Title: Aneuploidy and recombination in the human preimplantation embryo – A detailed analysis using techniques for detection of copy number variation and genome-wide polymorphism genotyping

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Declaration: At the time the study was conducted all authors apart from ZG were employees of or affiliated with Reprogenetics (CooperGenomics), a company specializing in provision of preimplantation genetic diagnosis clinical services. DW is currently (at time of publication of the study) employed by IVI-RMA, a company specializing in provision of preimplantation genetic diagnosis clinical services.

Abstract

Research Question: Aneuploidy and recombination are important biological phenomena. Can a detailed exploration of these phenomena be carried out in the human preimplantation embryo and novel information acquired of research and clinical importance?

Design: Single nucleotide polymorphism (SNP) microarrays and array comparative genomic hybridization (aCGH) were carried out on a total of 1,442 blastocyst stage embryos derived from 269 fertile couples (average maternal age 34±0.3) undergoing preimplantation genetic diagnosis for the purposes of avoiding transmittance of a known genetic disorder to their offspring; 24-chromosome aneuploidy screening was carried out in parallel.

Results: 100% of meiotic trisomies identified in these embryos were of maternal origin and their incidence increased significantly with advancing maternal age. 55.8% of meiotic trisomies arose during meiosis I and 44.2% during meiosis II. Certain chromosomes suffered aneuploidy more often during meiosis I, while others experienced it more frequently in meiosis II, suggesting that the relative contribution of mechanisms causing/contributing to aneuploidy differ for individual chromosomes. A detailed recombination analysis was carried out for 11,476 chromosomes and 17,763 recombination events were recorded. Considering only the 22 autosomes in euploid embryos, the average number of recombination sites was 24.0±0.3 for male meiosis and 41.2±0.6 for female meiosis. Additionally, there were sex-specific differences in the locations of recombination sites. Comparative analysis conducted between 190 euploid embryos and 69 embryos presenting meiotic trisomies showed similar global recombination rates and non-recombinant chromosome rates between the two categories (1.87±0.02 vs. 1.85±0.03 and 0.13±0.02 vs. 0.14±0.02, respectively) when comparing euploid chromosomes alone; differences

however were observed when analyzing embryos affected with specific meiotic trisomies (i.e. trisomy 16 and 21).

Conclusions: This study yielded unique data concerning recombination and the origin of aneuploidies observed during the first few days of life and provides a novel insight into these important biological processes.

Keywords: aneuploidy; recombination; preimplantation embryo; preimplantation genetic diagnosis; female meiosis; Karyomapping

Introduction

It is estimated that 30-60% of human preimplantation embryos are aneuploid. A vast majority of these abnormalities are either monosomies or trisomies and their frequency varies according to stage of development and increases markedly with advancing maternal age (Franasiak et al., 2014; McCoy et al., 2015; Nagaoka et al., 2012). The prominence of maternal meiotic errors in human reproductive material, relative to those of paternal origin, has been demonstrated in numerous studies (Handyside et al., 2012; Hassold et al., 2007; Nicolaidis and Petersen, 1998). The aneuploidy rate detected in human sperm is reported to be approximately 10-fold lower than that observed in oocytes, hinting that sex-specific differences in meiotic processes could influence the risk of chromosome malsegregation (Hassold and Hunt, 2001; Pacchierotti et al., 2007). Female meiosis begins in the fetal ovary, but the oocyte undergoes a prolonged arrest at the end of prophase (dictyate arrest), potentially lasting over four decades. Many studies have considered how the drawn-out female meiotic process influences the incidence of chromosomal abnormalities (Fragouli and Wells, 2011; Handyside et al., 2012; Lee et al., 2011; Nagaoka et al., 2012). In addition to female age, several other factors have been considered as potential contributors to aneuploidy risk, such as the frequency and chromosomal location of recombination sites (Nagaoka et al., 2012).

Mechanisms of meiotic error occurring during human gametogenesis include: nondisjunction of homologous chromosomes during meiosis I (MI)[1], with both homologues segregating to the same pole of the meiotic spindle; nondisjunction of sister chromatids in meiosis II (MII) resulting in the

[1] aCGH (array comparative genomic hybridization); DHC (detailed haploblock chart); IVF (*in vitro* fertilization); MI (meiosis I); MII (meiosis II); MDA (multiple displacement amplification); PGD (preimplantation genetic diagnosis) SNP (single nucleotide polymorphism)

two chromatids segregating together; premature separation of homologues or sister chromatids at the first meiotic division leading to their random segregation, potentially causing a segregation error (Handyside et al., 2012; Hassold and Hunt, 2001; Jones 2008).

Other than the well-known association of aneuploidy with increasing female age, altered recombination is the most important etiological factor linked with meiotic chromosome malsegregation (Handyside et al., 2012; Hassold et al., 2007). Apart from generating genetic diversity, recombination is known to be fundamental to the successful completion of meiosis (Cheung et al., 2007; Fledel-Alon et al., 2009). Sites of recombination (chiasmata) provide physical connections between homologues, helping to facilitate their successful division during meiosis I (Fledel-Alon et al., 2009). The general consensus is that a reduction in the number of meiotic recombination events, or the occurrence of crossover events located too close to or too far from the centromere, increase the risk of aneuploidy (Cheng et al., 2009; Handyside et al., 2012). A complete failure to establish chiasmata between homologues, or the premature loss of crossovers, are thought to make oocytes particularly vulnerable to chromosome segregation errors. Achiasmate homologs that fail to remain together during meiosis I, and therefore segregate randomly at anaphase, account for nearly one half of MI error-derived cases of trisomy 21 (Down's syndrome) (Jones 2008; Lamb et al., 1997). Selection against chromosomally abnormal embryos (e.g. by developmental arrest, implantation failure or early pregnancy loss) means that studies of aneuploidy and its origins at later developmental stages can only ever provide a partial view of the situation at conception. Some aneuploidies are so lethal that they never produce a clinical pregnancy and consequently cannot be studied in the prenatal samples, miscarriages and affected children/adults that have usually been the subject of such research.

To date, most studies into aneuploidy and recombination have involved analysis of short tandem repeat polymorphisms or high-density single nucleotide polymorphism (SNP) genotyping, establishing patterns of recombination by attempting to trace the inheritance of haplotypes through living families (Chowdhury et al., 2009; Kong et al., 2002). Other investigations have examined female and male gametes (Cheng et al., 2009; Lamb et al., 2005; Lu et al., 2012; Wang et al., 2012), polar bodies (1^{st} and 2^{nd}) and a few fertilized pronuclei or preimplantation embryos (Hou et al., 2015; Ottolini et al., 2015). For the current study, comprehensive chromosome screening and genome-wide genetic linkage analyses were performed on a large number of preimplantation embryos [derived from fertile patients undergoing preimplantation genetic diagnosis (PGD) for single gene disorders] for the purposes of obtaining a better understanding of the recombination process in human preimplantation embryos and its relationship to aneuploidy, particularly maternally-derived meiotic trisomies. The strategy employed simultaneously provides an insight into both male and female meiotic processes. Furthermore, the data obtained reflects the situation at a developmental stage close to conception. This is particularly important when considering the relationship between recombination and aneuploidy, given the early lethality of most chromosome abnormalities.

Materials and methods

A total of 1,442 blastocysts derived from 269 couples [average maternal age 34±0.3 years (± standard error of the mean)] undergoing preimplantation genetic diagnosis for single gene disorders were included in this study. Each embryo was also assessed for aneuploidy. It is

important to note that patients included in this study were not infertile and the reasoning for undergoing IVF procedures was to gain access to preimplantation genetic diagnosis for the purposes of avoiding transmittance of a known genetic disorder to their offspring. None of the disorders tested is expected to have any influence on incidence of aneuploidy or recombination.

Sample processing

Three-to-ten trophectoderm cells were removed at time of biopsy for analysis. The cells were placed in a single 0.2ml microcentrifuge tube, lysed and their DNA subjected to whole genome amplification using multiple displacement amplification (MDA). This was achieved using the Repli-g Single Cell kit (Qiagen, USA) according to the manufacturer's instructions. Aliquots of amplified product from each sample were used to carry out the Infinium Karyomapping assay protocol (Illumina, San Diego, USA) following the manufacturer's protocol (Natesan et al., 2014a, 2014b). These embryo samples were processed alongside DNA samples from the parents and DNA samples from a family member, often a child of the couple or at least one grandparent on either or both sides of the family. Results obtained from Karyomapping were analyzed using the BlueFuse Multi v4.2 (Illumina). The Karyomapping analysis allowed genotyping of SNPs and phasing of alleles on the parental chromosomes (i.e. deduction of parental haplotypes composed of alleles existing in contiguous blocks on the same chromosome) as previously described (Handyside et al., 2010; Konstantinidis et al., 2015; Natesan et al., 2014a). Briefly, the method investigates SNP loci in the genomes of the parents where the one parent is homozygous (AA or BB) and the other heterozygous (AB) and identifies the allele that is unique among the four parental chromosomes; phasing in the embryo is carried out at each SNP locus relative to the reference based on the

presence or absence of this unique allele. The use of a grandparent as a reference allows the SNP loci in the embryo to be phased for that specific parent only. When using a grandparent as a reference, Karyomapping uses those SNP loci in which the grandparent is homozygous and this allows for unambiguous determination of phase in the embryo. The quantity of DNA produced from embryo biopsy samples using MDA was sufficient to allow array comparative genomic hybridization (aCGH) analysis in addition to Karyomapping. This involved analysis using 24Sure microarray (Illumina) as previously described (Fragouli et al., 2011a); results were analyzed using BlueFuse Multi v4.1.

Aneuploidy Analysis to Determine Incidence, Type and Origin of Errors

Aneuploidy analysis for the purposes of determining the incidence, type and origin of errors was carried out on a total of 1,098 embryos derived from 199 patients processed over a defined period of time. All embryos with results were included in analysis. Aneuploidy analysis was focused on embryos affected by trisomies, detected using aCGH, which were determined to be of meiotic origin, as revealed by SNP genotype analysis providing genetic linkage data. Even though based on our experience Karyomapping can often distinguish monosomies of meiotic vs. mitotic origin, aCGH cannot readily distinguish the two. Therefore, to err on the site of caution and to avoid any mis-representation of errors of meiotic vs. mitotic origin regarding monosomies, we purposefully excluded this type of error from our analysis. Trisomies of mitotic origin were also excluded from analysis for the purposes of this study. Careful analysis of Karyomapping data related to chromosomes that aCGH had shown to be trisomic, allowed determination of parental origin and the stage of meiosis at which the error occurred (i.e. Meiosis I or Meiosis II). Determination of

meiosis I vs. meiosis II errors was carried out as previously described through examination of pericentromeric SNPs (Middlebrooks et al., 2014). Specifically, for errors happening during meiosis I the extra chromatid copy is expected to have come from a different homologue and therefore, increased allelic heterozygosity in the pericentromeric region is anticipated; this indicating different chromatid copies of different grandparental origin (i.e. one from grandmother and one from grandfather). For errors happening in meiosis II we expect to see regular allelic heterozygosity in the pericentromeric region (as such seen in euploid chromosomes) and presence of only one (grand)parental haplotype since the error would involve sister chromatids from the same homologue; some areas on the chromosome would be expected to be different and show increased allelic heterozygosity due to recombination events that took place during meiosis I between the two different homologues but since recombination cannot happen too close to the centromere the immediate region surrounding the centromere is expected to be the same for the two chromatids (same parental haplotype). Based on the above, regarding meiotic trisomies detected with the Karyomapping dedicated software, the same parental haplotype present in the pericentromeric region would support a meiosis II error and presence of two different parental haplotypes would support meiosis I error (Fig 1C, D).

Recombination analysis

Karyomapping reveals inheritance of parental haplotypes and identifies recombination sites. These are visualized in the BlueFuse Multi software in the detailed haploblock chart (DHC) as a sudden swap from the haplotype associated with one parental homologue to the haplotype of that parent's other homologue (Fig 1). The Karyomapping SNP array platform and analysis method has been

shown to be highly accurate in determining recombination events in single cells and has been determined to offer a median resolution of 331Kb for crossovers in embryos which is noted to be similar to the one observed in population-based studies employing SNP arrays (Ottolini et al., 2015). Sites of recombination were visualized on the DHC generated by the BlueFuse Multi software and mapped by recording the genomic coordinates (GRCh37) of the SNP closest to the cross-over event or by dividing the distance between the two closest SNPs located either side of the cross-over event and then recording it.

Recombination data were obtained from 389 blastocysts derived from 98 couples for which grandparental DNA was available for analysis. Twenty-eight of the patients were common between this cohort and the patient cohort that was included in the aneuploidy analysis described in the previous section; an additional 70 patients (with available grandparental DNA) were included in this part of the study for purposes of recombination analysis. Some patients had embryos of either chromosomal status (i.e. euploid and aneuploid) included in the analysis while other patients had only embryos that were euploid or only embryos that were aneuploid analyzed. In terms of aneuploid embryos, recombination analysis was focused on embryos carrying maternal meiotic trisomies. Nevertheless, embryos with monosomies were also included in certain analyses and specifically when calculating overall rates of recombination and of non-recombinant chromosomes and making correlations with chromosome size (Fig 2 and Fig 3). Recombination rate was calculated as the number of recombination events divided by number of chromosomes assessed. We were also interested in the rate at which crossover events failed to occur. Non-recombinant chromosome rate was therefore calculated as the number of chromosomes that had zero recombination events out of the total number of chromosomes assessed. The determination of recombination and non-recombinant chromosome rates in aneuploid (and euploid) embryos in

this study was calculated taking into consideration the overall number of chromosomes available for analysis [i.e. for each embryo, chromosome(s) affected by aneuploidy where excluded from analysis and the overall number of chromosomes considered for analysis was adjusted accordingly when calculating rates of recombination); this was important to carry out in order to account for variability and normalize the data accordingly. It is important to note however, that by testing day-5 embryos it was only possible to determine the rate of non-recombination for one of the two chromatids in a homologue (i.e. the chromatid that segregated in the gamete which gave rise to the embryo). Each of the two chromatids in a homologue can be involved in recombination with a chromatid from the other homologue in a bivalent during Meiosis I. As such, the non-recombinant chromosome rate reported in this study provides only a partial image of non-recombination occurrence at time of gamete formation.

A comparison of generalized genome-wide recombination events was carried out between euploid and aneuploid embryos affected by maternally-derived meiotic trisomies. A more targeted comparison of genome-wide recombination events was carried out between euploid embryos and embryos determined to carry maternally-derived meiotic trisomy 16 alone (i.e. no other chromosomal abnormalities were present in these embryos other than trisomy 16), trisomy 21 alone and trisomy 22 alone. Additionally, rates of recombination and of non-recombinant chromosome were correlated with chromosome size, while recombination event cytoband frequency was determined for each chromosome of euploid embryos and embryos with maternally-derived meiotic trisomies. An analysis to determine the correlation of recombination rate with parental age was also performed. Finally, we looked at embryos derived from patients undergoing PGD for BRCA1 or BRCA2 testing. Recombination events in BRCA1 euploid embryos were compared to recombination events in euploid embryos from the rest of the cohort.

Statistical analysis

Statistical analysis of meiotic trisomy data was conducted using Fisher's Exact Test (GraphPad, USA). Non-parametric (Mann-Whitney U, Kruskal-Wallis H) and parametric (one-way ANOVA) tests (IBM SPSS® Statistics) were utilized as indicated following assessment of data for normal distribution, to analyze recombination data. A p-value of <0.05 was considered to be statistically significant with $p<0.001$ indicating high significance.

The raw counts of meiotic trisomy and recombination events were used in vast majority of instances to carry out statistical analyses. When making comparisons of recombination counts between embryos carrying specific trisomies alone (i.e. trisomy 16, 21, 22) and euploid embryos, the specific chromosome affected by trisomy was excluded from recombination count analysis in both cohorts in an effort to eliminate variances. Recombination rate per euploid embryo was utilized to do a comparison between different parental ages.

Ethics approval

Singed consent for provision of PGD clinical services was obtained from patients. Specific informed consent was not obtained for this research study. Clinical data included in this study was de-identified i.e. stripped of all patient identifiers. The study protocol was reviewed by Aspire Independent Review Board (a WIRB-Copernicus Company) and was exempted from IRB review/approval.

Results

A total of 1,442 embryos were included in this study. All embryos were assessed for aneuploidy; recombination data were collected in 389 of these embryos.

Aneuploidy Analysis to Determine Incidence, Type and Origin of Errors

One thousand ninety-eight embryos derived from 199 patients processed over a defined period of time were included in aneuploidy analysis for the purpose of delineating incidence, type and origin of errors. Overall, 432/1,098 (39.3%) embryos were determined to carry one or more aneuploidies, including 2 embryos determined to be triploid. Three hundred and forty trisomies (including 15 segmental chromosome gains) and 383 losses (including 47 segmental chromosome losses) were observed.

In total, 122/1,098 (11.1%) embryos were determined to carry one or more meiotic trisomies. Gain of informative ('key') SNPs visualized on the DHC (Fig 1) confirmed that a total of 163 distinct trisomies were of meiotic origin. Further investigation revealed that all of these events (100% or 163/163) had occurred during maternal meiosis with 55.8% (91/163) of errors originating during MI and 44.2% (72/163) during MII (Table 1).

Chromosomes 15, 16, 19, 21, and 22 were the most frequently affected with meiotic trisomies; the highest incidences of meiotic gains were recorded on chromosomes 15, 16 and 22 (Table 1).

The specific meiotic division at which each trisomy arose was determined based upon a detailed analysis of informative SNPs flanking the centromere of the affected chromosome (Fig 1). The data was assessed in order to evaluate whether there is a tendency for specific chromosomes to

experience malsegregation in a particular meiotic division. The results (Table 1) demonstrated that chromosomes, 16, 19, 21, and 22 were more likely to undergo meiosis I errors, although statistical significance was only reached for chromosomes 16 and 22 ($P<0.0001$). Meiosis II errors predominated for chromosomes 2, 14 and 20 ($P<0.05$). Most other chromosomes showed no significant bias towards either meiotic division with similar number of events being attributed to each stage.

The incidence of meiotic trisomies for each maternal age category was calculated as the number of embryos with meiotic trisomies vs. overall number of embryos per age group. As expected, the frequency of meiotic trisomies increased with advancing maternal age with the highest frequency being at ages ≥40 years. Specifically, the incidence was determined to be 5.3% for ≤34 age group, 15.2% for 35-39 age group and 35.5% for ≥40 years (Table 2) ($P<0.0001$). The same trend was observed when considering MI derived trisomies and MII derived trisomies separately. The incidence of MI trisomies was calculated to be higher than the incidence of MII trisomies for all age groups, although for the youngest and oldest age groups (≤34 years and ≥40 years) the difference is marginal and no statistical significance was observed. Of the embryos found to be affected by meiotic trisomies, 9% were observed to exhibit a combination of MI and MII errors.

Overall recombination analysis

Recombination data were collected for 389 embryos derived from a total of 98 patients. Analysis was carried out on a total of 11,476 individual chromosomes and 17,763 independent recombination events were recorded. To locate the sites of recombination events, we determined the haplotypes attributable to each parental homologue and searched for points along the length of

chromosomes in embryos where there were transitions in the haplotype associated with one homologue to that associated with the other (Fig 1). It is important to note that for the purposes of recombination analysis, for all embryos assessed, DNA from the maternal and/or paternal grandparents was available. This was essential in order to ensure the most accurate detection of recombination events. For some embryos maternal and paternal grandparents were both available and recombination analysis was therefore possible for both sides, while for other embryos only DNA from maternal or paternal grandparent(s) was available and therefore recombination analysis was only possible for the maternal or paternal side, respectively.

The disorders being tested were not expected to have any influence on incidence of recombination or aneuploidy. To evaluate this, we separately analyzed recombination events recorded in embryos derived from patients undergoing PGD for BRCA1/BRCA2 testing. In total, there were 44/389 embryos [32 euploid, 12 aneuploid (but only 7 with meiotic trisomies)] being analyzed for recombination that were derived from 12/98 couples. For the purposes of this analysis we focused on euploid embryos derived from BRCA1 patients which represented the vast majority of embryos (n=24). Comparison between euploid BRCA1 embryos to euploid embryos in the rest of the cohort showed no significant difference in incidence of recombination events (P=0.158). The mean recombination rates for BRCA1 euploid embryos and euploid embryos in the rest of the cohort were determined to be 1.93±0.05 vs. 1.85±0.02, respectively.

Considering only the autosomes from euploid embryos, the average number of recombination events was calculated to be 24.0±0.3 for male meiosis and 41.2±0.6 for female meiosis. Thus, maternal recombination rates were 1.7-fold higher than paternal recombination rates. The frequency of recombination events and the incidence of non-recombinant chromosomes were also calculated per chromosome. Both of these measures varied across all chromosomes. Chromosomes

21 and 22 for female meiosis and chromosomes 21, 22, X and Y for male meiosis recorded the lowest number of cross-over events (Fig 2A). The same chromosomes also exhibited the highest rates of non-recombinant chromosomes (Fig 2B). Additionally, chromosomes involved in male meiosis exhibited higher non-recombinant chromosome rates than their female counterparts.

Further analysis was carried out to assess the relationship between chromosome length/size and the measures of recombination rate and non-recombinant chromosome rate. Unsurprisingly, this analysis confirmed that chromosome length is strongly correlated with maternal recombination rate ($R^2 = 0.95$) and also, with paternal recombination rate (when looking at autosomes and Y chromosome; excluding X chromosome) ($R^2 = 0.90$) (Fig 3). Correlation was also observed between chromosome length and maternal/paternal non-recombinant chromosome rates, but to a lower degree [$R^2 = 0.68$ for maternal; $R^2 = 0.71$ (when excluding X chromosome)].

Patterns in recombination activity

Locations of cross-over events were mapped for all embryos and all chromosomes where recombination data were available. In general, sites of recombination tended to be more common towards the distal ends of chromosomes when considering male meiosis. Regarding female meiosis, cross-over events showed a wider distribution. As with male meiosis there was a tendency for telomeric regions to show higher degrees of recombination, but spikes in recombination activity were also observed in chromosome interstitial regions and regions closer to the centromere. In both sexes, some recombination hotspots were apparent. Fig A1 highlights the frequency of recombination events per chromosome separated by chromosomal band for both male and female meiosis. Overall trends were more obvious for certain chromosomes than others.

Crossover events in the sex chromosomes involved in male meiosis were localized at specific regions. As expected, recombination activity of paternal origin was restricted to the short arms of X and Y chromosomes; the p22.3 band in the X-chromosome and p11.3 band in the Y-chromosome.

Recombination activity in aneuploid and euploid embryos

A side by side comparison of recombination rates was conducted between 190 euploid embryos and 69 aneuploid embryos presenting with maternally-derived meiotic trisomies. The overall genome-wide incidence of recombination in female meiosis was very similar between euploid embryos and embryo with maternally-derived meiotic trisomies (mean= 1.87±0.02 vs. 1.85±0.03, respectively; P=0.425). We also calculated the average non-recombinant chromosome rate in both euploid and aneuploid embryos with maternally-derived meiotic trisomies. Similar to recombination rate, no differences were observed in the overall non-recombinant chromosome rates between euploid and embryos with maternally-derived meiotic trisomies in female meiosis [0.13±0.02 vs. 0.14±0.02, respectively]. We then decided to carry out a more targeted analysis of the genome-wide incidence of recombination in embryos affected by specific meiotic trisomies (i.e. trisomy 16, 21, 22) and compare to euploid embryos. Embryos affected with either trisomy 16 alone (n=8) or trisomy 21 alone (n=5) were determined to have lower incidence of recombination compared to euploid embryos (P=0.003 and P=0.004, respectively) [mean= 1.89±0.02 (euploid) vs. 1.57±0.09 (MatTri16) and mean= 1.93±0.02 (euploid) vs. 1.57±0.12 (MatTri21). On the contrary, incidence of genome-wide recombination for embryos carrying

meiotic trisomy 22 alone (n=7) was similar to euploid embryos (P=0.700) [mean= 1.92±0.02 (euploid) vs. 1.92±0.11 (MatTri22)].

It is important to note that due to limitations of the analysis software, for all aneuploid embryos, recombination events could only be counted for the euploid chromosomes. As such, the analysis presented in this study demonstrates that overall recombination rates in euploid embryos are similar to embryos with maternally-derived meiotic trisomies. However, it does not examine recombination rates on the chromosomes specifically affected by trisomy or other aneuploidy.

The next step in the analysis was to determine differences in recombination patterns between euploid and aneuploid embryos with maternally-derived meiotic trisomies. As described earlier, each crossover event was mapped according to its chromosomal band location. The spread of recombination events in aneuploid embryos was similar to that of euploid embryos (Fig A1).

Incidence of recombination and parental age

The rate of recombination events in euploid embryos was analyzed in association with maternal and paternal age. No correlation was observed between maternal or paternal age and recombination rates in female or male meiosis; P=0.251 and P=0.431, respectively (Fig 4).

Discussion

The intertwined relationship between chromosome abnormalities and advancing maternal age has been well described (Fragouli et al., 2011b, 2013; Franasiak et al., 2014; Hassold and Hunt, 2009; Hunt and Hassold 2010; Jones 2008; McCoy et al., 2015). The role of female meiosis, particularly alterations in recombination frequency or location, has also been implicated in the aneuploidy risk (Cheng et al., 2009; Chiang et al., 2012; Hassold and Hunt, 2001). Most studies into aneuploidy and recombination have utilized genotyping data from DNA repositories of populations and families, all of which involve analysis of samples from liveborn individuals or have examined male and female gametes separately (Bleazard et al., 2013; Kong et al., 2002; Lee et al., 2011; Martin, 2008; McVean et al., 2013). Studies examining recombination events in female and male gametes have usually involved immunostaining to visualize the synaptonemal complex and associated proteins of the recombination machinery. For example, MLH1, a protein that binds at sites where cross-overs form, has been utilized extensively by many studies to investigate recombination events (Hassold and Hunt, 2009). Immunostaining techniques, although useful, are labor-intensive, are not likely to detect 100% of chiasmata, and as concluded after comparing results to results obtained from genetic linkage analyses, precision may be limited (Cheng et al., 2009). A few studies have been carried out before, utilizing methods for genome-wide genotyping and analysis of a small number of male and female gametes, polar bodies, fertilized pronuclei and preimplantation embryos (Hou et al., 2015; Lu et al., 2012; Ottolini et al., 2015; Wang et al., 2012).

In contrast to above studies, the current investigation was able to evaluate recombination and aneuploidy in a large number of human embryos derived from couples undergoing *in vitro* fertilization (IVF). This analysis involved the application of a combination of molecular genetic methods developed for the analysis of minute samples, including single cells. It is noteworthy that

patients in this cohort were not infertile and were undergoing IVF solely for the purpose of accessing preimplantation genetic diagnosis and avoiding transmittance of a known genetic disorder to their offspring. The disorders being tested are not considered to have any influence on recombination or the incidence of aneuploidy. To provide evidence of this, embryos derived from patients undergoing PGD for BRCA1 testing were analyzed for recombination events and compared to embryos from the rest of the cohort. BRCA1 has been shown to be present on developing synaptonemal complexes in meiotic cells (Chen et al., 1998) and has been suggested to be involved in monitoring and modulating meiotic recombination (Li et al., 2018) and have an essential role in DNA double-strand break repair during gametogenesis (Janisiw et al., 2018). No significant difference in incidence of recombination was observed between the two cohorts. Unique data was obtained, revealing aneuploidy (particularly meiotic trisomies) affecting any chromosome at one of the earliest developmental stages (5-6 days after fertilization) and highlighting sites of recombination across the genome. The vast majority of the abnormalities detected are not compatible with formation of a pregnancy and consequently cannot be observed at later stages (e.g. in miscarriages, prenatal samples or after birth). It should be noted that even though day-5 blastocyst stage is an early stage in embryo development, results obtained in this study do not necessarily equate those at conception since considerable selection may have already occurred during the first few days of development from fertilization to day-5.

For technical reasons, aneuploidy analysis in this study focused on meiotic trisomies. It was determined that errors occurring during meiosis I were, in general, more common than those occurring during MII. Particularly interesting was the observation that while MI errors were significantly more common than MII errors for younger patients ($P<0.05$), the difference narrowed as patient age increased; for ages ≥ 40, MI and MII errors were seen at almost the same frequency

(Table 2). This observation is in agreement with previous studies where MII errors were seen at similar or even higher frequencies compared to MI errors for patients of advanced maternal age (Fragouli et al., 2011b; Kuliev et al., 2003; Kuliev and Verlinsky, 2004). This suggests that MII error and/or premature separation of chromatids are mechanisms of aneuploidy that are particularly sensitive to, and impacted by, increasing female age.

The most common aneuploidies at the blastocyst stage involve chromosomes 15, 16, 21, 22 and X - with 22 and 16 displaying the highest frequencies of abnormality (Fragouli and Wells, 2011; McCoy et al., 2015). Some of these aneuploidies are also amongst those most often associated with spontaneous abortions (e.g. trisomies for chromosomes 16, 21, 22 and X) (Hassold and Hunt, 2001). In the current study, the vast majority of errors leading to aneuploidy (trisomy) of chromosomes 16, 21 and 22 were shown to arise during meiosis I, in agreement with findings from previous studies (Hassold and Hunt, 2001). While the majority of chromosome 21 trisomies were derived from MI (61.5%), the bias towards the first meiotic division was much more pronounced for chromosomes 16 (95.5% MI errors) and 22 (84% MI errors). Chromosomes displaying a preponderance of trisomies arising in MII were identified (i.e. chromosomes 2, 14 and 20), although these chromosomes were found to have a generally low frequency of abnormality, in agreement with previous observations (Fragouli and Wells, 2011; McCoy et al., 2015).

It is important to note that some errors determined to have occurred during meiosis II in this study may in fact have arisen due to problems originating during meiosis I; errors occurring during MI may predispose the oocyte to MII error. For example, premature separation of homologues or sister chromatids during MI will not always lead to a segregation error during that division; in these situations, the MII oocyte will be at elevated risk of becoming aneuploid following the completion of MII. Ottolini et al. (2015) in their study described for the first time a novel, non-canonical

474 segregation pattern which they termed ‘reverse segregation’. According to this pattern, sister
475 chromatids from both homologues separate from each other during meiosis I giving rise to meiosis
476 I products with normal chromosomal complement but with each containing non-sister chromatids.
477 Such non-canonical segregation could predispose to misegregation during meiosis II.

478 Not unexpected, but still quite interesting, was the fact that the all of the meiotic trisomies observed
479 in this study were of female origin. It has long been recognized that aneuploidy in human oocytes
480 occurs at high frequencies and that most of the autosomal trisomies compatible with pregnancy or
481 at birth have a maternal origin (Handyside et al., 2012; Hassold and Hunt, 2001; Fragouli et al.,
482 2011c; McCoy et al., 2015). Examining meiotic trisomies affecting all 23 pairs of chromosomes,
483 shortly after conception, the extent of the skew observed in the current study was dramatic. While
484 most chromosome abnormalities arise during female meiosis, it is important to note that a paternal
485 contribution still exists, especially for certain chromosomes (e.g. X and Y) (Hassold and Hunt,
486 2001, 2009; McCoy et al., 2015; Nicolaidis and Petersen, 1998). Our results are along the lines of
487 a previous study carried out on a large number of embryos (28,052 day-3 and 18,387 day-5
488 embryos) which concluded that paternal meiotic errors were rare and paternal meiotic trisomies
489 were only detected in 1% of embryos analyzed (McCoy et al., 2015). The fact that McCoy et al
490 detected a small percentage of paternal meiotic trisomies compared to our study that did not detect
491 any could be attributed to different factors. McCoy et al tested a considerably larger number of
492 embryos which is expected to confer higher likelihood of detecting rare abnormalities.
493 Furthermore, the majority of embryos in the McCoy et al study was day-3 embryos compared to
494 our study which was solely carried out on day-5 embryos. As documented by McCoy et al and
495 other studies in the past (Sandalinas et al., 2001), the incidence and type of chromosomal

abnormalities in day-5 embryos is considerably different from day-3 embryos due to processes such as self-correction and selection/arrest of aneuploid embryos before reaching day-5.

Several mechanisms have been proposed to explain the incidence of maternally-derived aneuploidy, including loss of sister chromatid cohesion in eggs (especially with advancing age), altered recombination activity (e.g. recombination failure and suboptimal location of recombination events/cross-overs) and inefficient spindle assembly checkpoint (Hassold and Hunt, 2001, 2009; Nagaoka et al., 2012).

In this study, frequencies and locations of recombination events were recorded for 389 blastocysts allowing an investigation into the relationship between overall levels of recombination and the incidence of aneuploidy (particularly maternally-derived meiotic trisomies). Maternal crossover events eclipsed those occurring during paternal meiosis (averaging 41.2 vs. 24.0 events per haploid genome, respectively). These results are concordant with other similar studies performed at much later developmental stages or after birth (Cheung et al., 2007; Chowdhury et al., 2009; Coop et al., 2008; Lee et al., 2011) and provide evidence for the precision and accuracy of the Karyomapping method in determining recombination events on minute amounts of DNA derived from a few cells of preimplantation embryos. The incidence of non-recombinant chromosomes (i.e. chromosomes with no evidence of any recombination with their homologue) was clearly higher in male meiosis than female meiosis for every chromosome analyzed. Based on our current knowledge of the human meiosis process, high levels of recombination failure would be expected to predispose to errors during meiotic divisions which in turn would result in high levels of aneuploidy. However, the situation is more complex as evidenced by the fact that aneuploidies detected in human embryos/fetuses are mainly maternally derived despite the lower incidence of non-recombinant chromosomes during female meiosis. Sex-specific differences in checkpoint mechanisms have

been suggested as a possible explanation for this observation, with checkpoint controls in spermatogenesis being more effective than oogenesis (Nagaoka et al., 2012; Templado et al., 2013). The huge difference in the age of the female gamete with respect to those of the male is also likely to be of great relevance. The ageing of the oocyte is potentially associated with a declining ability to process chromosomes with suboptimal recombination, or a decline in the cohesins binding them together, predisposing to aneuploidy. It is important to note however that the rate of non-recombinant chromosomes presented in this study cannot be used to draw definitive conclusions about complete recombination failure for a specific chromosome. Since day-5 embryos were used in this study, only one of the four chromatids (i.e. the chromatid present in the gamete that gave rise to the embryo) is assessed for absence of recombination and the rest of the chromatids could have still undergone recombination but cannot be examined. Cheng et al (2009) reported in their study with human oocytes that the proportion of recombination-less bivalents (as assessed via the absence of MLH1 foci) ranged from 0-6% depending on the chromosome, with a general incidence of 1.4%.

Perhaps unsurprisingly, higher maternal and paternal recombination rates per chromosome (when considering only autosomes and Y chromosome) were found to be strongly correlated with increasing chromosome length (Fig 3). Chromosomes 21 and 22 exhibited some of the highest rates of non-recombinant chromosomes - both of these are amongst the chromosomes displaying the highest levels of aneuploidy at the blastocyst stage (Fragouli and Wells, 2011; MCoy et al., 2015). However, chromosome 16, which is also subject to high aneuploidy rates, was not associated with elevated levels of non-recombinant chromosomes. In accordance with previous studies (Hassold and Hunt, 2009), this reinforces the idea that there are different mechanisms producing aneuploidy and a range of factors contributing to aneuploidy predisposition. The data

from the current study suggests that non-recombination and/or low recombination rates may be associated with aneuploidy affecting some chromosomes but may have a lesser effect on others.

The availability of a large amount of recombination data allowed us to map sites of crossover activity for all chromosomes including the sex chromosomes. Autosomes in male meiosis typically showed increased recombination activity towards the telomeres, while for female meiosis recombination activity appeared to be more widely dispersed, including telomeric, centromeric and interstitial chromosomal regions. Crossover activity for the sex chromosomes in male meiosis was restricted to the short arms (PAR1 region) as expected; no recombination activity was seen for PAR2 region. X-Y pairing in the PAR and particularly PAR1 region, is thought to be an important process for human spermatogenesis (Mangs and Morris, 2007; Weissenbach et al., 1987).

Different studies have reported correlations between altered recombination activity and occurrence of non-disjunction leading to trisomy for different chromosomes. Specifically, a reduction in recombination activity of affected chromosomes has been associated with the occurrence of non-disjunction (e.g. chromosomes 15, 16, 18, 21) while suboptimal location of cross-over events has also been noted for certain affected chromosomes (e.g. chromosomes 16 and 21) (Cheng et al., 2009; Hassold et al., 2007; Hassold and Hunt, 2001). Ottolini et al (2015) in their study, utilizing similar methodology to this study, had determined that global incidence of recombination was significantly higher in euploid oocytes/preimplantation embryos when compared to aneuploid ones. However, their study was carried out mainly on oocytes derived from healthy donors and day-3 embryos derived in their majority from patients of advanced maternal age and a patient affected by recurrent miscarriage. Similarly, Hou et al. (2015) determined a lower crossover activity in aneuploid oocytes compared to euploid. Yet, they noted that when they tried to

normalize their data set in order to account for crossover detection defects in aneuploid chromosomes, the difference in recombination activity was small between the two groups and was not at the level previously reported. In the current study, only day-5 embryos were examined and as discussed earlier, some self-correction and selection/arrest of aneuploid embryos is expected to happen by day-5 of embryo development resulting in a cohort of embryos with a different range of chromosomal abnormalities compared to mature oocytes or day-3 embryos reported in the above two studies. Furthermore, the current study was carried out on fertile patients from a wide range of ages undergoing PGD to prevent a known genetic disorder from being inherited in their offspring. No significant differences between euploid embryos and embryos carrying maternally-derived meiotic trisomies were observed in the current study regarding overall rates of recombination or distribution of recombination sites. Overall rates of non-recombinant chromosomes also appeared to be essentially identical between euploid embryos and embryos with maternally-derived meiotic trisomies. Nevertheless, a more targeted analysis of the incidence of recombination in embryos carrying specific maternally-derived meiotic trisomies identified differences with euploid embryos. Specifically, embryos carrying meiotic trisomy 16 alone or meiotic trisomy 21 alone were determined to have significantly lower incidence of genome-wide recombination compared to euploid embryos (P=0.003 and P=0.004, respectively). On the contrary, embryos affected by meiotic trisomy 22 alone did not show altered global recombination counts compared to euploid embryos (P=0.700). Results from this analysis should be considered with caution since only a small number of embryos carrying these specific trisomies alone was available for analysis. Nevertheless, these results are in concordance with another study carried out on living individuals with trisomy 21 which concluded that such probands had reduced genome-wide recombination counts compared to controls, with statistical significance being

588 reached for MI errors (Middlebrooks et al., 2014). The current study did not investigate
589 recombination sites or rates for the chromosomes specifically affected by trisomy, but rather
590 examined global recombination rates in disomic chromosomes (i.e. chromosomes not involved in
591 aneuploidy) in aneuploid and euploid embryos. Taken together the results of this study suggest
592 that generalized atypical recombination activity may be associated with meiotic aneuploidy in
593 certain occasions when specific chromosome errors occur (i.e. trisomy 16, 21) but in other
594 occasions it is likely restricted to the affected chromosomes and gametes that experience
595 chromosome malsegregation do not typically display a generalized alteration in recombination
596 activity.

597 Reports of significant correlation between recombination rates and maternal or paternal age have
598 been conflicting; some studies have reported no significant effects (Cheung et al., 2007), while
599 other studies found an increase in number of recombination events with increasing maternal age
600 (Coop et al., 2008; Kong et al., 2004). A more recent study (Bleazard et al., 2013) was able to
601 demonstrate a negative relationship with advancing maternal age after extensive regression
602 analysis, while no such correlations were observed with paternal age. Martin et al (2015) carrying
603 a meta-analysis of results in nine different cohorts (including above studies) concluded that
604 collectively there is a small and significant positive effect with maternal age (i.e. increase of
605 maternal crossovers with advancing age). However, they note that even after applying the same
606 analysis methods in all cohorts there was significant heterogeneity between cohorts, with some
607 showing a positive effect and others a negative effect. They found no significant effect of the
608 father's age on the number of paternal crossovers. The data presented here, involving analysis of
609 more than 17,000 recombination sites did not reveal any significant effects of maternal or paternal
610 age on recombination activity, in terms of the number of recombination events. This suggests that

recombination frequency in sperm remains constant as men age. For women, recombination has already been initiated in utero, but the data indicates that oocytes reaching maturity in early adulthood are equivalent, in terms of recombination frequency, to those that mature towards the end of the reproductive lifespan.

In conclusion, this study provides a significant and novel insight into the processes of aneuploidy and recombination in human, achieved through utilization of powerful methodologies that allowed genome-wide examination of embryos at the earliest stages of life. Further research involving preimplantation embryos and such comprehensive methodologies will be beneficial for increasing the understanding of the important biological phenomena of aneuploidy and recombination.

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Figure Titles and Legends

Fig 1. Types of analysis carried out using dedicated Karyomapping software. *(please print in color)*

(A) Log R and B-allele frequency charts of a normal female (46,XX) embryo as generated by the Bluefuse Multi software. Sites of recombination are determined as areas where genotypes are exchanged on the detailed haploblock chart (DHC). SNPs above each chromosome represent 'key' SNPs and SNPs below each chromosome are known as 'non-key'. 'Key' SNPs are those that provide a clear indication of which parental allele was inherited through presence of a unique SNP allele; 'non-key' SNPs involve non-unique alleles and although accurate in vast majority of times they could be affected by phenomena such as allele dropout and therefore include occasional erroneous results. (B) Log R, B-allele and detailed haploblock charts showing a maternal meiotic gain on chromosome 21 (47,XY,+21). Gain of key SNPs on the DHC indicates chromosome copy gain. (C) DHC illustrating meiosis I error on chromosome 19. (D) DHC showing a meiosis II error on chromosome 18.

Fig 2. Incidence of recombination events per chromosome. *(please print in color)*

(A) Recombination rate per chromosome. (B) Non-recombinant chromosome rate per chromosome. Recombination rate was calculated as the number of recombination events divided by number of chromosomes assessed. Non-recombinant chromosome rate was calculated as the number of chromosomes that had zero recombination events out of the total number of chromosomes assessed.

Fig 3. Association of chromosome length to recombination rate and non-recombinant chromosome rate. *(please print in color)*

Orange circles are used to show data points for the X chromosome on the paternal recombination and non-recombinant chromosome rate plots against chromosome length. When X chromosome is excluded (i.e. looking at autosomes and Y chromosome), the R^2 values of the regression line analysis equal 0.8997 and 0.7114 for paternal recombination rate and paternal non-recombinant chromosome rate, respectively. As can be deduced from the regression line analysis, 95% of the variability in maternal recombination rates between different chromosomes and 90% (when excluding X chromosome) of the variability in paternal recombination rates can be explained by the differing chromosome sizes. When considering non-recombinant chromosome rates, a lower degree of variability between chromosomes [68% for maternal and 71% for paternal (when excluding X chromosome)] can be explained by differing chromosome size. Chr = chromosome

Fig 4. Recombination rates by Maternal and Paternal Age. *(please print in color)*

Recombination rates per euploid embryo were used to make correlations of maternal and paternal age at conception with incidence of recombination. Maternal ages examined ranged at 26-45 years while, paternal ages examined ranged at 27-50 years. No significant difference in recombination rates between different maternal or paternal ages could be calculated (P=0.251 and P=0.431, respectively).

Appendix

Fig A1: Recombination rates per cytoband obtained from euploid embryos vs. embryos with maternally derived meiotic trisomies *(please print in color).* Bioinformatic analysis of recombination data points for determination of cytoband frequencies was carried out using a script in R. Chromosomal bands (cytobands) surrounded by red boxes are bands for which only 0 – 2 SNPs were available on the SNP array utilized for this study and as such, no recombination events could be collected. CEN = centromere

Tables B1-B10: Collected raw data