chromosomes. Each chromosome will then consist of two chromatids that should still be attached at the centromere. Many of the chromosome-specific probes used for FISH hybridize to the centromere region and should appear as a doublet signal but this is not always clear. Therefore the distinction between the presence of one or two chromatids may be difficult, especially as the 1st PB rapidly degenerates. Assuming the

primary oocyte is normal disomic, loss of a chromosome or a chromatid from the 1st PB should be mirrored by a gain in the secondary oocyte, as these are the two haploid products of the first meiotic oocyte division. While an extra whole chromosome will always lead to a trisomic embryo, an extra chromatid in the secondary oocyte will lead to a trisomic embryo in 50% of cases, since it is likely to segregate randomly at AII after fertilization. This in turn highlights a second problem with 1st PB analysis – namely that events at AII will determine the eventual outcome. Ideally, therefore, both 1st and 2nd PBs should be analyzed where possible. If the 1st PB alone is analyzed this will lead to the discarding of oocytes with chromatid anomalies that could lead to a chromosomally normal embryo if the additional chromosome material is passed to the 2nd PB.

In cases of PGD for maternal carriers of chromosomal rearrangements, usually reciprocal or Robertsonian translocations, there are additional problems posed by crossing-over during the prophase of meiosis I. As oogenesis progresses in the mother (occurring during fetal development), crossing- over within the chromosome segment between the centromere and the breakpoint (the interstitial segment) will create a 'hybrid' chromosome with one normal chromatid and the other unbalanced due to the translocation. This may be detected by the use of 'chromosome paints' – collections of probes that hybridize along each chromosome arm – but once again it is not possible to predict whether the normal or the abnormal chromatid will pass to the oocyte ([52]). The corresponding oocytes complementary to such 1st PBs can thus not be used for fertilization. In summary, unless

analysis of the 2nd PB is also possible, many of the oocytes will unfortunately have to be discarded due to the uncertainty of the outcome after AII segregation.

5.5 Problems with single blastomere analysis

A single blastomere will provide an interphase nucleus for analysis. FISH analysis of interphase chromatin is less efficient than that of metaphase chromosomes, especially when locus-specific probes are used. Hybridisation failure is one source of error. Probes that detect multiple copies of a sequence, e.g. alpha satellite DNA associated with the centromere, give larger signals and are less prone to this type of technical error. Another major problem is that of chromosomal mosaicism; the cell chosen for biopsy and analysis may not be representative of the embryo as a whole. In the case of specific PGD for chromosomal rearrangements it would be advisable to take two cells for analysis, as embryos that are a mosaic of balanced and abnormal cells occur quite frequently ([49]). Unfortunately, however, the indications for PGS are such that removal of two cells is not considered advisable as this may retard embryonic development.

Embryo mosaicism is of two basic types, firstly, diploid/aneuploid mosaicism where the embryo began with a normal diploid complement and an aneuploid cell line developed during post-zygotic cleavage divisions, usually due to loss or gain of a chromosome (or similarly diploid/aneuploid mosaicism where the embryo began with an aneuploid complement and a normal cell line developed

during post-zygotic cleavage divisions, due to loss of the extra chromosome); and secondly a mixture of different aneuploid cells. Clearly, it is the first type where there is a mixture of normal and abnormal cells that has the most potential to cause misdiagnosis. The outcome of diploid/aneuploid mosaicism cannot then be predicted with certainty. In cases where an aneuploid cell is detected, the chances are that many of the remaining cells are normal. Equally, when a normal cell has been biopsied, it is still possible that a large proportion of the remaining cells are aneuploid. Analysis of a single blastomere out of eight available at 3 days post-conception is not likely to detect aneuploidy that affects one or two cells only. However, such embryos do have a reasonable chance of implantation and progression to a normal pregnancy.

5.6 Controversies over PGS

Given the high rates of chromosome abnormalities seen in embryos created by IVF, screening a cell from each embryo for aneuploidy should improve the outcome, especially for high risk couples. There is a large amount of evidence to indicate that PGS improves implantation and reduces miscarriage ([53-57]). However, these studies were retrospective and non-randomised. Others using randomised control trials (RCTs) were not able to demonstrate a significant difference between couples treated by PGS and a control group ([61,58]) at least when there was no restriction on the number of embryos to be replaced per cycle. Most recently, a RCT demonstrated a negative effect ([59]). There are crucial differences between these studies, quite apart from being randomised or not

being so; the methodology used in the RCTs has been widely criticised ([60]). For example in the studies by Staessen et al. ([61]) and Mastenbroek et al. ([59]) chromosomes 15 and 22 were not tested – the latter is a particularly important omission as it is one of those most frequently involved in the production of aneuploid oocytes originating from parental meiotic segregation errors. Most striking is the poor diagnostic success rate in the Mastenbroek et al. ([59]) study with 20% of embryos remaining undiagnosed, far higher than in any other reported study. This fact alone suggests that the group was not experienced at carrying out PGS, and other data presented indicates that the biopsy technique used was detrimental ([59]). In conclusion, evidence to date suggests that if PGS is carried out efficiently and is applied to appropriately chosen groups of patients who are at high risk of producing aneuploid embryos then it is beneficial ([37]). Couples who are likely to benefit from aneuploidy screening include those where the maternal age is over 40, couples experiencing repeated miscarriage (where the parents have normal chromosomes) and couples with recurrent implantation failure. The former two groups have been shown to have an increased risk of parental meiotic errors and the third group to have a very high rate of post-zygotic anomalies ([37,51])

5.7 Screening the whole chromosome complement

At the preimplantation embryonic stage, trisomy or monosomy can affect any chromosome; this has led to the development of technology which enables the whole chromosome complement to be screened for aneuploidy. The most effective method is that of CGH. This was first applied to analyse

single blastomeres from cleavage stage embryos ([43,44]) and provided concrete support for the findings of the interphase FISH studies. Namely that mosaicism was very common (60%) and that some of it was 'chaotic' in nature, with differing abnormalities in cells from the same embryo. The most striking finding from both studies was that only 25% of embryos were normal euploid.

The problem with diagnostic CGH analysis of single blastomeres is that the time taken precludes transfer of the embryo in the same cycle, requiring it to be frozen while analysis is carried out ([62]). With more efficient (vitrification) freezing protocols there is hope that this may now be feasible. In the meantime, others have approached the problem by subjecting the 1sr PB to CGH analysis ([34]). This provides an extra two days for analysis, allowing embryo selection and transfer in the same cycle, albeit with a very labour intensive protocol. As with any analysis involving the 1st PB, there needs to be confirmation either with the 2nd PB or by analysis of a blastomere. Some recent reports have failed in this respect ([63]; see also commentary by Fragouli et al. [64]). However, post-conception diagnosis is banned in Italy and Germany; for those countries 1st PB analysis is all that is available.

When dealing with extremely high risk patient groups the level of embryonic aneuploidy is so high that screening the whole chromosome set is not necessary since much of the mosaicism is chaotic ([37]). Additionally, certain groups of chromosomes are more likely to be affected by aneuploidy caused by parental meiotic chromosome segregation errors ([30]) and these are the ones chosen for PGS.

Paradoxically, the net effect of these considerations is that when dealing with groups of patients at lower risk, a more thorough screen of a wider range of chromosomes is needed to demonstrate any beneficial effect.

Summary: Evidence-Based Guidelines

Mammalian oogenesis, leading up to mature eggs having half the chromosome number in comparison to somatic cell nuclei, is a complex process. Uniquely, in human females this process is also highly error-prone, with a high proportion of mature eggs showing an abnormal chromosome number, aneuploidy. Superimposed on the segregation errors taking place during the meiotic cell divisions at oogenesis, there is a high amount of mitotic segregation errors during early embryogenesis, so that a high proportion of embryos are aneuploidy mosaics. The underlying reasons for these problems are not yet fully understood. Much work has been devoted within Assisted Conception Units to the development of schedules for pre-selection of embryos that, following IVF treatment, have the optimal chance of being implanted and the successful outcome of the pregnancy. This includes analysis of chromosome copy number in the 1^{st} and the 2^{nd} polar bodies as well as in individual cells, blastomeres, micro-manipulated from the embryo at the eight cell stage. Both types of investigation are labor intensive with limited success rate. Nevertheless, there are indications that these methods, if carried out efficiently and applied to appropriately chosen groups of patients, who are at high risk of producing aneuploid embryos, then they are beneficial.

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FIGURE LEGENDS

Figure 1 Schematic illustration of meiosis with homologous chromosome synapsis, and crossing-over at the Pachytene stage of Prophase I and the derivative bivalents at Metaphase I, followed by progression through Metaphase I to Anaphase I, Metaphase II to Anaphase II, and Telophase II showing the four potential haploid gametes

Figure 2 Changes in human oocyte number during prenatal and postnatal development

There is a very rapid increase in human female germ cell (oocyte) number early during fetal development with a peak at 7 months gestational age, followed by a relatively rapid decline before birth and postnatally before puberty, but a slower depletion during reproductive years until menopause.

Figure 3 Schematic illustration of the proteinaceous Synaptonemal Complex (SC)

During meiosis I, taking place in fetal oocytes, the two homologs are paired along their entire length, held together by a number of meiosis-specific proteins (cohesins, green and red). The process of crossing-over (black dot) takes place within the centre of the SC. Note that most of the chromatin (blue) is not exposed within the SC.

Figure 4 Imaging the Synaptonemal Complex (SC) in a spermatocyte (a) and an oocyte (b)

MLH1 foci are seen in yellow, the centromeres in the spermatocyte in magenta, and the SCs by SYCP3 staining in red. Note that the chromosome pairs (bivalents) are shorter in the spermatocyte than in the oocyte; and there are also a lower number of MLH1 foci/crossover points/chiasmata/recombination foci.

Figure 5 Cartoon illustrating the different types of meiosis I segregation that may take place in a normal disomy 21 oocyte

a) Normal chromosome pairing and crossing-over, attachment of the movement centres (kinetochores) at Metaphase 1 and separation at Anaphase 1

b) Lack of crossing-over and chiasma formation may lead to primary non-disjunction at Anaphase 1

c) Lack of a chiasma can also lead to the same type of segregation at Anaphase 1 as during mitosis (precocious meiotic disjunction)

Figure 6 Chromosomes at Metaphase I in a spermatocyte (a), and an oocyte (b)

Chiasmata can be reliably identified and counted in the spermatocyte, but this presents more difficulty in the oocyte.

Figure 7 Birth rate of trisomy 21 Down syndrome in relation to maternal age

The so-called maternal age effect was first recognized by Penrose in 1934, and has since been seen without much variation in different countries around the world.

Figure 8 FISH analysis of an MII oocyte to show an extra chromatid 16

Chromosomes are stained with DAPI and successively hybridized to probes from chromosomes 13 and 21, then 16, 18 and the X.

(Reproduced with permission from Mahmood et al., 2000, Hum Genet 10: 620-626; Copyright Springer Verlag Ltd)

Figure 9 Protocol for CGH analysis of individual MII oocytes and corresponding 1st PB

(Provided by Elpida Fragouli, Oxford University)

Figure 10 Outcome of meiotic non-disjunction of chromosome 12 visible in an oocyte/polar body complex after CGH analysis

The oocyte has lost one chromosome 12 and the PB has gained an extra chromosome 12. Reference DNA was from a 46, XX source, labelled in red in both cases; test DNA was labelled in green. (Provided by Anna Mantzouratou, University College London Centre for PGD)

Figure 11 Three probe strategy for detection of reciprocal translocations

Probes are located on both sides of the breakpoint on one of the chromosomes involved, and the third maps to the second involved chromosome. This example illustrates the FISH probe strategy for a carrier of 46XX, t(8;12)(q11.2;q12) for use in PGD. Probes are shown hybridised to the chromosomes of carrier parent (left) and to an embryonic nucleus, which is balanced for the translocation.

(Provided by Anna Mantzouratou, University College London Centre for PGD)

TABLES

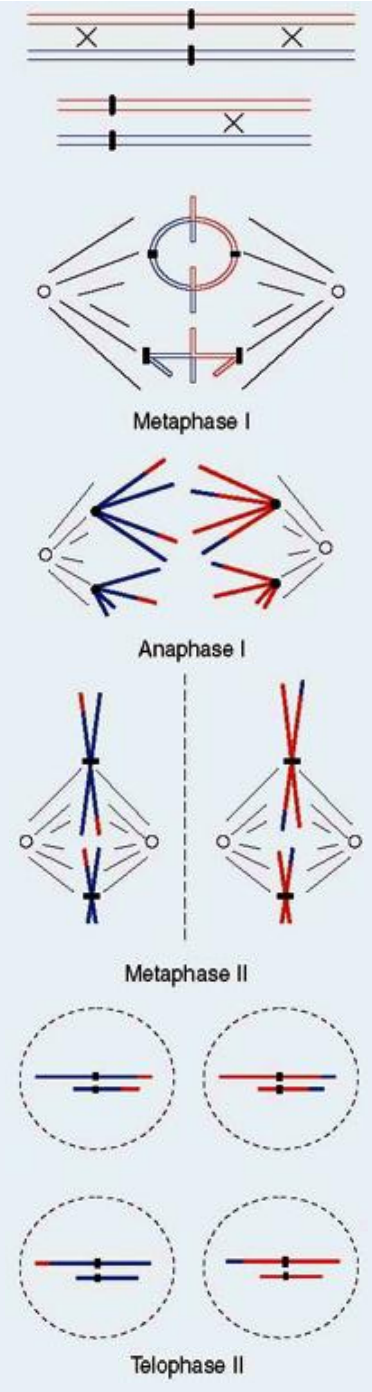
Table 1 Features and timing of meiotic stages in oogenesis (adapted from Hultén et al. [65])

		Stage	**Subdivision**	**Features**
FOETAL DEVELOPMENT	MEIOSIS I	Prophase I		DNA replication of each chromosome produce sister chromatids joined at the centromere
			Leptotene	Chromosome condensation begins, formation of the lateral elements of the synaptonemal complex
			Zygotene	Chromosomes pair, completion of the synaptonemal complex
			Pachytene	Chromosomes fully paired, crossing-over completed and chiasmata fully established
			Diplotene	Initiation of separation of the synapsed chromosomes, meiosis arrested at this stage until ovulation
ADOLESCENCE OVULATION		Prometaphase I	Diakinesis	Chromosomes condense and separate, except at chiasma sites
		Metaphase I		Homologous chromosomes align on the equatorial plate, the kinetochores attach to the spindles
		Anaphase I		Homologous pairs separate, sister chromatids remain together
		Telophase I		Two daughter cells form
	MEIOSIS II	Prophase II		Nuclear envelope dissolves, new spindle created
		Metaphase II		Chromosomes align on the spindle
FERTILISATION		Anaphase II		Centromeres separate, sister chromatids migrate to opposite poles
		Telophase II		Cell division results in four potential haploid gametes from each parent cell

Table 2 Main differences between oogenesis and spermatogenesis (adapted from Hultén et al. [65])

Oogenesis	**Spermatogenesis**
Can take over 40 years, few oocytes progress to the final stages, most are lost before birth	Continuous process from puberty throughout life
Two unequal cell divisions with most cytoplasm retained in the oocyte and only a minor part forming the1st and 2nd PBs. Thus only one mature egg is produced from each parent cell.	All divisions are equal and each parent cell produces four gametes.
Oocyte numbers appear to be limited to those present at birth, with approximately 350 ovulating between puberty and the menopause.	Continual supply of gametes
Interstitial initiation of chromosome synapsis is more common	Chromosome synapsis is initiated telomerically
Poor efficiency of obligate chiasma formation	Efficient obligate chiasma formation
Higher chiasma frequency	Lower chiasma frequency
Synaptonemal complexes are relatively decondensed, associated with higher chiasma frequency	Condensed synaptonemal complexes, associated with lower chiasma frequency

Figure 1



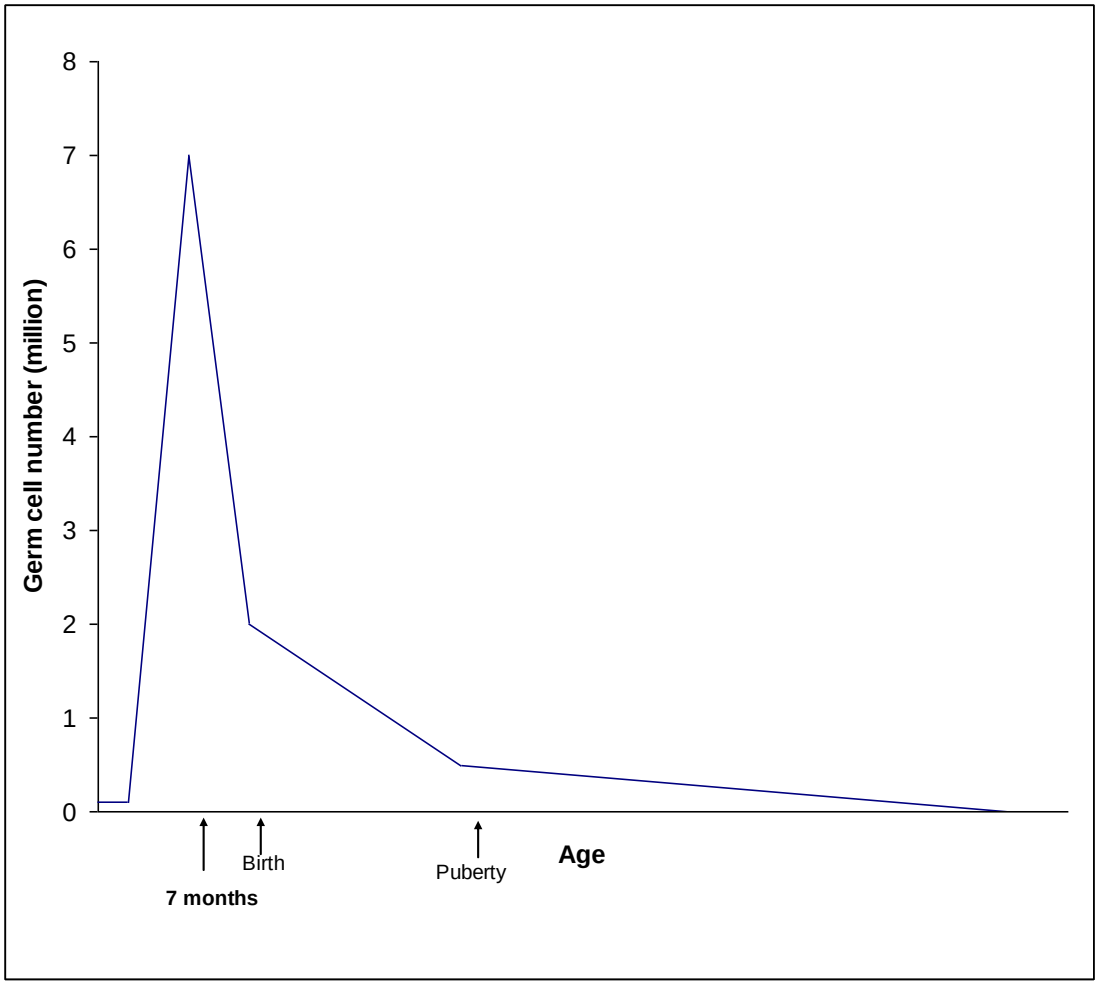

8
7
6
5
4
3
2
1
0
Germ cell number (million)
Birth
Puberty
Age
7 months

Figure 3

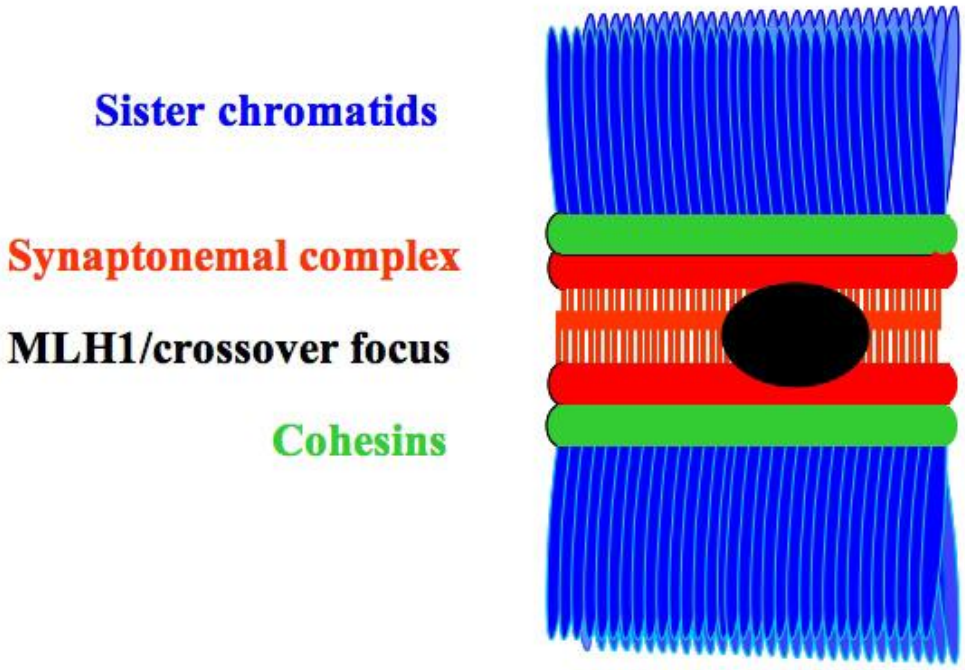

Sister chromatids
Synaptonemal complex
MLH1/crossover focus
Cohesins

Figure 4

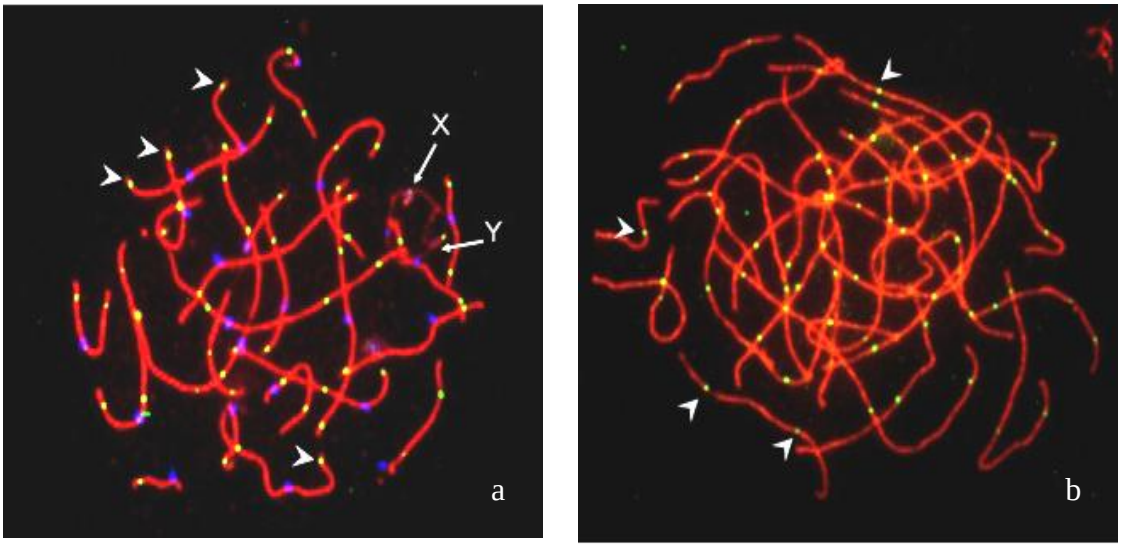


Figure 5

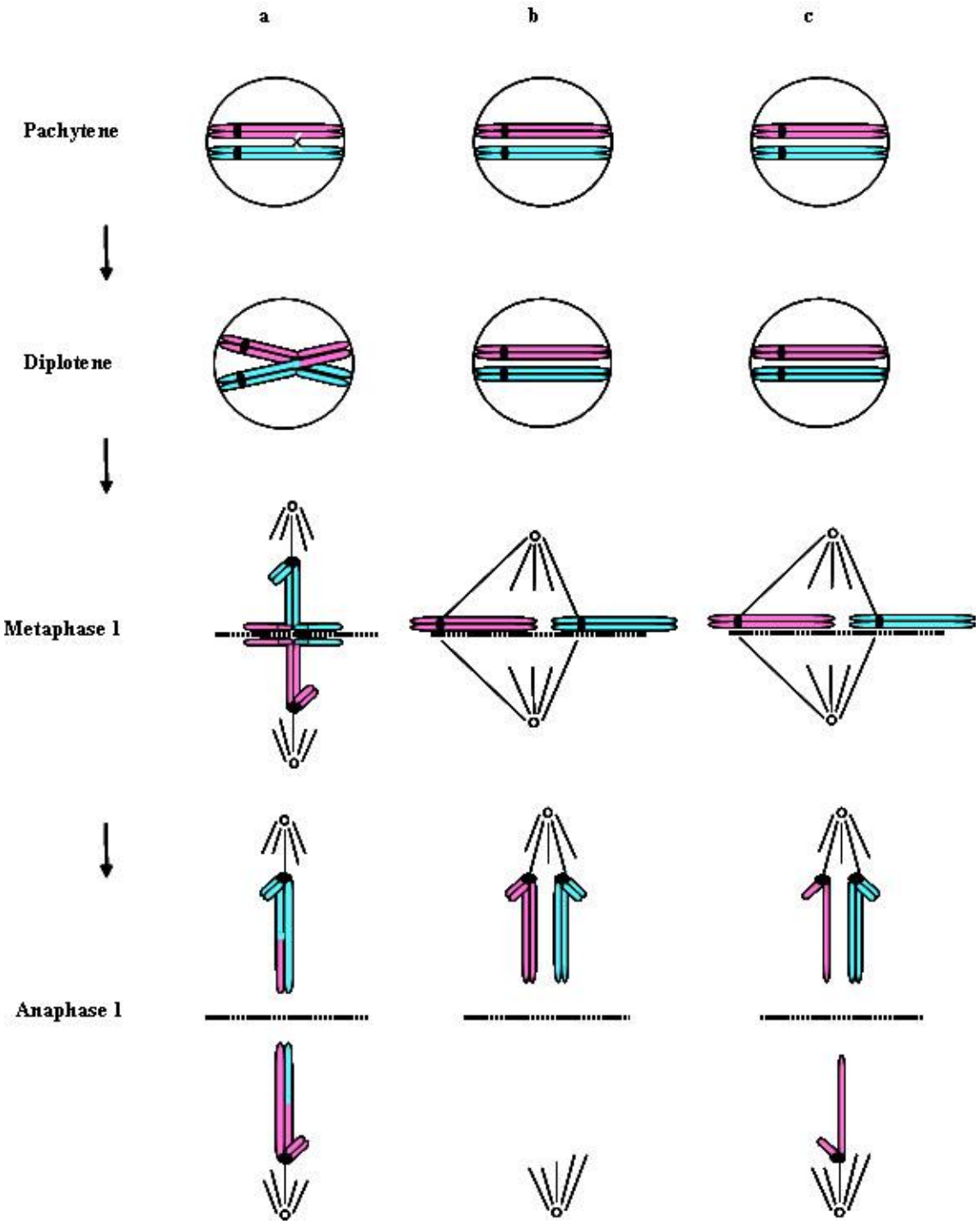
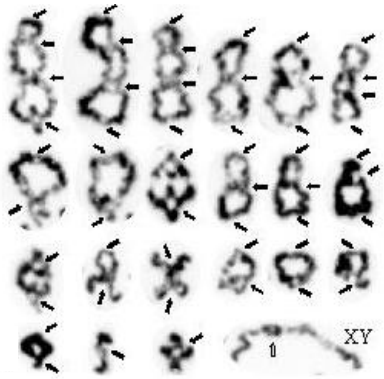
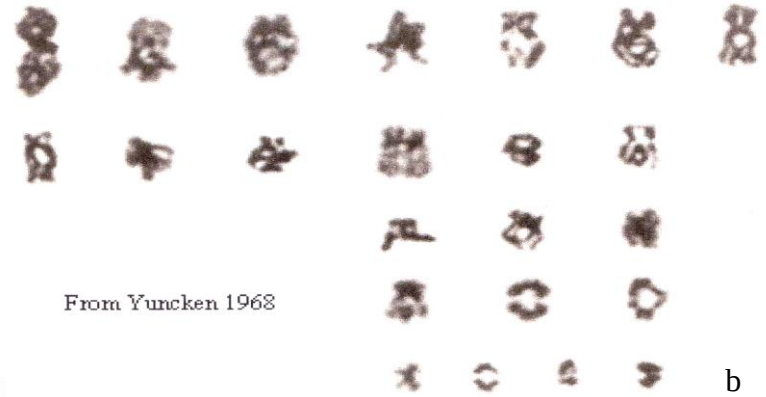


Figure 6



a



b

Figure 7

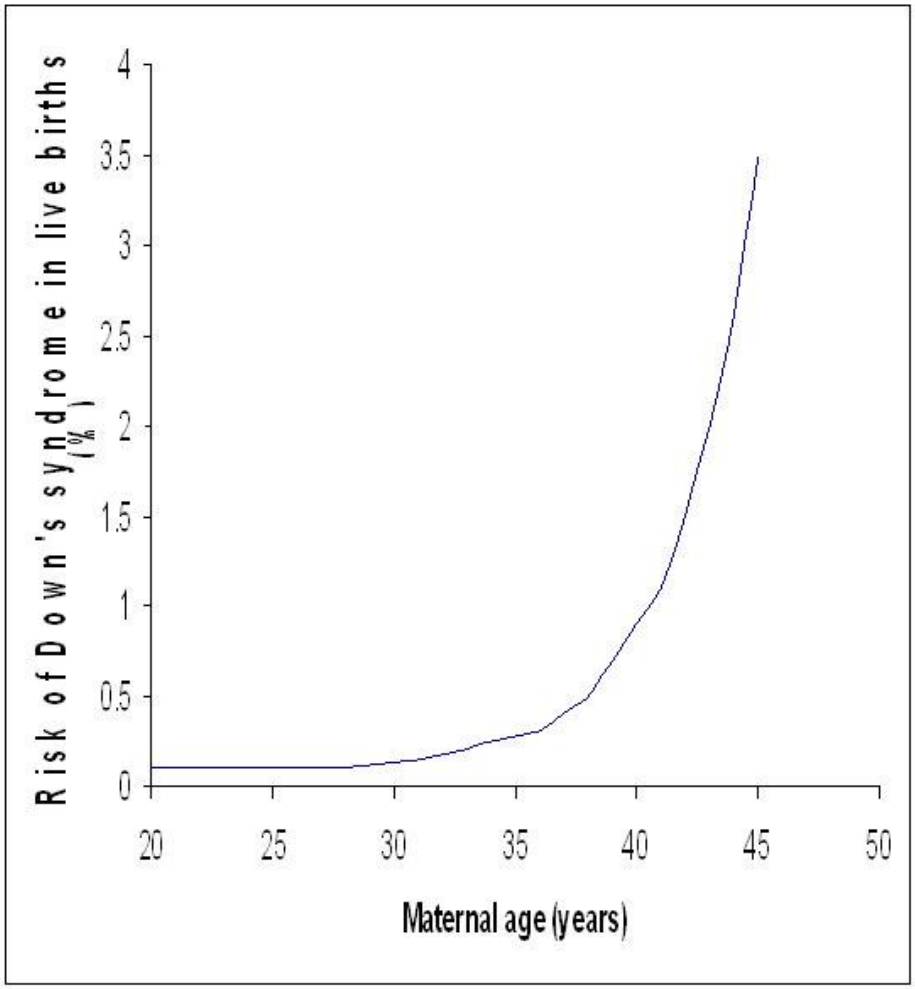

Risk of Down's syndrome in live births (%)
4
3.5
3
2.5
2
1.5
1
0.5
0
20
25
30
35
40
45
50
Maternal age (years)

Figure 8

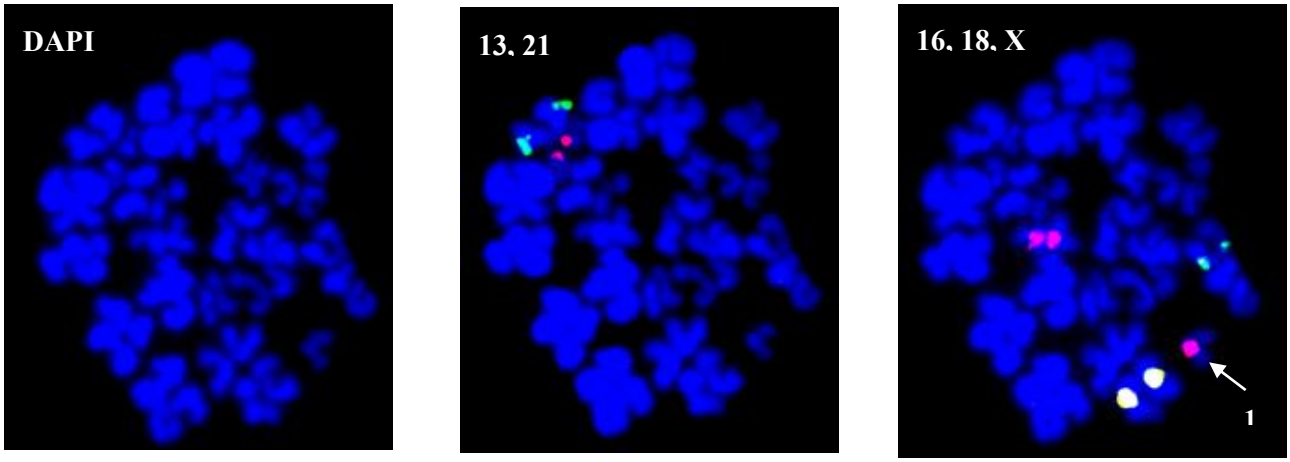


Figure 9

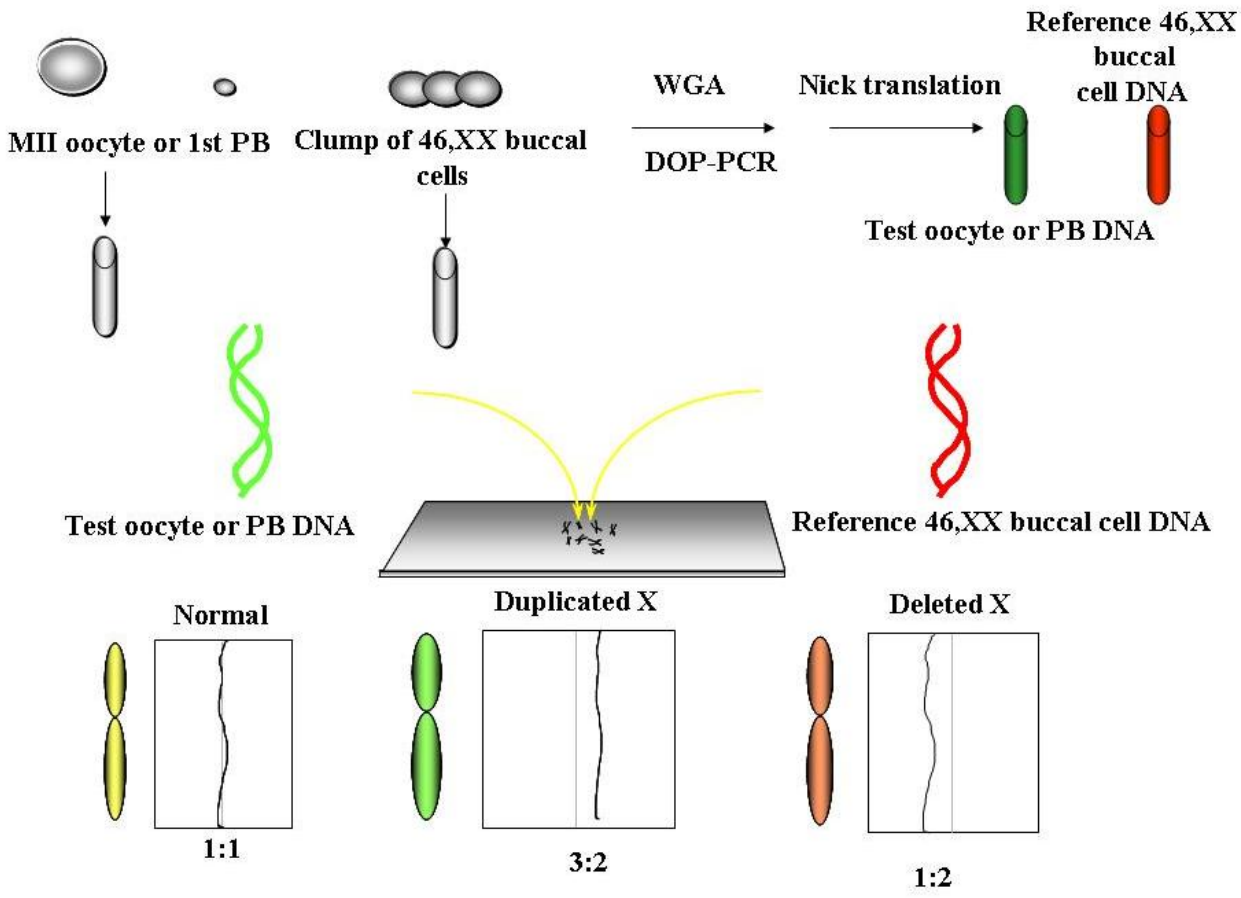
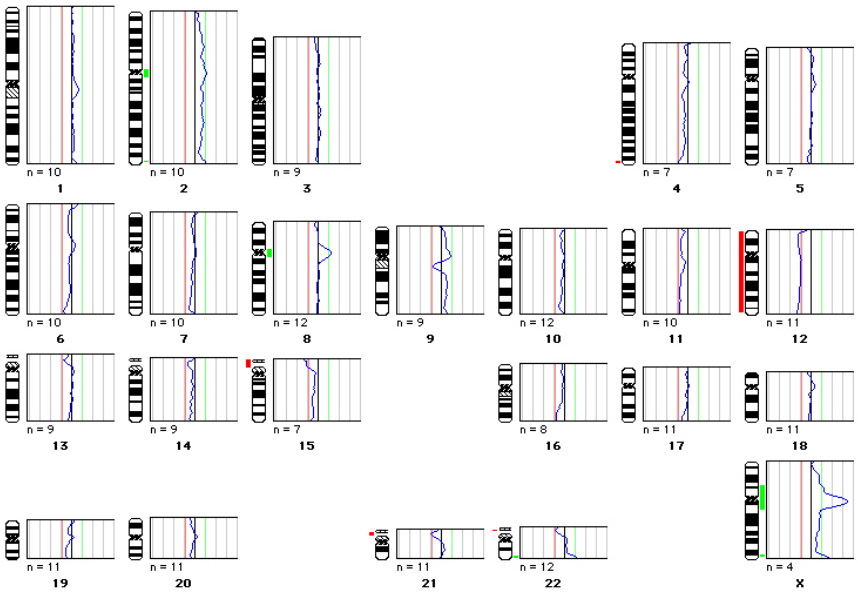

MII oocyte or 1st PB
Clump of 46,XX buccal cells
WGA
DOP-PCR
Nick translation
Reference 46,XX buccal cell DNA
Test oocyte or PB DNA
Test oocyte or PB DNA
Reference 46,XX buccal cell DNA
Normal
Duplicated X
Deleted X
1:1
3:2
1:2

Figure 10

Oocyte

.



1st polar body

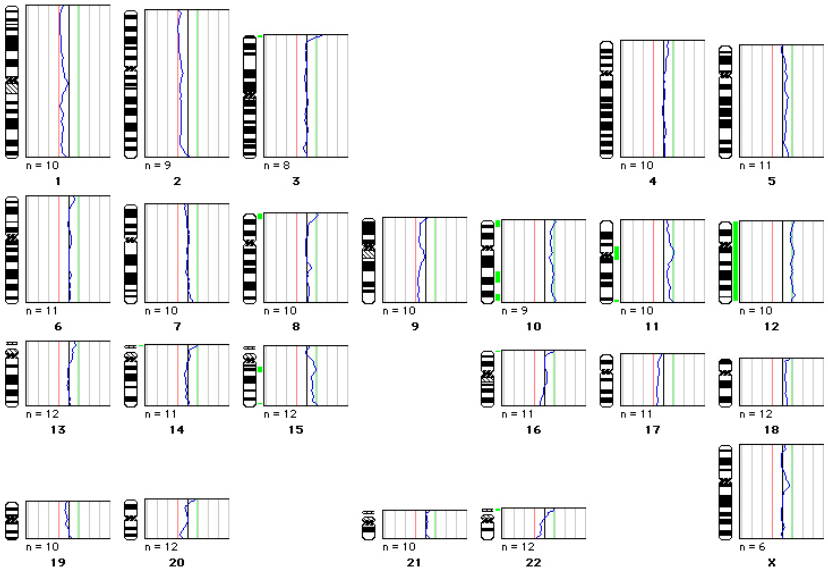


Figure 11