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1 **Array CGH on first polar bodies suggests that non-disjunction is *not* the**
2 **predominant mechanism leading to aneuploidy in humans**

3

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20

21 **Abstract**

22 Aneuploidy (the presence of extra or missing chromosomes) arises primarily through
23 chromosome segregation errors in the oocyte at meiosis I, but the details of
24 mechanism by which such errors occur in humans are the subject of some debate. It
25 is generally believed that aneuploidy arises primarily as a result of segregation of a
26 *whole chromosome* to the same pole as its homologue (non-disjunction).
27 Nonetheless, classical cytogenetic studies suggest that this model does not fully
28 account for the patterns observed in human oocytes. An alternative model
29 (precocious separation of sister chromatids) has thus been proposed but recurring
30 criticism of this model purports that technical issues may have led to interpretation
31 errors. To resolve this question we used array CGH on 164 human first polar bodies
32 to distinguish between whole chromosome (non-disjunction) and chromatid
33 (precocious separation) errors. Single chromatid errors were over 11 times more
34 common than whole chromosome errors, consistent with prior classical cytogenetic
35 and FISH studies. The received wisdom that non-disjunction is the primary
36 mechanism leading to human aneuploidy should therefore be reconsidered.

37

38 Introduction

39 Aneuploidy, the presence of extra or missing chromosomes in a gamete, embryo,
40 fetus or live born individual is the major cause of human pregnancy loss, mental
41 retardation and, most likely, IVF failure¹⁻⁴. Although errors can occur in male
42 gametes and post-meiotically, aneuploidy arises primarily through chromosome
43 segregation errors in the first division of female meiosis (maternal MI)¹⁻⁵. It is well
44 established that advanced maternal age and perturbed genetic recombination at MI
45 can affect the levels of aneuploidy; differences in segregation error rates for
46 individual chromosomes are also reported⁶⁻⁷. Despite decades of study, the precise
47 mechanism by which chromosome segregation errors occur (and lead to aneuploidy)
48 is the subject of some debate.

49 It is generally believed that the origin of human aneuploidy (at MI) involves the
50 segregation of a *whole chromosome* to the same pole as its homologue i.e. non-
51 disjunction (see figure 1); this is because studies on model organisms such as yeast,
52 *Drosophila* and mouse largely point to this as the primary mechanism²⁻⁴. In contrast,
53 several investigations involving the generation of chromosome preparations from
54 human oocytes suggest that the non-disjunction model does not fully account for the
55 pattern of aneuploidies seen^{1; 8-13}. As a result, Angell and colleagues offered an
56 alternative explanation, variously described as 'pre-division', 'premature separation of
57 sister chromatids' and/or 'precocious separation'^{8-9; 11-12; 14} to explain the majority of
58 aneuploidy seen in humans. This model proposes that *meiosis I errors result in extra*
59 *or missing chromatids, not whole chromosomes* in the daughter cells (see also figure
60 1). Recurring criticism of such a model, suggest that poor oocyte quality, use of
61 "failed IVF" oocytes, prolonged time in culture, metaphase preparation technique and
62 lack of rigour in the analysis may have led to interpretation errors¹⁵⁻¹⁸. In other words,
63 critics of the Angell model suggest that these confounding factors cause the
64 chromatids to separate prematurely for technical, rather than biological reasons.

65

66 With the above in mind, experiments that derive results independent of these
67 confounding factors are key to understanding how this fundamental phenomenon in
68 medical genetics arises. In particular, it is important that we reconcile the relative
69 contributions to human aneuploidy of the two models illustrated in figure 1. The aim
70 of this study was therefore to distinguish whole chromosome from single chromatid
71 errors in freshly harvested human first polar bodies using a methodology less prone
72 to the aforementioned problems, i.e. array comparative genomic hybridization
73 (aCGH)¹⁹.

74

75

76 **Materials and Methods**

77 Human oocytes from 25 patients aged 29-50 were harvested 43-45 hours after
78 administration of Human Chorionic Gonadotrophin and 169 first polar bodies were
79 biopsied from them by micromanipulation. They were subjected to whole genome
80 amplification (WGA) using SureplexTM then aCGH using a commercial service (both
81 BlueGnome, Cambridge). Briefly, WGA products from biopsied polar bodies and
82 control (male) DNA were labelled with Cy3 and Cy5 fluorophores by random priming.
83 Labelling mixes were combined and co-precipitated with human Cot-1 DNA,
84 resuspended in hybridisation buffer and hybridized onto “24sure” microarray slides
85 (BlueGnome) followed by stringency washes to remove unbound DNA. Scanned
86 images of hybridised fluorophores were then exported to the “BlueFuseMulti”
87 software (BlueGnome, Cambridge) for ratio analysis¹⁹ in which per-chromosome
88 Cy3/Cy5 ratios were examined.

89

90 Representative amplification of genomic DNA from single cells is a technically
91 challenging procedure susceptible to variation in the quality of the biological material
92 as well as upstream methods, including polar body biopsy, culture and storage

93 conditions. Hybridisation plots of WGA products are therefore typically “noisy”
94 compared to those of high quality genomic DNA samples and this is manifested as
95 variation in the log₂ratio of the Cy3 vs Cy5 signals. We compensated for this by
96 technically matching samples to a co-hybridised reference and by the recent
97 introduction of smoothing algorithms during data analysis. Specifically, the latest
98 version of the “BlueFuseMulti” software incorporates the generation of “green lines”
99 drawn automatically based on the result of the aneuploidy calling algorithm that the
100 software employs (see figure 2). The algorithm classifies each whole chromosome as
101 either “copy number neutral” or showing evidence of gain or loss by estimating the
102 probability of each outcome. The software then automatically selects the most likely
103 status for each chromosome through the generation of a horizontal green line. When
104 a chromosome has been determined to be copy number neutral, the green line is
105 drawn across the chromosome at zero on the log₂ ratio scale. Where a chromosome
106 is determined to have an abnormal copy number the green line is drawn across the
107 chromosome at the level of the median log₂ ratio of the set of probes that map to the
108 chromosome. In our experience, this approach generates an excellent “first
109 visualization” of the data, detecting over 95% of errors accurately. In addition, three
110 independent observers examined each graph carefully to identify any chromosomes
111 for which the software may not have called aneuploidy within the criteria set out below.
112 When all three observers agreed that that the green line should be re-drawn (as, in
113 our opinion, the software had not called copy number accurately) then this was done
114 manually. Although this approach inevitably leads to a degree of subjectivity in the
115 interpretations, analysis of several thousand single cells in this way has led us to be
116 confident about the assignments in each case.

117

118 Single chromatid errors (precocious separation) were distinguished from whole
119 chromosome (non-disjunction) errors through examination of the mean per-
120 chromosome hybridisation ratios. For most chromosomes (i.e. not the sex

chromosomes nor the aneuploid chromosomes) a consistent 1:1 ratio was observed along the chromosome length. As all samples were co-hybridised with male genomic DNA, we first examined the graphs to establish a hybridisation pattern representing a 2:1 ratio (typically $\log_2 = 0.4$ to 0.6 ; figure 2a) for the X chromosome, and a “0:2” ratio for the Y chromosome (typically $\log_2 = -0.6$ to -1 however the Y chromosome displayed more variation than the X; figure 2a). Whole chromosome gains were consistent with a 2:1 ratio. In practice we generally scored a whole chromosome gain (non-disjunction error) if the mean hybridisation ratio had a \log_2 value greater than 0.6 and greater or equal to that of the X chromosome. Single chromatid gains (precocious separation) were consistent with a 3:2 (or 1.5:1) ratio, which was manifested as \log_2 ratios of between 0.25 and 0.45 (and lower than that of the X chromosome). Whole chromosome losses (non-disjunction errors) were generally scored when the mean hybridisation ratio was lower than -0.8 , whereas chromatid losses (precocious separation) were consistent with a 1:2 hybridisation ratio, i.e. typically between -0.4 and -0.7 (Figure 2b-c). Visual comparisons (for losses) were also noted for with the Y chromosome but, because of the aforementioned variation compared to the Y, was used only as a guide. In any event, the ratios for the X and Y chromosome were somewhat closer to zero than the whole chromosome gains and losses. We believe this is due of the presence of X/Y homologous genes.

Only rare “intermediate” assignments (e.g. between 0.45 to 0.6 for gains; -0.7 to -0.8 for losses) were the cause for some discussion and consensus. The vast majority were given the same diagnosis by all three observers and, where consensus could not be reached (e.g. where there were multiple errors or the sex chromosomes were not easily read) the data was disregarded. For both gains and losses therefore there were two clear groups (with rare intermediates) that we could interpret as either “whole chromosome” or “single chromatid” gain/loss and thereby infer the mechanism of chromosome segregation error.

149

150

151 **Results**

152 Of the 169 oocytes biopsied, a total of 164 (97.0%) first polar bodies were
153 successfully amplified and analysed, the remaining 5 (3.0%) were discarded for
154 technical reasons (e.g. failed to amplify). Of those analyzed, 78 (47.6%) had no
155 detectable chromosome segregation error (e.g. figure 2a), 86 (52.4%) had at least
156 one gain or loss of a chromosome/chromatid (e.g. figure 2b-c). The total number of
157 errors was 256 giving a per-polar body error rate of 1.56. The per-patient error rate
158 was then compared to maternal age (see below). Of those aneuploid polar bodies, a
159 further 7 were not used for the chromatid/chromosome differentiation as the three
160 independent observers were not confident of making a diagnosis. The main reason
161 for this was that the polar bodies had multiple (up to 20) errors or, on occasion, the
162 X/Y ratios were not clear. With these removed, the per-polar body error rate became
163 1.21. There was no statistical difference overall between the frequency of losses (110
164 = 55.3%) compared to gains (89 = 44.7%) but whole chromosome losses were twice
165 as common as gains (figure 3). Most notably, *single chromatid errors were 11.5 times*
166 *more common than whole chromosome errors* (92.0% vs. 8.0%; figure 3). Figure 3
167 also shows that errors involving chromosomes 15, 21 and 22 were the most frequent
168 and those involving chromosomes 3, 4, 5, 8 11, 12 and 16 the least frequent. Finally,
169 a Pearson correlation coefficient of 0.4 ($p=0.02$ by one tailed t-test) indicated a
170 positive association between the frequency of segregation errors (all types) and
171 maternal age, albeit with apparent inter-individual differences (table 1, figure 4).

172

173

174 **Discussion**

175

176 Our observations are consistent with previous studies on metaphase preparations of
177 human oocytes^{1; 8; 14; 20}, and mouse model systems²¹ supporting the hypothesis that
178 precocious separation of sister chromatids is the predominant mechanism leading to
179 aneuploidy in humans. The more often cited non-disjunction model on the other hand
180 appears a relatively minor player. It has been suggested that reduction of function of
181 cohesin proteins holding the chromatids together is involved in the aetiology of
182 aneuploidy^{20, 21} and further studies combining the approaches outlined here
183 combined with 3D imaging of the oocytes themselves will help to confirm this
184 hypothesis.

185

186 The notion of chromosome specific error rates is also supported, but those observed
187 in this study as being most prone to error were not necessarily those represented
188 most frequently among spontaneous abortions^{2; 4; 11; 22-24}. For instance, chromosome
189 16, the most frequent trisomy observed in human abortus material, was apparently
190 one of the least likely to undergo a segregation error in this data set. It is possible
191 that the absence of aneuploidy for chromosome 16 in this data set is a mathematical
192 anomaly, this will be confirmed or refuted when larger studies are analysed.

193

194 Unsurprisingly, our data supports the well-established association of advanced
195 maternal age with aneuploidy²⁵⁻²⁶. Nonetheless, even in this small data set, we found
196 preliminary evidence that some older women may segregate chromosomes normally
197 in their oocytes. Thus identification of such key individuals may be a route by which
198 we might understand what specific factors associated with maternal age can lead to
199 aneuploidy and, ultimately, what clinical interventions can be implemented to
200 alleviate it.

201

202 Finally, our findings raise some immediate practical concerns regarding the use of
203 polar bodies for preimplantation genetic screening. The presence of extra or missing

204 chromatids in the first polar body can lead to both euploid and aneuploid outcomes in
205 the oocyte, reinforcing the need to screen both polar bodies when a definitive specific
206 diagnosis is required (e.g. for positive selection of euploid oocytes)¹⁹⁻²⁰. The
207 observation that meiosis II errors are not infrequent in female meiosis also supports
208 the notion that both polar bodies should be in a clinical setting.

209

210

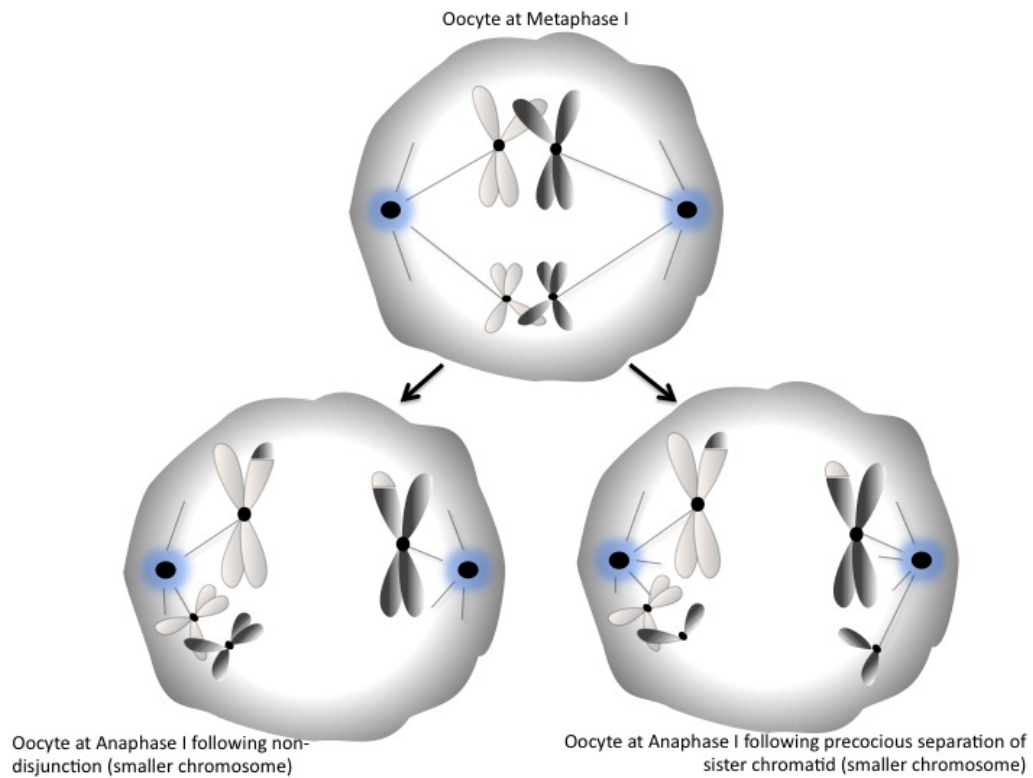
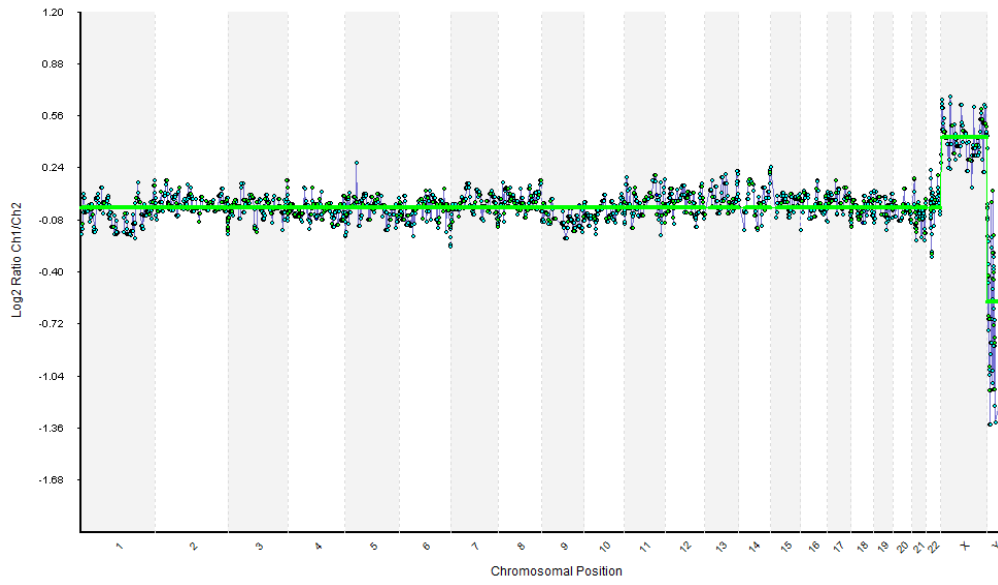
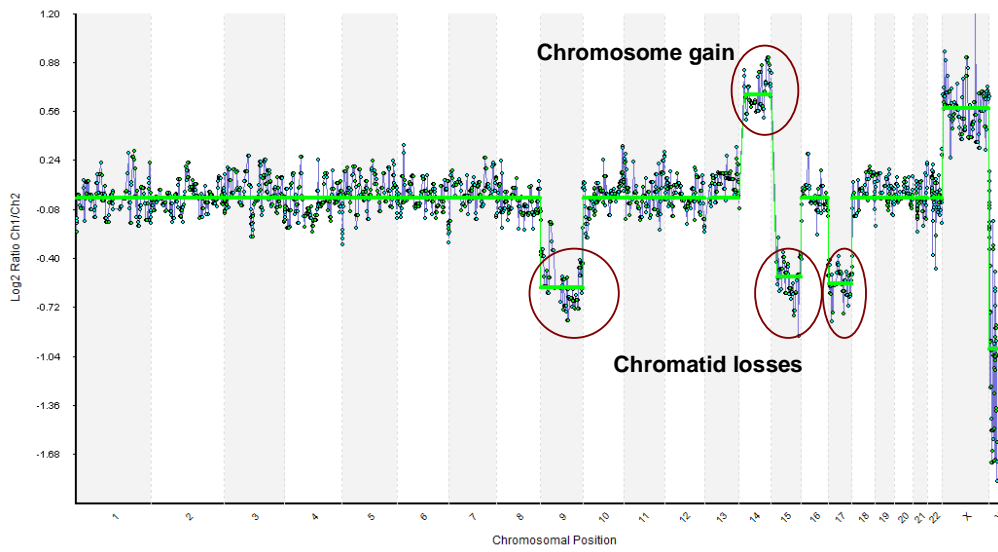


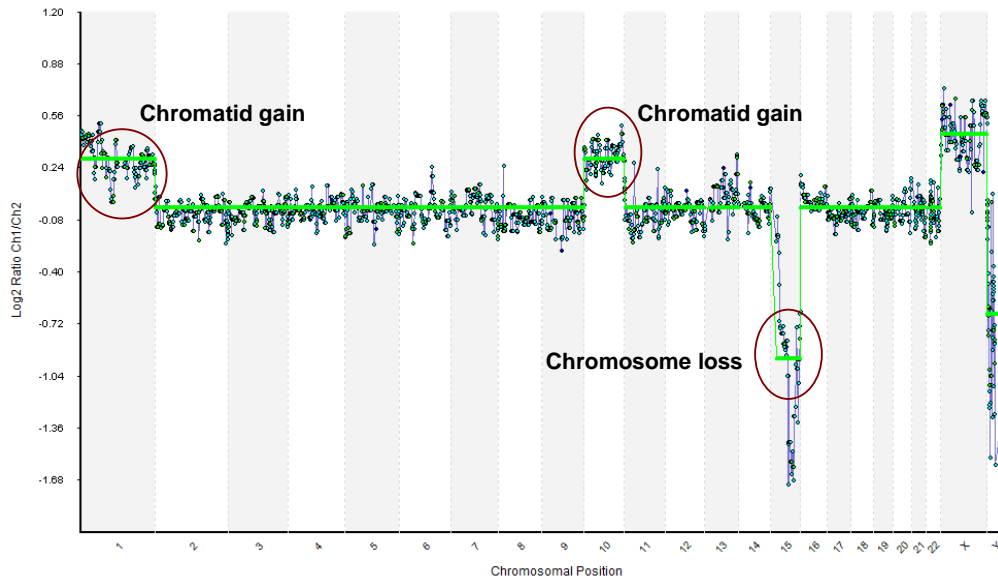
Figure 1. A metaphase I oocyte about to undergo division (top). The larger chromosome is represented as undergoing normal disjunction (bottom); the smaller chromosome is shown undergoing classical non-disjunction (bottom left) and precocious separation (bottom right). Both mechanisms can lead to aneuploidy.



218



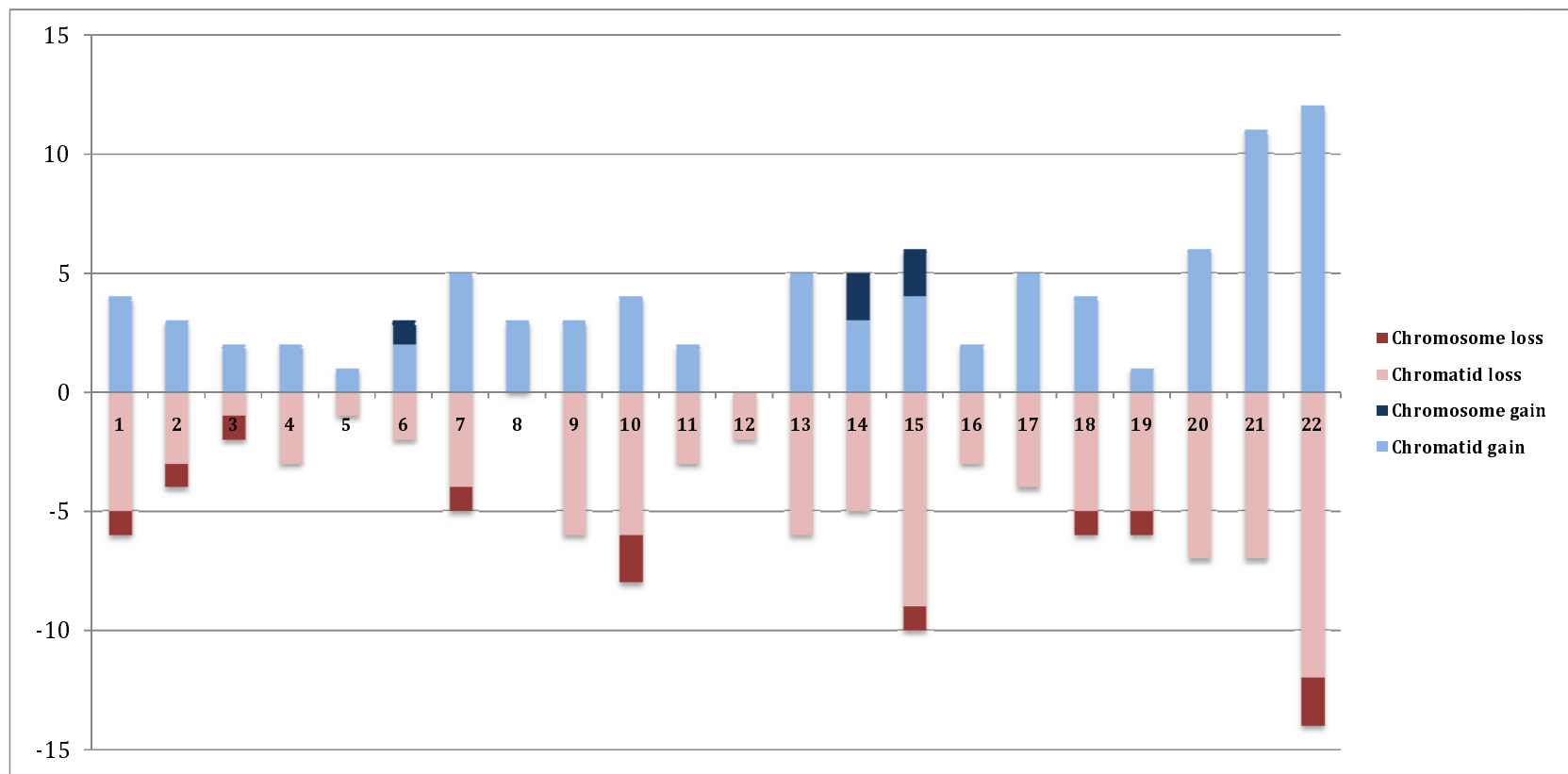
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220

221 Figure 2. aCGH hybridisation ratio plot showing gains and losses from first polar
 222 bodies and comparisons with sex chromosomes. a) Euploid polar body compared to
 223 male reference. b) Aneuploid polar body demonstrating chromatid losses on
 224 chromosomes 9, 15 and 17, and a gain of whole chromosome 14. c) Aneuploid polar
 225 body demonstrating chromatid gains on chromosomes 1 and 10, and a loss of whole
 226 chromosome 15.

227



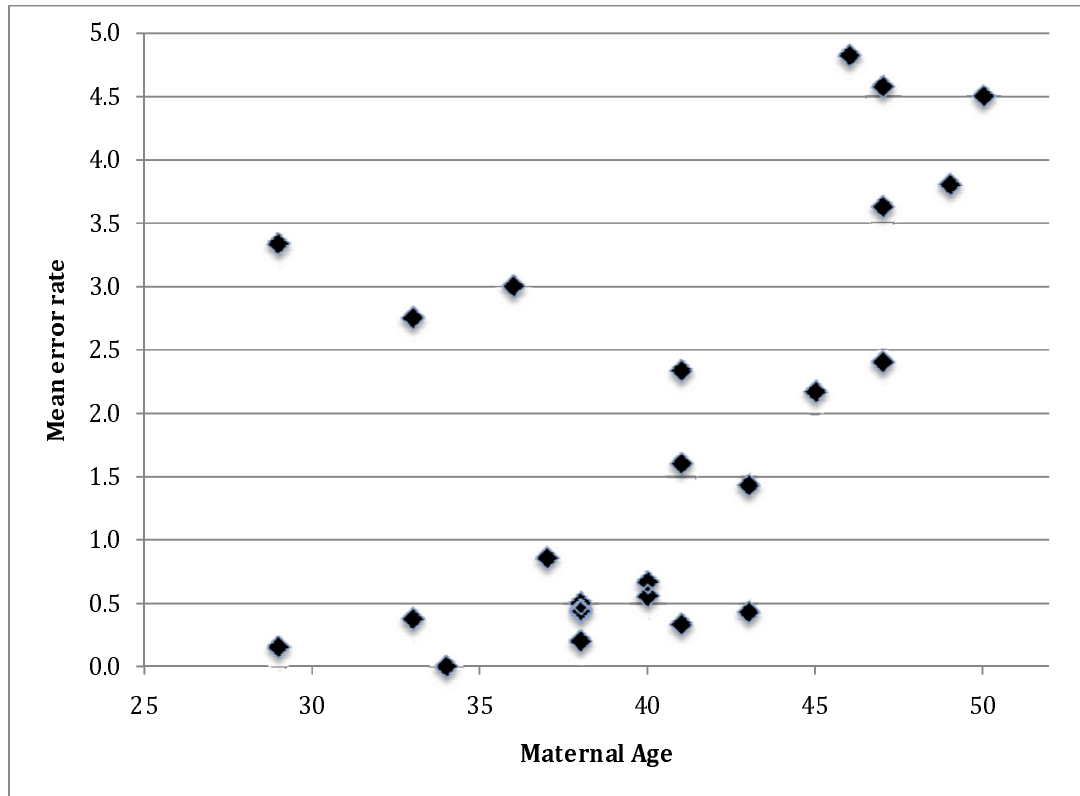
228

229 Figure 3. Summary of aCGH experiments from human 1st polar bodies plotted against number of observed chromosomal abnormalities.

230 Losses for chromatids and chromosomes are shown in light and dark red respectively. Gains for chromatids and chromosomes are shown in

231 light and dark blue respectively.

232



233

234

235

236 Figure 4: Maternal age plotted against per-polar body chromosome segregation rate

237

| Maternal age | Number of polar bodies | Total number of errors | Error rate per polar body |
|--------------|------------------------|------------------------|---------------------------|
| 29 | 3 | 10 | 3.3 |
| 29 | 13 | 2 | 0.2 |
| 33 | 8 | 3 | 0.4 |
| 33 | 4 | 11 | 2.8 |
| 34 | 9 | 0 | 0.0 |
| 36 | 2 | 6 | 3.0 |
| 37 | 7 | 6 | 0.9 |
| 38 | 9 | 4 | 0.4 |
| 38 | 5 | 1 | 0.2 |
| 38 | 7 | 3 | 0.4 |
| 38 | 4 | 2 | 0.5 |
| 40 | 9 | 5 | 0.6 |
| 40 | 6 | 4 | 0.7 |
| 41 | 6 | 2 | 0.3 |
| 41 | 5 | 8 | 1.6 |
| 41 | 3 | 7 | 2.3 |
| 43 | 7 | 3 | 0.4 |
| 43 | 7 | 10 | 1.4 |
| 45 | 6 | 13 | 2.2 |
| 46 | 11 | 53 | 4.8 |
| 47 | 8 | 29 | 3.6 |
| 47 | 7 | 32 | 4.6 |
| 47 | 10 | 24 | 2.4 |
| 49 | 5 | 19 | 3.8 |
| 50 | 4 | 18 | 4.5 |

Table 1: Ages, number of oocytes, number of errors, and per oocyte errors rates of patients in this study

Supplementary material 1: 50 genome plots illustrating our results. The diagnosis is given in the file name. For instance “+1,+10 tid; -15 some” (figure 2c) should be interpreted as a gain of single chromatids for chromosomes 1 and 10 and a loss of whole chromosome 15.

Supplementary material 2: 13 genome plots focussing exclusively on whole chromosome gains and losses. Footnotes explain the intervention of the independent observers.

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