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# The Role of Aneuploidy Screening in Human Preimplantation Embryos

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## 1. Introduction

Aneuploidy can be defined as extra or missing whole chromosomes in the nucleus of a cell and occurs during cell division when chromosomes do not separate equally between the two new daughter cells (Hassold & Hunt 2001). Chromosome imbalance typically results in non-viability, manifesting as developmental arrest prior to implantation, miscarriage or stillbirth. Depending on the chromosomes involved, aneuploidy can also result in viable but developmentally abnormal pregnancies e.g. Down or Klinefelter Syndrome. In all cases, aneuploidy results in a non-favourable outcome for the family in question and is undoubtedly a major contributing factor to the relatively low fecundity of humans when compared with other species.

Aneuploidy is a particularly frequent event during both human gametogenesis and early embryogenesis in humans and arises due to mal-segregation of the chromosomes. The most cited mechanism is classical "non-disjunction" however this has been challenged in recent years and alternative mechanisms have been proposed (see subsequent sections). It is estimated that at least 20% of human oocytes are aneuploid, a number that increases dramatically with advancing maternal age over the age of 35 years (Dailey *et al.* 1996a; Hassold & Hunt 2001). Conversely, the incidence of aneuploidy in sperm cells from a normal fertile male is estimated to be as low as 4-7% (Martin *et al.* 1991; Shi & Martin 2000). However, this can significantly increase in some cases of severe male factor infertility.

The idea of screening pre-implantation embryos to eliminate the aneuploid ones is not new, but the ability to do this effectively has required rapid evolution of diagnostic technologies to combine speed, accuracy and reliability. To date, only direct analysis of chromosomes from cells in gametes and pre-implantation embryos (rather than indirect methods such as metabolic analysis) has proved successful in accurately detecting aneuploidy. Performing Preimplantation Genetic Screening (PGS) in this way involves the biopsy of cellular material from the embryo or oocyte at different stages of development. Since embryo biopsy is too invasive a procedure for routine embryo selection, PGS remains a test for high-risk patient groups only rather than for routine universal application. Worldwide, the test is only offered routinely to patients presenting with advanced maternal age, recurrent miscarriage, recurrent implantation failure and in some cases of severe male factor infertility. Due to the invasive nature of embryo biopsy and the complexity of human aneuploidy in the human IVF embryo,

cost benefit analysis is crucial to achieve positive outcomes. It could be argued that, in the past, the practice of PGS has not given proper concern to these issues and thus, going forward, patient selection and understanding the mechanisms of aneuploidy should be central to an effective PGS strategy. This chapter explores the premise underpinning the use of PGS in human embryos, its clinical applications, current methodologies and future applications.

## 2. Origin of aneuploidy

Aneuploidy in pre-implantation IVF embryos (and presumably also those naturally conceived) primarily arises during three developmental stages: (i) pre-meiotic divisions of gametogenesis; (ii) meiotic divisions of gametogenesis and (iii) early mitotic divisions of embryogenesis. Understanding the mechanism behind the mal-segregation of chromosomes at these stages gives insight into the limitations of PGS when applied clinically.

### 2.1 Gonadal mosaicism

Errors in germ cell proliferation or errors inherited in an otherwise somatically normal individual resulting in germ cell aneuploidy (gonadal mosaicism) can also contribute to aneuploidy of the gametes. This is perhaps the least studied of the three stages. In any event, the outcome is a hyper- or hypo- gamete and thus can be considered in the same way as a meiotic error.

### 2.2 Meiosis

Meiosis is the production of a haploid gamete by two specialised cell divisions in which the diploid chromosome complement of normal somatic cells is reduced (a requisite for sexual reproduction). Errors in chromosome segregation during these divisions typically result in gamete aneuploidy and subsequent 'uniform' aneuploidy in any resulting embryo. Although the basic principle of chromosome mal-segregation holds for both male and female meiosis in humans, the processes and resulting gametes are vastly different. Female meiosis, the process by which a single diploid germ cell develops into a single haploid ovum, involves two unequal meiotic divisions producing a mature ovum and two non-functional products containing mirror images of the chromosomes present in the ovum. These are known as polar bodies (PB) and, once extruded, take no further part in development thus making them a useful sample for inferring chromosome constitution of the oocyte itself. Failures in female meiosis make, by far, the biggest contribution to aneuploidy in human pre-implantation embryos. Cytogenetic studies on oocytes and first polar bodies (PB1) from assisted conception cycles have shown more than 20% of oocytes from patients with an average age under 35 to be aneuploid (Selva *et al.* 1991; Fragouli *et al.* 2006). The percentage of aneuploid oocytes increases significantly with age and has been shown to affect an average of around 70% of oocytes for patients of advanced maternal age (Van Blerkom 1989; Angell *et al.* 1993; Kuliev *et al.* 2003; Gutierrez-Mateo *et al.* 2004; Kuliev *et al.* 2005).

There is conflicting evidence on the frequency of errors in both the first and second meiotic division with groups showing errors in both the first meiotic division-MI (Kuliev *et al.* 2003) and more recently in the second meiotic division-MII (Fragouli *et al.* 2011; Handyside *et al.* 2012) occurring more frequently. This discrepancy may be due in part to differences in

patient maternal age of the study groups and the difference in resolution of the cytogenetic techniques used. Either way, it is clear that chromosome segregation errors occur at significant rates during both the first and second meiotic divisions of oogenesis.

Based on studies of yeast, drosophila and mouse models it was generally believed that aneuploidy arose as a result of classic nondisjunction and involved the segregation of a whole chromosome to the same pole as its homologue during meiosis. Studies of human oocytes led to an alternative model for the origin of aneuploidy (Angell 1991) suggesting that errors in meiosis can result in extra or missing chromatids (known as premature or precocious separation of sister chromatids - PS), as well as whole chromosomes in the daughter cells (See figure 1). Early studies of human oocytes supporting the hypothesis that precocious separation was the predominant mechanism leading to human aneuploidy were subject to recurring criticism (Angell 1991; Angell *et al.* 1993; Angell *et al.* 1994; Pellestor *et al.* 2002; Kuliev *et al.* 2003). It was argued that use of 'failed IVF' oocytes' prolonged time in culture, sub-optimal metaphase preparation technique, and lack of rigour in the analysis may have led to interpretation errors (Dailey *et al.* 1996b; Lamb *et al.* 1996; Lamb *et al.* 1997; Mahmood *et al.* 2000). Recently several groups, including our own, performed analyses using methodology less prone to these confounding criticisms - the results of which support the hypothesis. Quantitative analysis of loss or gain of all 24 chromosomes on PB1 (Gabriel *et al.* 2011b) and sequential 24 chromosome analysis of PB1, PB2 and zygote performed on freshly harvested oocytes used in IVF treatments (Geraedts et al. 2011) have shown PS to be the predominant mechanism of chromosome mal-segregation in assisted reproduction derived oocytes. This is consistent with recent data exploring the decline of adhesion molecules holding sister chromatids together during anaphase arrest leading to increased PS events (Chiang *et al.* 2010; Lister *et al.* 2010).

In contrast to oogenesis, male meiosis results in four equivalent functional spermatozoa from a single progenitor germ cell. The presence of typically millions of sperm per ejaculate make them easy to study *en masse* however it is impossible, with current technology, to screen a sperm head for aneuploidy then subsequently use it for PGS. This is because aneuploidy assessment of a sperm cell inevitably results in its destruction, and unlike in the ovum, there are no by-products available from which a determination of chromosome complement can be inferred.

The overall incidence of aneuploidy in sperm is estimated to be around 4-7% (Martin *et al.* 1991; Shi & Martin 2000) although some studies suggest it is as high as 14% in some infertile men (Johnson 1998; Shi & Martin 2001). Spermatogenesis can theoretically continue unchanged throughout the life of a man however several studies have shown there to be a correlation between increased sperm aneuploidy and advanced paternal age (Griffin *et al.* 1995; Robbins *et al.* 1995) albeit not as dramatic as in the female. Other factors such as male factor infertility, smoking and chemotherapy can however increase sperm aneuploidy levels, making individual couples in which these risk factors are present possible candidates for PGS.

### 2.3 Mitosis

Mitosis is the process by which a diploid cell usually divides into two chromosomally identical daughter cells. It is the primary mechanism by which a multicellular individual

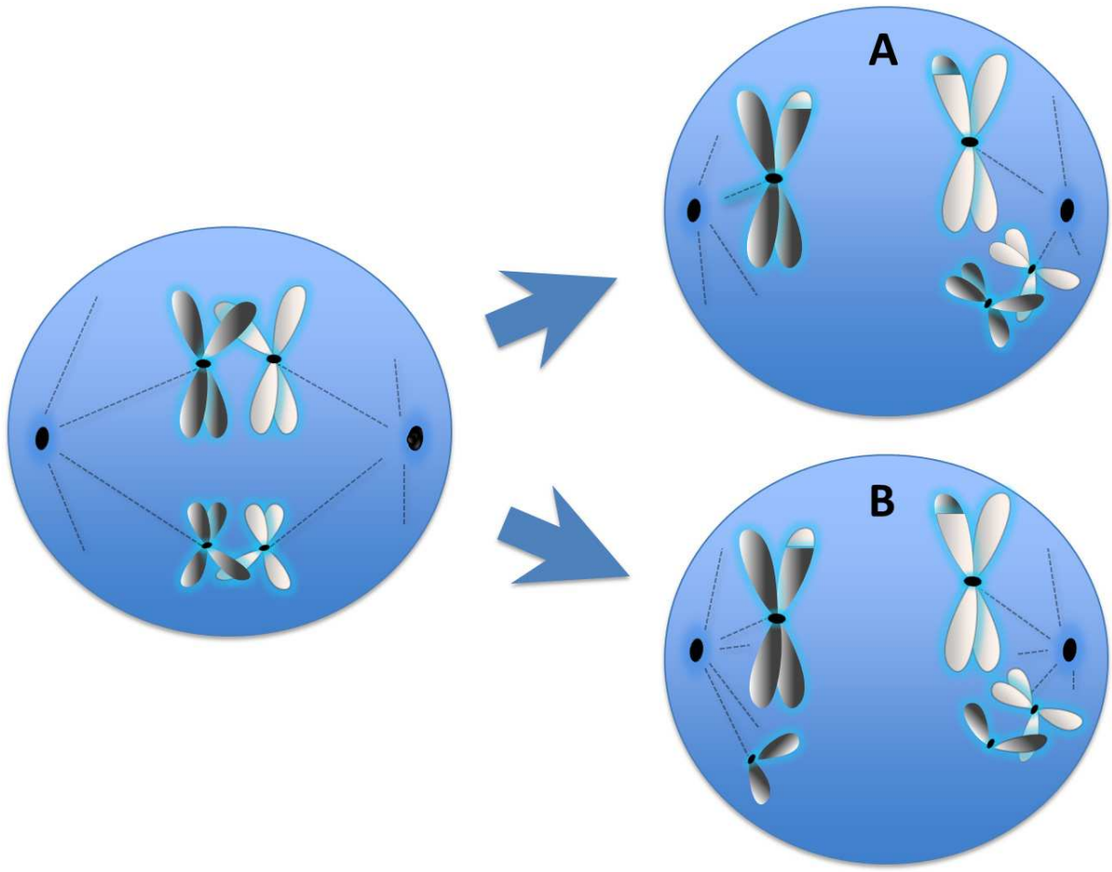


Fig. 1. Diagrammatic representation of classic nondisjunction (A) and premature separation of the sister chromatids (B), the two predominant mechanisms by which aneuploidy can arise in the first meiotic division in humans.

develops from a single fertilised oocyte (zygote). Human mitotic divisions are generally not prone to chromosome segregation errors to any great extent, except in the case of early embryo cleavage stages where cells are thought to be exquisitely prone to segregation errors (Bean *et al.* 2001). Indeed, recent studies using a variety of cytogenetic techniques on early IVF human embryos have demonstrated that over 50% are subject to some form of mitotic error (Bielanska *et al.* 2002; Munne *et al.* 2004; Delhanty 2005; Munne 2006).

Most mitotic errors in early embryo development will lead to chromosomal mosaicism which is defined as the presence of two or more chromosome complements within an embryo developed from a single zygote. There are three hypothesised mechanisms by which mitotic aneuploidy can arise: (i) chromosome loss (presumably from anaphase lag resulting in chromosome loss in one cell line), (ii) chromosome duplication (the underlying mechanisms of which are not well understood) or (iii) reciprocal chromosome loss and gain (resulting mainly from a mitotic nondisjunction event or potentially anaphase lag creating one cell line with chromosome loss and one with a reciprocal gain) (see figure 2). Following observations of increased incidence of chromosome loss in pre-implantation embryos compared to gains and the relative paucity of reciprocal events -which would indicate non-disjunction (Daphnis *et al.* 2005; Delhanty 2005) the predominant mechanism leading to post-zygotic errors in human

embryos is likely to be chromosome loss resulting from anaphase lag (Coonen *et al.* 2004). Anaphase lag is described as the delayed movement during mitotic anaphase of a homologous chromosome resulting in it not being incorporated into the nucleus of the daughter cell. Often the lagging chromosome is lost creating one euploid daughter cell and a daughter cell with a monosomy for the chromosome in question.

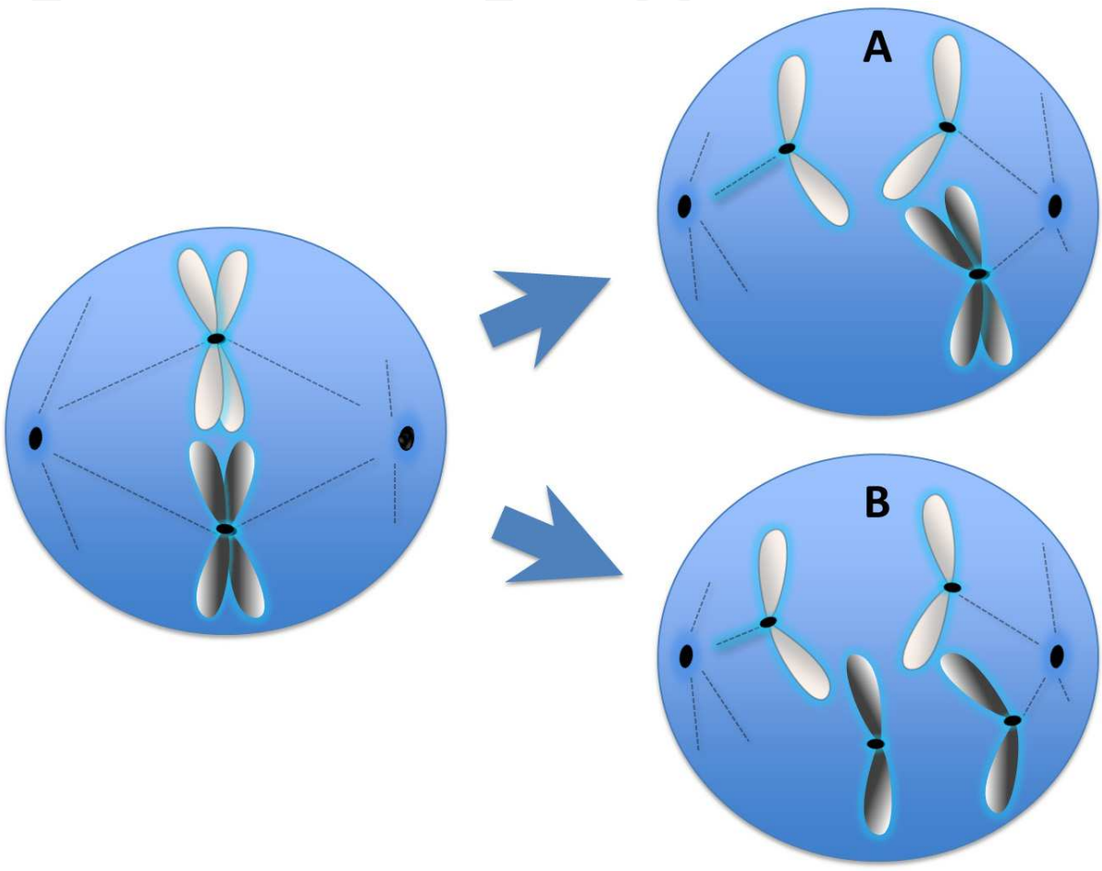


Fig. 2. Diagrammatic representation of anaphase lag (A) and mitotic nondisjunction (B), the two predominant types of mitotic errors in humans resulting in embryo mosaicism.

Mosaicism is considered to be largely independent of age (Delhanty 2005). However, it has been shown that mosaicism originating by the mechanism of mitotic non-disjunction could perhaps be related to advanced maternal age (Munne *et al.* 2002). Results also suggest that mosaicism involving multiple chromosomes and a high proportion of cells (chaotic embryos) appear to impair early embryo development considerably.

The general consensus for the viability of mosaic embryos is that, if more than half of the cells at day 3 post fertilization are aneuploid, the embryo is unlikely to be viable. Conversely, if a small proportion of cells are aneuploid in an otherwise healthy and euploid background, it is likely to be viable (Delhanty 2005).

Clearly mosaicism affects embryo development making it a key element in the selection of embryos in clinical IVF cases (Bielanska *et al.* 2002; Delhanty 2005).

### 2.4 Abnormal fertilisation

Abnormal fertilisation can also contribute to chromosome errors in pre-implantation embryos. Approximately 1% of conceptions contain more than two paired homologous sets of chromosomes-referred to as polyploidy rather than aneuploidy (Hassold 1986). There are two ways in which a polyploid embryo can arise: Firstly, if a diploid (2n) sperm or oocyte is involved in the fertilisation event and secondly, if two or more haploid sperm are involved in the fertilisation of a haploid oocyte (polyspermy). The majority of all polyploid embryos are the result of polyspermy and account for around 60% of polyploid conceptions (Egozcue *et al.* 2002). Following IVF with ICSI in which only a single sperm is inserted into each oocyte, the main mechanism leading to polyploidy in the embryo is the failure of the oocyte to extrude the second polar body (Grossmann *et al.* 1997). This results in a triploid embryo when fertilisation is achieved with a haploid sperm. Non-reduced or diploid sperm have also been shown to be involved in as many as 8.3% of polyploid conceptions (Egozcue *et al.* 2002).

## 3. Aneuploidy and IVF development

Since the first human IVF success in 1978 (Steptoe & Edwards 1978), advances in morphologic embryo grading and technologies aiding morphologic embryo selection have contributed to vastly improved IVF outcomes (Figure 3). Unfortunately, the morphological selection criteria for human gametes and embryos across all developmental stages have shown only weak correlations with aneuploidy (Munne 2006; Gianaroli *et al.* 2007; Alfarawati *et al.* 2011a). Karyotypic analysis indicates that there is a higher rate of chromosome abnormalities in morphologically abnormal monospermic embryos than morphologically normal embryos (Pellestor 1995; Almeida & Bolton 1996). However, clear distinctions cannot be made between chromosomally normal and abnormal human embryos by morphological assessment alone (Zenzes & Casper 1992). This may be because chromosome abnormalities detected at the early stages of embryogenesis cannot induce dysmorphism, since embryonic gene expression has not yet commenced (Braude *et al.* 1988; Tesarik *et al.* 1988). There is evidence from 24 chromosome copy number analysis that morphology and aneuploidy are linked at the later stages of pre-implantation embryo development (blastocyst stage). However, again the association is weak, and consequently, morphologic analysis can still not be relied upon to ensure transfer of chromosomally normal embryos. A significant proportion of aneuploid embryos are capable of achieving the highest morphologic scores even at the later stages of pre-implantation development, and, conversely, some euploid embryos achieve only poor morphological scores or even fail to develop (Alfarawati *et al.* 2011a).

Other indirect aneuploidy screening methods have been trialled in the past with limited success. More recently, proteomic studies have shown to be a potentially useful tool in prenatal aneuploidy screening (Cho & Diamandis 2011; Kolialexi *et al.* 2011). By applying the same principle to pre-implantation embryos, one study has identified the first protein secreted by human blastocysts that is associated with generic chromosome aneuploidy (McReynolds *et al.* 2011). Although promising, this technology is still some way from becoming a routine aneuploidy screening test and oocyte or embryo biopsy with molecular cytogenetic analysis is still the preferred technique for PGS.

All molecular cytogenetic techniques involving gametes and embryos require direct access to the nuclear material of the gametes or blastomeres themselves. This process is achieved by cell biopsy and inevitably results in the destruction of the cells involved. With this in mind, it is important that fertilisation or embryogenesis is not compromised and the biopsy procedure impacts minimally on developmental potential.

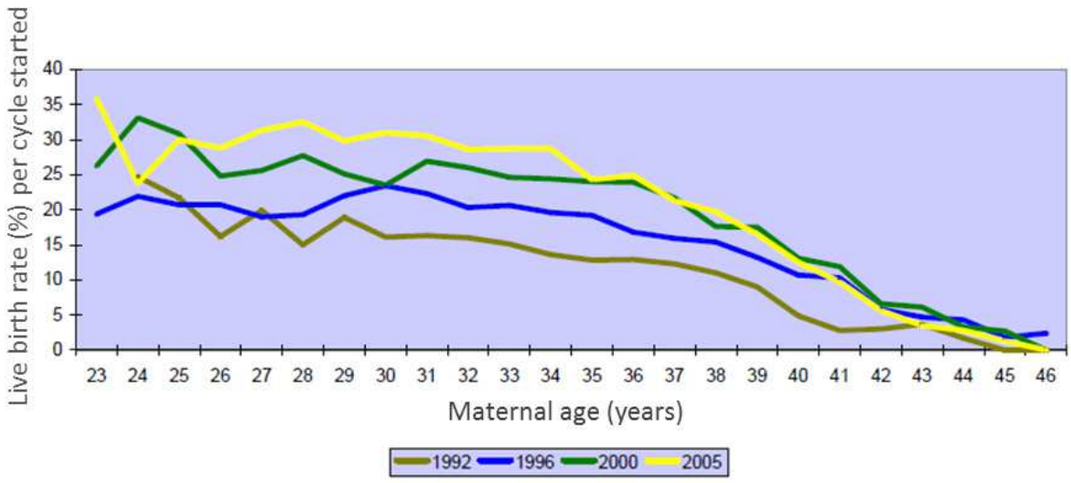


Fig. 3. Overall IVF and IVF/ICSI success rates by maternal age in the UK from 1992 – 2005. Figure adapted from Human Fertilisation and Embryology Authority website [ HFEA] (2008a)

Recent clinical trials and meta-analyses of cases have suggested no benefit, and in some cases worse IVF pregnancy outcomes following PGS- presumably the result of discard of normal embryos (diagnosed as abnormal – false positives), detrimental effects of the biopsy including reduction of cellular mass and excessive micromanipulation outside of the incubator (Mastenbroek *et al.* 2007; Twisk *et al.* 2008). These results have however been dismissed by many PGS practitioners due to questionable experimental design (Munne *et al.* 1999; Handyside & Thornhill 2007). Nevertheless, at the very least, these trials have reinforced the idea that embryo biopsy can only be justifiable when the benefit of the testing outweighs the cost to the embryo, since the ultimate aim of PGS is to identify chromosomally competent embryos without compromising embryo viability.

## 4. Oocyte/embryo biopsy

Biopsy for PGS is currently a two-step micromanipulation process involving the penetration of the zona pellucida followed by the removal of one or more cells for chromosome analysis. Breaching the zona is generally performed by laser ablation as it has been shown, when used appropriately, to have no detrimental effects on embryo development in both animal and human studies (Montag *et al.* 1998; Park *et al.* 1999; Han *et al.* 2003). Specialised micromanipulation pipettes are then used to separate the required cells from the oocyte or embryo. Theoretically, PGS can be accomplished at any developmental stage from the mature (MII) oocyte to the blastocyst stage. To date only three discrete stages have been proposed for clinical use: (i) polar body (oocyte and/or zygote), (ii) cleavage stage (day 3

embryo) and (iii) blastocyst (day 5 or 6 embryo). Each of these stages is biologically different thus having different diagnostic limitations in terms of information to be gained and impact on embryo viability.

### 4.1 Polar body biopsy

The removal of PB1 and/or PB2 from a human oocyte should have no deleterious effect on subsequent embryo, foetal and infant development as neither is required for successful fertilisation or embryogenesis (Gianaroli 2000; Strom *et al.* 2000). Biopsy and subsequent analysis of the first and second polar bodies allows the indirect interpretation of the chromosome complement of the corresponding oocyte thereby allowing the detection of maternally derived aneuploidy in resulting embryos (Verlinsky *et al.* 1996). While biopsy of PB1 alone and a combined PB1 and PB2 strategy have been used clinically for PGS, it is becoming increasingly evident that PB1 alone has limited applicability to PGS as only errors in MI can be detected and even MI chromatid segregation errors may not all be detected without analysis of both polar bodies. Indeed, as much as 30% of aneuploidy of maternal origin will not be diagnosed if only PB1 is sampled (Handyside *et al.* 2012). It is therefore the authors opinion that biopsy of both first and second polar body is essential for optimal detection of oocyte aneuploidy if used as an embryo selection tool. A further limitation is that cytogenetic analysis of either polar body does not allow the detection of aneuploidies of paternal origin nor aneuploidies arising after fertilisation in the embryo.

The process of polar body biopsy is relatively labour intensive and may involve the micromanipulation of oocytes that ultimately do not develop into therapeutic quality embryos. Sometimes up to four manipulations - ICSI, PB1, PB2 and blastomere biopsy (as a reflexive test following test failure or an ambiguous PB result) may be required. However, in experienced hands, even 3 independent biopsy manipulations appear to have no deleterious effect on development (Magli *et al.* 2004; Cieslak-Janzen *et al.* 2006). Although simultaneous removal of PB1 and PB2 is possible on day 1 of embryo development (Magli *et al.* 2011) there may be advantages to sequential biopsy where PB1 is removed on day 0 (day of insemination) followed by the removal of PB2 on day 1. This is to avoid any degeneration of PB1 leading to possible diagnostic failure and also to allow for the distinction between polar bodies, thereby allowing accurate identification of errors in the first and second meiotic divisions.

### 4.2 Cleavage stage embryo biopsy

Historically cleavage stage biopsy was the most widely practiced form of embryo biopsy for PGS worldwide. This biopsy strategy is now becoming less popular however due to its potential detrimental effect on embryo viability and the problem of mosaicism in human cleavage stage embryos. A typical procedure for cleavage stage biopsy involves the removal of one or two blastomeres from an embryo on day 3 post-fertilization – usually those of suitable quality with at least 5 cells having entered the third cleavage division. Although cleavage stage biopsy allows the detection of maternally and paternally derived aneuploidy as well as post-zygotic errors, they are not always distinguishable. The main problem affecting cleavage stage biopsy is chromosomal mosaicism resulting in an increased rate of false positive and negative results from single cell (or two cell) analysis (Figure 4).

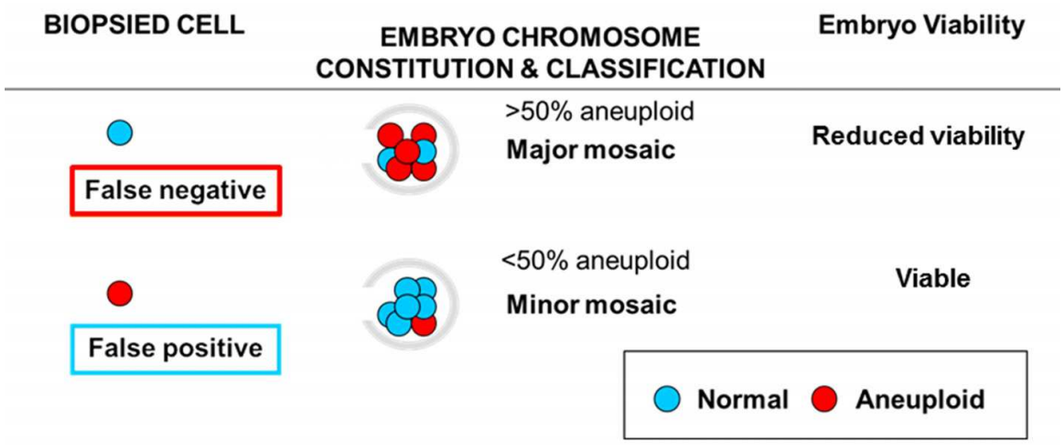


Fig. 4. Schematic representation of possible misdiagnosis following cleavage stage biopsy of a single cell

Data from studies comparing undiagnosed biopsied embryos and non-biopsied control embryos showed a detrimental effect of biopsy on implantation (Cohen & Grifo 2007; Mastenbroek *et al.* 2007), most evident in embryos of suboptimal quality. Animal models have shown that the potential for the embryo to continue to develop and implant is progressively compromised the greater the proportion of the embryo is removed (Liu *et al.* 1993). While such evidence provides fuel for the argument against performing biopsy at early cleavage stages at all, evidence from frozen-thawed embryo transfers (as a proxy for biopsied embryos) in which successful implantations and live births can be achieved even following embryonic cell death demonstrates that a certain degree of cell loss is tolerated (Cohen *et al.* 2007). However, just as in the animal models, success is inversely correlated with the amount of cellular mass lost.

The successful application of cleavage stage biopsy minimising cell removal from good quality embryos shows it is compatible with normal embryo metabolism, blastocyst development and foetal growth (Hardy *et al.* 1990). Moreover, studies of pregnancies and children born after cleavage stage biopsy have identified no significant increase in abnormalities above the rate seen in routine IVF (Harper *et al.* 2006; Banerjee *et al.* 2008; Nekkebroeck *et al.* 2008).

A general estimate therefore is that cleavage stage biopsy of 1 cell may reduce the implantation potential of an IVF embryo of around 10% although this figure would inevitably increase in less experienced hands (Cohen & Grifo 2007). The challenge for any future application of cleavage stage biopsy PGS therefore is to ensure that any benefits outweigh these costs and it remains a question whether this will be possible even with more accurate and reliable tests given the high levels of mosaicism.

### 4.3 Blastocyst stage biopsy

Blastocyst biopsy involves the sampling of trophectoderm (TE) cells, the spherical outer epithelial monolayer of the blastocyst stage embryo. Just as at cleavage stage, TE biopsy is

able to detect aneuploidy arising in either gamete or post-fertilisation. It is more akin to early prenatal diagnosis when compared to the other biopsy stages as it involves the removal of up to 10 cells without depleting the inner cell mass (ICM) from which the foetus is derived. TE biopsy is most commonly achieved by partial zona dissection followed by a period of culture in which time the expansion of the blastocyst will cause herniation of several cells through the artificial breach. The herniating cells (~4-10 cells) are then easily removed by excision or aspiration using micromanipulation tools with or without the aid of a laser. Sampling of several cells at this stage lessens the likelihood that mosaicism will produce false positive results also overcoming the limitations of extreme sensitivity apparent with conventional single cell diagnosis.

As with cleavage stage biopsy, it has been suggested that the removal of cells may negatively impact on the embryo's developmental potential. However, skilled biopsy practitioners are able to remove TE cells and achieve comparable implantation rates to non-biopsied blastocyst stage embryos (Kokkali *et al.* 2007). It has also been proposed that sampling of the TE may not reflect the genetic composition of the ICM (Kalousek & Vekemans 1996). Recent data however comparing TE to ICM suggests 100% concordance with the exception of structural abnormalities (Johnson *et al.* 2010a).

Currently the main limitation of blastocyst biopsy is the low number of embryos that reach the blastocyst stage; a number that significantly decreases with advanced maternal age (Pantos *et al.* 1999). If very few blastocysts are available, particularly in older patients, biopsy for selection purposes may be of no benefit. Also, time constraints at the blastocyst stage dictate, in many cases, the need to cryopreserve biopsied blastocysts awaiting diagnosis. Thus, the effect of cryopreservation and subsequent thawing on embryo viability must be taken into account. Nonetheless, improved culture techniques, possible vitrification and rapid molecular analysis regimes are making blastocyst biopsy an increasingly attractive option (Schoolcraft *et al.* 2010).

## 5. Molecular cytogenetics – The rise and fall of fluorescence *in situ* hybridization (FISH) in PGS

Following embryo or oocyte biopsy, PGS requires cytogenetic techniques with high sensitivity and specificity to establish the chromosome composition of the embryo via the analysis of one or very few cells. Classic karyotyping techniques are not suitable for pre-implantation testing due to the difficulty of achieving good metaphase spreads with the limited cells available for testing (Angell *et al.* 1986; Papadopoulos *et al.* 1989). In 1993 the application of Fluorescent In-situ Hybridisation (FISH) for the single cell detection of the sex chromosomes in pre-implantation embryos provided a springboard for aneuploidy detection and clinical application of PGS soon followed (Griffin *et al.* 1992). FISH is a highly sensitive, relatively inexpensive molecular cytogenetic tool enabling the determination of chromosome copy number at the single cell level. Its successful application rapidly led to the implementation of PGS as a clinical adjunct to IVF globally. To date tens of thousands of PGS cases have been performed globally, attesting to its popularity. Nonetheless, advances in technology are making FISH for PGS in oocytes and embryos a less attractive option due to a range of technical and biological considerations that are becoming increasingly apparent (Table 1).

| Test | Chromosomes detected | Resolution | Parental DNA required | Polyploidy | Recombination mapping | Origin of aneuploidy | |
|---|---|---|---|---|---|---|---|
| | | | | | | parent of origin | MI or MII |
| Fluorescence in situ hybridization (FISH) | 5 to 12 | low | no | yes | no | no | no |
| Array comparative genomic hybridisation (aCGH) | 24 | high | no | no | no | no | no |
| Single nucleotide polymorphism (SNP) array | 24 | highest | yes | yes | yes | yes | limited to hyperploidy |

Table 1. Scope and limitations of different molecular cytogenetic techniques after embryo biopsy

### 5.1 Fluorescent *In-situ* Hybridisation (FISH)

FISH requires the fixation of biopsied cells to a glass slide before visual analysis of hybridised fluorescent chromosome specific DNA probes. The advantage to the observer of being able to view the presence of chromosome copy number directly is considerable. Technical issues however include the fact that FISH signals can overlap (making two signals appear as one, or three as two) or "split" according to the stage of the cell cycle making a single signal appear as two (Cohen *et al.* 2009). Initially only five different fluorescent probes attached to different chromosomes (typically 13, 16, 18, 21, 22 or 13, 18, 21 X and Y) were used, however a recent study analysing twelve chromosomes (X, Y, 2, 4, 13, 15, 16, 18, 19, 20, 21, 21) at the cleavage stage described detection of 91% of chromosomally abnormal embryos reaching the blastocyst stage. In this case, if the misdiagnosis rate of each probe averaged 1%, over the two rounds of hybridisation required, an accuracy of only 88% per embryo could be achieved. The test's ability to diagnose only 91% of aneuploid embryos compounded by the 12% misdiagnosis rate per embryo would result in only 80% efficiency of the test in its ability to diagnose aneuploidy per embryo. This would inevitably result in the transfer of aneuploid embryos (false negative) or the discarding of euploid embryos (false positive). It has been widely accepted that the efficiency of each probe is reduced in subsequent hybridisation rounds (Harrison *et al.* 2000) however a 24 chromosome FISH assay has recently been applied to preimplantation human embryos with no apparent loss of signal, even after four rounds of hybridization (Ioannou *et al.* 2011).

The importance of low error rates on the diagnostic efficiency of PGS is strongly argued (Summers & Foland 2009; Munne *et al.* 2010), as is the need to detect all chromosomes simultaneously for aneuploidy. Notwithstanding the ability now to detect all 24 chromosomes by FISH, the issues of mosaicism, signal interpretation, clinical trial data and the development of microarray based methods for detecting 24 chromosome copy number are now signalling the demise of FISH based PGS approaches. Microarray based tests are now becoming the standard and these have been made possible through the advancement of whole genome amplification (WGA) technology.

### 5.2 Whole genome amplification (WGA)

The introduction of whole genome amplification (WGA) techniques has led to new more efficient 24 chromosome molecular karyotyping tests. WGA brought with it the potential to increase the amount of cytogenetic information that can be obtained from a single nuclear

genome contained in one cell. A single cell contains 6pg of DNA, far less than the 0.2-1.0μg usually required for microarray analysis and thus the need for amplification is paramount (Wells & Delhanty 2000). The process simply involves the transfer of the cell(s) to a microfuge tube followed by cell lysis prior to genome amplification either by polymerase chain reaction based methods or, more recently, multiple displacement amplification (MDA) to yield quantities of DNA in excess of 20 μg from a single cell. These products can in turn be used for genome wide analysis studies to establish chromosome copy number with impressive accuracy. One of the biggest drawbacks of single cell DNA amplification is a phenomenon known as allele dropout (ADO) where only one of the two alleles at a locus successfully amplifies (Walsh *et al.* 1992; Findlay *et al.* 1995; Piyamongkol *et al.* 2003). This proved a limiting factor on the resolution and reliability of PGD for single gene disorders where individual gene sequences are analysed but is less of an issue for array based PGS where many probes along each chromosome are used (Ling *et al.* 2009). Further problems involving the extreme sensitivity of single cell analysis still exist in the form of failed or poor amplification. However, these failure rates can be maintained at under 3% in experienced laboratories (Gutierrez-Mateo *et al.* 2011).

### 5.3 Comparative genomic hybridisation (CGH)

Originally designed for molecular karyotyping of tumour cells (Kallioniemi *et al.* 1992; Kallioniemi *et al.* 1993), comparative genomic hybridisation (CGH) has been successfully adapted for the analysis of human polar bodies and pre-implantation embryonic cells (Voullaire *et al.* 2000; Wells *et al.* 2002). Originally a labour intensive and time consuming procedure involving hybridization to and analysis of standard cytogenetic metaphase chromosome preparations, CGH was adapted for use in microarray technology, which allowed streamlining of the process. Recent successful applications of the technology have enabled array CGH (aCGH) to become the current platform of choice for PGS at all biopsy stages in the majority of laboratories around the world (Hellani *et al.* 2008; Alfarawati *et al.* 2011b).

The process involves the separate labeling of the amplified DNA and normal reference sample using different fluorescent dyes followed by co-hybridization to several thousand probes derived from known regions of the genome printed on a glass slide. Using quantitative image analysis, differences in the fluorescence ratio are interpreted to identify gained or lost regions along all chromosomes simultaneously with an error rate of less than 2% (Gutierrez-Mateo *et al.* 2011). The main technical limitations of this process are (i) that it does not supply information about chromosomal ploidy *per se*, only deviations from the most frequent level of the combined fluorescence signal and (ii) the origin of the error is not determined. Thus haploid and polyploid embryos will appear diploid or 'normal' and meiotic errors are not distinguished from post-zygotic ones. Despite these limitations, aCGH is rapidly establishing itself as the "gold standard" for PGS, replacing FISH based approaches in most laboratories.

### 5.4 Single Nucleotide Polymorphism (SNP) arrays

Single Nucleotide Polymorphisms (SNPs) are the most frequent form of DNA variation in the genome. To date over 6 million SNPs have been identified in the human genome (Javed & Mukesh 2010). SNPs are bi-allelic genetic markers that can be used in a variety of ways to

detect chromosome copy number. SNP micro-arrays are used to detect the specific alleles present in polar bodies or embryos at up to 500,000 SNP loci. This information can, in turn, be interpreted in several ways to obtain massive amounts of genetic information. Simple quantification of the SNP alleles and analysis of heterozygosity enables diagnosis of aneuploidy including uniparental isodisomy (Northrop *et al.* 2010; Brezina *et al.* 2011; Treff *et al.* 2011). Using this method, results can be difficult to interpret above the level of background 'noise' due to the problem of amplification from a single template. For this reason, methods involving comparison with parental DNA are under development (Handyside *et al.* 2010; Johnson *et al.* 2010b). Since all embryonic chromosomes are derived from parental chromosomes, predicted genotypes based on known parental data can be used to "clean" noisy single cell data resulting in a comprehensive and highly reliable molecular cytogenetic test for chromosome copy number (Johnson *et al.* 2010b). In addition to this, again with the aid of the known parental genotypes, our group has developed a test involving Mendelian inheritance analysis of SNPs known as 'Karyomapping'. By establishing the four parental haplotypes, only informative 'key' SNPs are analysed to establish chromosome copy number, parental origin and points of meiotic recombination of the tested cells can be 'Karyomapped'(Handyside *et al.* 2010). Karyomapping has the added advantage of being able to detect not only meiotic aneuploidy but also the presence of the chromosomes carrying the mutant allele for cases involving the risk of transmission of specific known inherited disorders.

SNP genotyping has the potential to be the most comprehensive platform for PGS. The interpretation of a SNP genotype allows diagnosis of all possible chromosome copy number aberrations. It has the capacity to perform as a high resolution molecular cytogenetic test at higher resolution than aCGH for all types of chromosomal gains and losses and with the added ability of linkage based analysis allowing diagnosis of inherited genetic disease (Handyside *et al.* 2010). Although largely clinically un-validated, comparative data with other platforms suggest better efficiency than both FISH and aCGH for aneuploidy screening (Johnson *et al.* 2010b; Treff *et al.* 2010a; Treff *et al.* 2010b). Recently presented clinical data of SNP array based PGS on cleavage stage embryos suggests significant improvement of pregnancy rates following embryo transfer (Rabinowitz *et al.* 2010).We anticipate that with further clinical validation, SNP genotyping will become the gold standard for PGS in the near future.

## 6. What we have learnt from PGS thus far?

The rationale for PGS is of course that if embryo ploidy could be determined and euploid embryos selected for embryo transfer, IVF pregnancy rates would increase and poor outcomes such as implantation failure and miscarriage would decrease. Few disagree with this premise underpinning PGS as scientifically and clinically sound.

Since its inception in the mid-90s, PGS has primarily involved the biopsy of one or two cells on the third day of embryo development followed by targeted chromosome analysis using FISH. Subsequently, diagnosed euploid embryos (for the limited number of chromosomes analysed) were transferred or cryopreserved with the remaining embryos diagnosed as aneuploid being discarded (with or without follow-up confirmation analysis). This work was based on the theoretical premise of PGS without the support of randomised controlled trials (RCTs). All recent RCTs using cleavage stage biopsy

followed by FISH analysis showed no improvement in delivery rates after PGS with some even suggesting adverse outcomes (Staessen *et al.* 2004; Mastenbroek *et al.* 2007; Blockeel *et al.* 2008; Hardarson *et al.* 2008; Mersereau *et al.* 2008; Debrock *et al.* 2010). The largest of these trials included over 200 patients in each of the experimental arms (control and treatment groups) and concluded that PGS resulted in a reduced delivery rate following IVF (Mastenbroek *et al.* 2007). These results, contrary to the original premise of PGS, sparked much debate with several institutions including the practice committees of the Society of Assisted Reproductive Technology and the American Society of Reproductive Medicine [SART & ASRM] (2008b) and the British Fertility Society (Anderson & Pickering 2008) issuing statements that PGS should no longer be performed. Meanwhile, several groups criticised the trials for their poor diagnostic efficiency, practical skill levels, inappropriate patient selection and generally low pregnancy rates. They claimed that the trials were performed by inexperienced practitioners thereby generating invalid or questionable results (Cohen & Grifo 2007; Simpson 2008).

What is not in question is that these trials have ultimately highlighted the complexity of considerations PGS requires when applied clinically. FISH of cleavage stage biopsies has clearly outlined that both technical and practical limitations exist when performing PGS to improve pregnancy outcomes. Furthermore, there is great importance and careful consideration needed in patient selection as well as effective test selection and implementation on a case-by-case basis (Handyside & Thornhill 2007).

The success of aneuploidy screening as a selection tool for IVF to improve pregnancy rates is dependent on the efficacy of the entire testing process. It is now clear that FISH, especially for cleavage stage biopsy, is not the optimal tool for PGS. The process is subject to the following technical limitations: (i) the efficacy of the cell preparation technique, (ii) the accuracy of the FISH test itself and its reliable interpretation. Biologically, we are constrained by the products we have to work with (embryo quality, mosaicism and nucleation) and the time in which to work with them. We believe that there is scope for PGS to improve pregnancy rates in ART but the test used must be optimised and tailored to suit the biological and technical limitations that exist to maximise benefit at the lowest possible cost to the embryo.

## 7. Clinical applications and decision making

Aneuploidy screening using 24 chromosome micro-array analyses should improve IVF outcomes with the implementation of case-by-case cost-benefit analysis. For best results, PGS should be performed with the most comprehensive cytogenetic platform available. PGS is considered too invasive to be employed as a routine embryo selection tool for IVF thus, at present, it should be offered only to patients at high risk of aneuploidy. The cost of the biopsy on embryo development is only justifiable if the information gained will outweigh the cost to the cohort of embryos as a whole. For this reason, false positive results due to mosaicism and the number of testable embryos in a cohort are important in the decision making process. Advanced maternal age (AMA) is the single largest indication for PGS as an adjunct to embryo selection to improve IVF success. Careful patient selection is still required within this group of patients to achieve the best results (see figure 5). There are a number of other indications for which PGS is likely to be of most benefit, all of which are associated with a potential increased risk of aneuploidy including patients with Repeated

Implantation Failure (RIF) and Recurrent Miscarriage (RM). Patients with diagnosed high levels of sperm aneuploidy or severe male factor infertility may also benefit.

PB biopsy theoretically has the lowest cost to embryo development but only gives information about maternally derived aneuploidy. PB biopsy is therefore of most benefit to patients of AMA with no other suspected aneuploidy input. Both PB1 and PB2 should be sampled to ensure that the majority of maternally aneuploidy is detected (Geraedts *et al.* 2011).

Theoretically, blastocyst stage biopsy is the optimal stage as it partially negates the problem of mosaicism and gives maximum aneuploidy information from maternal, paternal and post-zygotic events. In addition, the biopsy of 10 or more cells virtually eliminates the problem of ADO following WGA (Ling *et al.* 2009). However, the logistical downside is that embryos may need cryopreservation whist awaiting genetics results, a potential additional 'cost' to embryos. Furthermore, *in- vitro* blastocyst development may be limited in some patients leading to a limited cohort of blastocysts that can be biopsied simultaneously reducing the chance of a live birth and genetic information from the cohort (Janny & Menezo 1996). Thus it should only be considered for patients with RIF and RM, including male factor, with evidence of good blastocyst formation or proven fertility.

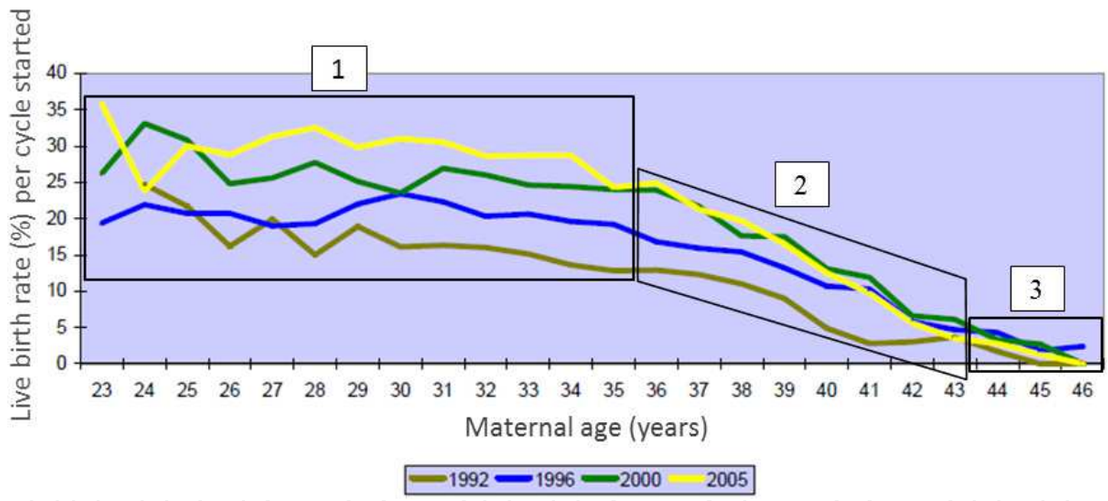


Fig. 5. Three distinct patient groups in relation to age and IVF success rates. Note the drop in success rates beyond maternal age 35 across all years (consistent with increasing rates of aneuploidy). High number of embryos and low rate of aneuploidy are expected in patient group under 35 years of age (1) thus PGS is not recommended - Cost outweighs benefit of PGS. Moderate embryo numbers and increased rate of aneuploidy consistent with reduced IVF success rates of patients above 35 years (2) indicate a target group for PGS - Benefit of PGS outweighs its cost. PGS is suggested to be of no benefit for embryo selection in patients of severe AMA due to low number of embryos and high rate of aneuploidy (3) - Cost outweighs benefit of PGS.
Figure adapted from Human Fertilisation and Embryology Authority website [ HFEA] (2008a)

The inherent problem of mosaicism and false positive results is a major problem for biopsy at the cleavage stage. This, paired with the cost of removing a significant amount of the cell mass (up to 25%), suggests that use of cleavage stage biopsy should be limited to cases of male factor aneuploidy with known poor ability for blastocyst development. Removal of only a single cell is recommended to minimize cost to the embryo and prevent the dilemma of discordant results due to mosaicism (Cohen *et al.* 2007). Cleavage stage biopsy may also be considered as follow-up for equivocal results from PB biopsy (Magli *et al.* 2004; Cieslak-Janzen *et al.* 2006). The relative pros and cons of all biopsy stages are summarised in table 2.

Biopsy, irrespective of stage, should only be performed if there are a sufficient number of oocytes or embryos to be tested. If there is limited or no embryo selection to be achieved by PGS then it (PGS) should be avoided as there will be no benefit to IVF success rates and may even be a detrimental effect (Summers & Foland 2009). An exception to this is when PGS is used not as an embryo selection tool but as a diagnostic tool to avoid or diagnose aneuploidy.

| | origin of aneuploidy | | | | | Detection of mosaicism | Day of biopsy - days post fertilisation | Associated costs and benefits | |
|---|---|---|---|---|---|---|---|---|---|
| | Maternal | | Paternal | | | | | | |
| **Biopsy stage** | **MI** | **MII** | **MI** | **MII** | **Post Zygotic** | | | **Benefit** | **Cost** |
| **PB1** | yes | no | no | no | no | no | Day 0 (day of fertilisation) | Minimal manpulations. No removal of viable cells. Maximum time for analysis prior to embryo transfer. | Only information from maternal MI. |
| **PB1 & PB2** | yes | yes | no | no | no | no | Day 0 and 1 | No removal of viable cells. Maximum time for analysis prior to embryo transfer. | Only information for maternal meiotic errors. |
| **Cleavage stage (blastomere)** | yes | yes | yes | yes | yes | no (Yes with limited sensitivity if >1 cell analysed) | Day 3 | Information for all origin of aneuploidy. Maximise number of embryos tested. Paternal aneuploidy detected. | Mosaicism resulting in false positive and negative results. Removal of significant proportion of cell mass. |
| **Blastocyst stage (trophectoderm)** | yes | yes | yes | yes | yes | yes (only with limitation on sensitivity) | Days 5 and 6 | Information for all origin of aneuploidy. Biopsy of several cells (~10 cells). No harm to ICM. Paternal aneuploidy detected. | Reduced number of embryos for testing (requires good blastocyst formation). Reduced time for diagnosis (embryo cryopreservation potentially necessary ). |

Table 2. Technical limitations, costs and benefits of the established biopsy stages for PGS

Some patients may require elimination of the possibility of aneuploidy resulting in poor outcomes such as miscarriage or birth of a child with a genetic defect. These 'must screen' patients for PGS should be considered more like diagnosis of inherited genetic disease and all embryos, irrespective of the number and quality, should be tested.

## 8. Research and future developments

It is now well documented when and how extra or missing chromosomes arise but the big question remains 'why'. Clearly, research into the origin of human aneuploidy will continue to provide new and exciting insights in the field of reproductive medicine. The introduction of new array technology, including SNP genotyping of embryos, will further improve PGS strategies. For example, recent evidence demonstrating that errors are equally likely during the two maternal meiotic divisions (Handyside *et al.* 2012) is rapidly leading to a shift in the

strategy for approaching polar body analysis for PGS with PB1 considered inadequate if embryo selection is the ultimate goal.

Continued efforts should be employed to use new technologies to correlate the rate and origin of aneuploidy with external factors in an effort to identify markers for aneuploidy risk. Patient assessment of these risk factors will lead to better personalisation of PGS treatment plans and provide an understanding of the underlying mechanisms behind chromosome segregation and the cause of aneuploidy.

Along with AMA, altered recombination in meiosis is the most important known aetiology related to aneuploidy and gives clues to the overall mechanism (Hassold *et al.* 2007). Algorithms applied to SNP genotyping data, including Karyomapping can be applied for high resolution pinpointing of recombination points (Handyside *et al.* 2010; Gabriel *et al.* 2011a). Patterns of recombination across the genome can then be correlated with chromosome mal-segregation in meiosis in an attempt to find aberrant patterns that predispose to aneuploidy. Similar strategies can be employed to different patient profiles to ascertain further aetiologies associated with aneuploidies of different origin. Further understanding of the predisposition to human aneuploidy will lead to specific patient treatment and more importantly guide the direction of studies on the molecular basis of aneuploidy. Once the mechanisms leading to aneuploidy are understood and there is an understanding of why it occurs, interventions to prevent aneuploidy could be usefully investigated.

It has been hypothesised that the high rates of aneuploidy and mosaicism following IVF procedures may in fact be an iatrogenic artefact of the procedure itself. Ovarian hyperstimulation (Baart *et al.* 2007), fertilisation in vitro (Bean *et al.* 2002) and in vitro culture environments (IVC) (Carrell *et al.* 2005; Sabhnani *et al.* 2011; Xu *et al.* 2011) appear to affect embryo aneuploidy. A recent RCT of ovarian stimulation protocols revealed that minimal stimulation, although associated with a reduced number of oocytes, results in higher proportions of chromosomally 'normal' embryos. It was hypothesised that conventional stimulation protocols result mainly in an increase of post zygotic chromosome segregation errors. Altered ovarian function (recruitment of follicles), gonadotrophin dose and GnRH analogue have been offered as potential correlates for further investigation. Furthermore, mouse studies have shown increased meiotic and post zygotic error rates following IVF and IVC respectively (Bean *et al.* 2002; Sabhnani *et al.* 2011). The sensitivity of mouse oocytes to different culture regimens resulting in differing aneuploidy rates corroborate the hypothesis that IVF affects aneuploidy (Carrell *et al.* 2005). In humans, FSH levels associated with in-vitro maturation correlate with chromosome mal-segregation in the first meiotic division (Xu *et al.* 2011). Animal models could be further employed for manipulation of IVF parameters in an effort to induce or suppress aneuploidy, although clinical IVF itself may provide the best 'experiment' to gain a better understanding of the mechanisms involved in human aneuploidy.

Full genetic sequencing seems the logical next technological advance for PGS and appears technically possible following successful genomic sequencing of microbial single cells (Zhang *et al.* 2006; Lasken 2007). Additional data at the highest possible resolution should inevitably prove more reliable for chromosome copy number analysis and, as with SNP genotyping, points of recombination as well as points of partial aneuploidy along

chromosomes could be analysed with more precision. Currently, increased resolution of PGS is limited by the WGA step (Ling *et al.* 2009). Achieving the highest possible resolution is directly restricted by the phenomenon of ADO when amplifying single or very few cells. Thus it necessary to invest effort in improving WGA technology before the full benefits of genomic sequencing could be realised.

With the increasing amount of data obtained from PGS technologies comes the issue of an increasing amount of 'incidental' findings of unknown pathological significance. Careful considerations of the social, ethical and legal aspects of these findings are required to combat potential problems prior to implementing higher resolution technologies.

The ultimate goal of PGS is to provide maximum benefit (in terms of information to the parent/healthcare provider) with minimal cost to the embryo. The possibility of gaining chromosome copy number information with no cost to the embryo would enable PGS to be used routinely as an embryo selection tool for IVF. An indirect aneuploidy screening test was first explored by associations with conventional embryo morphology scoring. However, morphological embryo grading is apparently at its limits to improve IVF success rates and has only shown very limited correlation with aneuploidy (Munne 2006; Gianaroli *et al.* 2007; Alfarawati *et al.* 2011a). The implementation of time-lapse imaging to embryo culture has facilitated high resolution morphokinetic analysis of embryo development in an attempt to improve IVF success rates and eliminate potentially abnormally developing embryos. Morphokinetic analysis involves continual analysis of the morphological state and rates of change during oocyte and embryo development and provides evidence of developmental milestones that can predict embryo implantation (Meseguer *et al.* 2011). Since, cells of different genotypes are known to have slightly different cell cycle times (Varrela *et al.* 1989), it follows that algorithms involving multiple developmental time points could be used to predict embryo aneuploidy at no cost to the embryo. Embryos with an abnormal karyotype (particularly those with multiple abnormalities) may have aberrant cell cycles, detectable by morphokinetic analysis, compared with normal embryos. New studies into the morphological rates of change including such developmental markers as PB extrusion, syngamy and early mitotic divisions could find more significant correlations with chromosome mal-segregation than embryo morphology alone.

Other approaches to non-invasive assessment of embryo viability include the measurement of what is used by or what is secreted by the oocyte or embryo. All culture media contain substances that are required for embryo development. Culture media will also contain all products secreted by the oocyte or embryo. Levels of these can be measured in a variety of ways to establish embryo viability (for review see Aydiner *et al.* 2010).

Analysis of spent culture media is an area already being explored as potential for a new indirect PGS platform. A recent study analysing uptake patterns of amino acids has shown that regulation of amino acid metabolism correlates with embryo aneuploidy. The study using FISH analysis of five chromosomes (13, 18, 21, X and Y) demonstrated altered amino acid turnover in embryos with grossly abnormal karyotypes when compared to genetically normal embryos (Picton *et al.* 2010). Although promising, these early data lack specificity and further work is needed to more accurately establish how embryo metabolism may be indicative of its chromosomal complement. Precise metabolic profiling of embryos with known copy number aberrations is proposed as a specific experiment to establish more meaningful correlations.

A further approach is based on the hypothesis of altered gene expression and protein synthesis of chromosomally abnormal embryos. One study, using a proteomic approach, has identified the first protein secreted by human blastocysts (Lipocalin-1) that is associated with generic chromosome aneuploidy (McReynolds *et al.* 2011) promising the biggest step to date towards a non-invasive PGS test.

## 9. Conclusion

PGS has proved to be one of the most controversial areas of reproductive medicine in recent times. The entire community is united in its collective will to improve IVF success, reduce miscarriage rates and ensure that couples avoid children with developmental abnormalities. The means by which this is achieved remains the subject of intense debate. What can be clear however is that the controversy will serve to increase the interest in PGS leading to new and radical future treatments.

## 10. List of abbreviations

| | |
|---|---|
| aCGH | Array comparative genomic hybridisation |
| ADO | Allele dropout |
| AMA | Advanced maternal age |
| CGH | Comparative genomic hybridisation |
| DNA | Deoxyribonucleic acid |
| FISH | Fluorescence *in-situ* hybridization |
| ICM | Inner cell mass |
| IVF | *In-vitro* fertilisation |
| IVC | *In-vitro* culture |
| MDA | Multiple displacement amplification |
| MI | First meiotic division (Meiosis 1) |
| MII | Second meiotic division (Meiosis 2) |
| PB | Polar body |
| PB1 | First polar body |
| PB2 | Second polar body |
| PGS | Preimplantation genetic screening (for aneuploidy) |
| PS | Premature/precocious separation of sister chromatids |
| SNP | Single nucleotide polymorphism |
| TE | Trophectoderm |
| WGA | Whole genome amplification |

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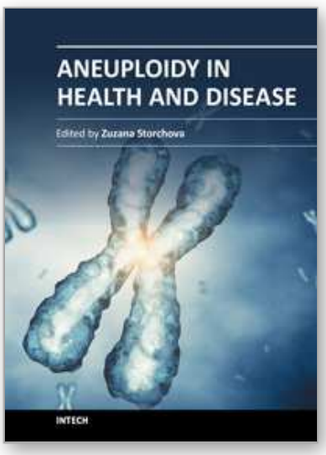


**Aneuploidy in Health and Disease**

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Aneuploidy means any karyotype that is not euploid, anything that stands outside the norm. Two particular characteristics make the research of aneuploidy challenging. First, it is often hard to distinguish what is a cause and what is a consequence. Secondly, aneuploidy is often associated with a persistent defect in maintenance of genome stability. Thus, working with aneuploid, unstable cells means analyzing an ever changing creature and capturing the features that persist. In the book Aneuploidy in Health and Disease we summarize the recent advances in understanding the causes and consequences of aneuploidy and its link to human pathologies.

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