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ARTICLE

Array CGH analysis shows that aneuploidy is not related to the number of embryos generated

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Abstract This study retrospectively analysed array comparative genomic hybridization (CGH) results of 7753 embryos from 990 patients to determine the frequency of embryonic euploidy and its relationship with the cohort size (i.e. the number of embryos available for biopsy and array CGH analysis). Linear regression analysis was performed to assess the effect of cohort size on euploidy rate adjusted for the effect of female age. While increasing female age was associated with a significant decrease in euploidy rate of day-3 and day-5 embryos ($P < 0.001$ for both groups), cohort size was not significantly associated with euploidy rate. Logistic regression analysis was performed to assess the effect of cohort size, adjusted for maternal age, on the likelihood of having at least one euploid embryo available for transfer. The odds of having at least one euploid embryo in an assisted cycle was significantly decreased by increasing female age ($P < 0.01$ for both day-3 and day-5 embryos) and was significantly increased by every additional embryo available for analysis ($P < 0.001$ for both day-3 and day-5 embryos).

KEYWORDS: aneuploidy, assisted reproduction, comparative genomic hybridization, IVF, preimplantation diagnosis

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Introduction

Aneuploidy in human cleavage-stage embryos increases with maternal age (Magli et al., 2001; Marquez et al., 2000; Munné et al., 1995), but even embryos from young women and egg donors can have high rates of aneuploidy (Munné et al., 2006; Reis Soares et al., 2003). Not surprisingly, aneuploidy in blastocyst-stage embryos also increases with maternal age although fewer studies have been performed at that stage (Fragouli et al., 2011). Aneuploidy seems to be one of the leading causes of implantation failure following assisted reproduction treatment.

Commonly used morphological criteria have proven inadequate to identify euploid embryos with high specificity (Munné et al., 1995; Marquez et al., 2000; Magli et al., 2001, 2007). Until recently, blastomere biopsy followed by determination of chromosome copy numbers with fluorescence in-situ hybridization (FISH) has been the only other widely used method for assessment of embryonic chromosomal status. Unfortunately, FISH only allows the analysis of a limited number of chromosomes. Moreover, technical difficulties resulted in a broad range of error rates between laboratories (Munné et al., 2010). These and other factors have led to FISH being increasingly abandoned as a preimplantation genetic screening (PGS) technique for improving clinical outcomes.

Contrary to FISH, more recent technologies, such as comparative genomic hybridization (CGH) (Schoolcraft et al., 2011; Wells et al., 2002; Wilton et al., 2001), array CGH (Gabriel et al., 2011; Gutierrez-Mateo et al., 2011) and single nucleotide polymorphism (SNP) arrays (Handyside et al., 2010; Johnson et al., 2010; Schoolcraft et al., 2011; Treff et al., 2010) allow assessment of the entire chromosomal complement of the embryo. Array CGH results for PGS have been previously validated and this technique yielded a result in 97.1% of all embryos tested and was highly specific with only 1.9% error rate (Gutierrez-Mateo et al., 2011). Therefore array CGH allows more precise analysis of chromosomal complement of the embryo and can provide valuable information for physicians and couples regarding prognosis.

This descriptive study determines the frequency of embryonic aneuploidy as assessed by array CGH across female age. Furthermore, it investigates whether embryonic euploidy is related to the number of embryos available for biopsy and array CGH analysis. Finally, it presents euploidy rates stratified for female age and the number of embryos available for biopsy and array CGH analysis.

Materials and methods

This is a retrospective analysis of PGS results of human embryos by array CGH. Embryonic biopsies from 7753 embryos of 990 patients visiting 70 North American assisted reproduction clinics were evaluated by Reprogenetics between January 2010 and July 2011.

The database used for this study prevented directly or indirectly identifying individual patients. Thus, the study was determined to be exempt from Institutional Review Board (IRB) approval by the Western IRB in Olympia, Washington. According to the common rule 45 CFR 46.101(b)(4), exemptions include 'research, involving the collection or study of existing data, documents, records, pathological specimens, if these sources are publicly available or if the information is recorded by the investigator in such manner that subjects cannot be identified, directly or through identifiers linked to subjects'.

Inclusion criteria

The study cohort included women who underwent PGS with array CGH. Embryos generated from oocyte donation cycles were also included but analysed separately. Women who underwent preimplantation genetic diagnosis for diagnosis of balanced translocations or single gene diseases were excluded.

Oocyte donors were selected from women $\leq$35 years of age who had a good ovarian reserve. Good ovarian reserve was defined according to endocrinological and sonographic markers including early follicular phase serum FSH, oestradiol and progesterone concentrations and antral follicle count.

Embryo biopsy

Embryo biopsy was performed on day 3 or at blastocyst stage. Briefly day-3 embryos with $\geq$4 blastomeres were exposed to biopsy media and blastomeres were removed by suction using a blastomere biopsy pipette or after exerting pressure on the zona pellucida. In some cases, blastomeres were removed by expelling fluid into the perivitelline space. In all cases, the cells were removed after drilling a hole with acidified Tyrode's solution or a non-contact commercial laser. Blastocyst biopsy involved hatching the embryo either on day 3 or day 5. Suction was applied to the hatched trophectoderm on day 5 followed by cutting a small piece of trophectoderm (3—10 cells) by laser.

Array CGH

Biopsied cells were analysed as described previously by Gutierrez-Mateo et al. (2011). Briefly, cells were washed and collected into sterile PCR tubes. The samples and reference male DNA were lysed, fragmented and amplified using the SurePlex whole genome amplification kit (BlueGnome, Cambridge, UK) according to the manufacturer's instructions. Amplified DNA samples and reference male DNA were labelled with Cy3 and Cy5, respectively, by using the BlueGnome fluorescent labelling system, according to the manufacturer's instructions. Amplification and labelling protocols used in this study are available at www.cytochip.com. Labelled samples and reference male DNAs were mixed and applied to a 24Sure (BlueGnome) microarray and co-hybridized for a minimum of 3 h, after which they were washed in x2 saline sodium citrate (SSC)/0.05% Tween 20 at room temperature for 10 min, followed by a wash in $\times$1 SSC at room temperature for 10 min and with $\times$0.1 SSC at 59°C for 5 min and finally washed for 1 min at room temperature in the same solution. Microarray slides were dried in a centrifuge for 3 min and scanned with a laser scanner (InnoScan 710AL; Innopsys, Carbonne, France). Scanned images were analysed using BlueFuse Multi software (BlueGnome). Once a specific amplification was observed, autosomal profiles were analysed for gain or loss of whole or partial

chromosomal ratios using a 3SD assessment, $\geq 0.3 \log_2$ ratio call or both. To pass hybridization quality controls, sex mismatched female samples had to show a consistent gain on chromosome X and a consistent loss of chromosome Y. Sex-matched male samples had to show consistently no change on either chromosome X or Y (Gutierrez-Mateo et al., 2011).

Array CGH can detect 2–6 Mb deletions and duplications (Alfarawati et al., 2011; Colls et al., 2011; Fiorentino et al., 2011) depending on the array used. In this case the 24sure has a resolution of 4–6 Mb (Colls et al., 2011). Because less than 3% of embryos were found to be abnormal due solely to structural abnormalities, for purposes of simplifying data analysis, they were grouped with the aneuploid embryos.

Outcome measures and statistical analysis

Each woman has been included in the study with only one assisted reproduction/CGH cycle. In case of multiple treatment cycles, only the chronologically first cycle has been included in the analyses. However, the first assisted reproduction/CGH cycle is not necessarily the first ever treatment cycle of a woman. In order to avoid multiplicity associated with multiple embryos being generated in a cycle, the proportion of euploid embryos over embryos biopsied was calculated per cycle and this value has been treated as a continuous variable. Linear regression analysis was performed to assess the effect of cohort size, i.e. the number of embryos available for biopsy and array CGH, on euploidy rate adjusted for the effect of female age. In this model, the dependent variable was euploidy rate and the independent variables were female age in years and number of embryos biopsied.

Secondly, women have been categorized into those who had at least one euploid embryo available for transfer and those who did not have any euploid embryos at all. A logistic regression analysis was performed to assess the effect of cohort size, adjusted for maternal age, on the likelihood of having at least one euploid embryo available for transfer. The dependent variable was presence of at least one euploid embryo per woman (binary variable coded as 0 or 1). Independent variables were female age in years and number of embryos biopsied.

All analyses were performed separately for day-3 and day-5 embryos, as the mean euploidy rate was significantly different between the groups ($P < 0.001$). In order to adjust for any differential effects of varying ovarian stimulation regimens and embryology laboratory practices across referring clinics, regression analyses were repeated after clustering the data for treating clinic and using robust standard errors.

Finally, the proportion of euploid embryos and percentage of women who had at least one euploid embryo were presented across arbitrarily defined categories of female age and the number of embryos biopsied in order to present the reader with figures that can be used to guide clinical decision making and to counsel patients.

Intercooled STATA 9 (StataCorp, TX, USA) was used for statistical analyses.

Results

The overall study cohort included 7753 embryos/blastocysts from 990 women who underwent PGS by array CGH. Array CGH yielded a result in 7345/7753 analysed embryos (94.7%) (6062/6404 (94.7%) and 1283/1349 (95.1%) for cleavage- and blastocyst-stage embryos, respectively) with the rest showing no analysable results either due to degraded DNA or amplification failure caused by anucleated biopsied cells or cells lost during the transfer to the tube. Female age (mean ± SD) was 36.7 ± 4.9 years and 36.5 ± 5.3 years for cleavage- and blastocyst-stage groups, respectively. Donor age was 26.6 ± 3.7 years for PGS cycles in which embryos were generated using donated oocytes.

Women using own oocytes

Cleavage-stage embryos biopsied on day 3

A total of 5918 cleavage-stage embryos obtained from 726 women were analysed with array CGH. The percentages of euploid and aneuploid embryos across female age are presented in **Figure 1**. Array CGH analysis results of cleavage-stage embryos stratified for age and the number of embryos are presented in **Table 1**.

Linear regression analysis revealed that for every year increase in female age, euploidy rate was decreased by 2.4 percentage points (95% CI −2.7% to −2.0%, $P < 0.001$), whereas analysable cohort size was not significantly associated with the euploidy rate (B 0.23%, 95% CI −0.10% to 0.55%). The association between female age and euploidy rate of day-3 embryos is presented in **Figure 1**.

Logistic regression analysis revealed that the odds of having at least one euploid embryo was significantly decreased by increasing female age (odds ratio (OR) 0.79, 95% CI 0.75 to 0.85, $P < 0.001$), while the odds of having at least one euploid embryo was significantly increased by every additional embryo available for analysis (OR 1.33, 95% CI 1.24–1.43, $P < 0.001$).

Introduction of the treatment centre into the regression model essentially did not change the estimates (data not shown).

Blastocysts

A total of 1218 blastocysts from 203 women were analysed with array CGH. The percentages of euploid and aneuploid blastocysts across female age are presented in **Figure 2**. Array CGH analysis results of blastocysts stratified for age and the number of embryos are presented in **Table 2**.

Similar to cleavage-stage embryos, linear regression analysis revealed that for every year increase in female age, euploidy rate was decreased by 2.9 percentage points (95% CI −3.8% to −2.0%, $P < 0.001$), whereas analysable cohort size was not significantly associated with the euploidy rate (B = −0.32%, 95% CI −1.4% to 0.8%). The association between female age and euploidy rate of blastocysts is presented in **Figure 2**.

Logistic regression analysis revealed that the odds of having at least one euploid embryo was significantly decreased by increasing female age (OR 0.82, 95% CI 0.70–0.94, $P = 0.006$), while the odds of having at least one euploid embryo was significantly increased by every additional embryo available for analysis (OR 1.55, 95% CI 1.25–1.93, $P < 0.001$).

Introduction of the treatment centre into the regression model essentially did not change the estimates (data not shown).

Figure 1 Euploidy status of embryos biopsied on day 3.

Table 1 Embryonic euploidy rate per cycle and the proportion of women who had at least one euploid embryo following day-3 biopsy and array CGH.

No. of day-3 embryos	*Oocyte donor*	*All women (age in years)*			
		<35	*35–39*	*40–42*	*≥43*
1–4					
Women (*n*)	3	27	43	60	24
Euploid embryos (%)	75.0	35.2	28.8	17.2	6.9
Women with ≥ 1 euploid embryo (*n*, %)	3 (100)	18 (66.7)	24 (55.8)	26 (43.3)	6 (25.0)
5–7					
Women (*n*)	9	48	98	59	16
Euploid embryos (%)	37.7	37.3	25.9	16.1	6.3
Women with ≥ 1 euploid embryo (*n*, %)	9 (100)	44 (91.7)	83 (84.7)	36 (61.0)	4 (25.0)
8–10					
Women (*n*)	6	42	71	40	17
Euploid embryos (%)	54.1	36.0	28.5	17.3	7.8
Women with ≥ 1 euploid embryo (*n*, %)	6 (100)	40 (95.2)	66 (93.0)	29 (72.5)	9 (52.9)
>10					
Women (*n*)	24	64	70	36	11
Euploid embryos (%)	42.7	44.7	32.4	13.6	14.8
Women with ≥ 1 euploid embryo (*n*, %)	24 (100)	64 (100)	68 (97.1)	30 (83.3)	10 (90.9)

Oocyte donation cycles

When cycles using donor oocytes were analysed separately, the linear regression analysis for day-3 (486 embryos from 42 women) and day-5 (131 blastocysts from 19 oocyte donors) embryos revealed that neither female age nor the number of embryos biopsied seemed to affect euploidy rate (data not shown). This was most likely due to the small sample sizes of these subgroups and narrow age range of oocyte donors. There was essentially at least one euploid embryo in each oocyte donation cycle and this prevented conducting logistic regression analyses. Oocyte donation cycles are presented in separate columns in **Tables 1 and 2**.

Discussion

These results demonstrate a negative correlation between female age and embryonic euploidy, similar to former studies (Marquez et al., 2000; Munné et al., 1995). The current

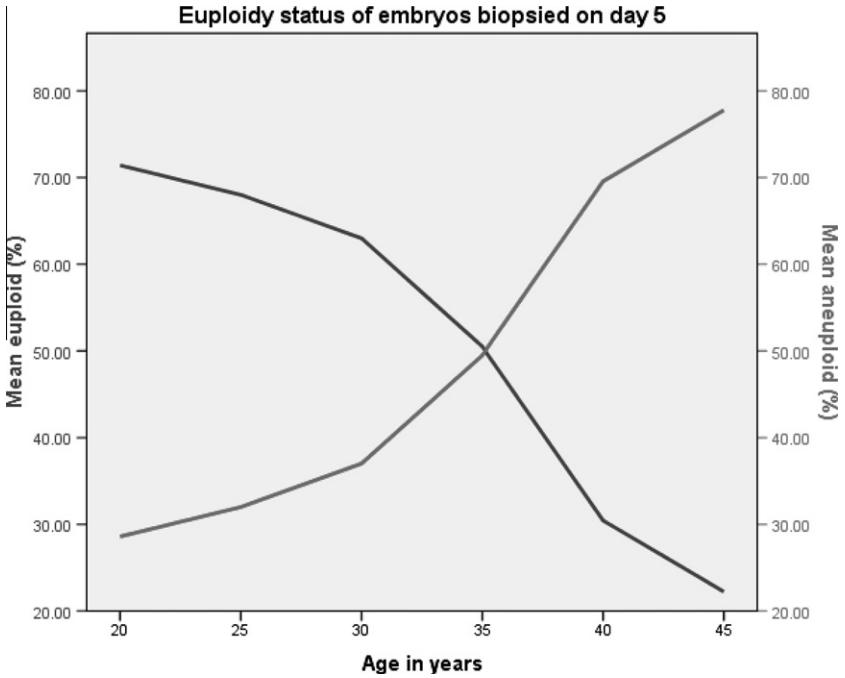

Figure 2 Euploidy status of blastocysts biopsied on day 5.

Table 2 Blastocyst euploidy rate and the proportion of women who had at least one euploid blastocyst following day-5 biopsy and array CGH.

No. of blastocysts	*Oocyte donor*	*All women (age in years)*			
		<35	*35–39*	*40–42*	*≥43*
1–4					
Women (*n*)	7	13	28	28	8
Euploid embryos (%)	70.2	66.0	49.1	34.2	16.7
Women with ≥ 1 euploid embryo (*n*, %)	7 (100)	12 (92.3)	22 (78.6)	17 (60.7)	3 (37.5)
5–7					
Women (*n*)	4	15	36	16	3
Euploid embryos (%)	77.5	69.9	52.3	31.0	13.3
Women with ≥ 1 euploid embryo (*n*, %)	4 (100)	15 (100)	35 (97.2)	13 (81.3)	2 (66.7)
8–10					
Women (*n*)	4	12	15	7	2
Euploid embryos (%)	62.4	56.7	48.3	27.4	22.5
Women with ≥ 1 euploid embryo (*n*, %)	4 (100)	12 (100)	15 (100)	6 (85.7)	2 (100)
>10					
Women (*n*)	4	5	7	7	1
Euploid embryos (%)	66.7	53.3	51.4	40.9	16.7
Women with ≥ 1 euploid embryo (*n*, %)	4 (100)	5 (100)	7 (100)	7 (100)	1 (100)

study presents the age-specific euploidy incidence assessed by array CGH. Euploidy rate seems unrelated to the number of analysable embryos generated per cycle. As far as is known, this is the largest study with complete chromosomal assessment reporting these relationships.

Although the proportion of euploid embryos remained unchanged with the number of embryos available, the proportion of women who had at least one euploid embryo increased when more embryos were generated. Arguably the number of embryos available reflects the ovarian reserve in this context as all women were stimulated with commonly used stimulation protocols and none had undergone mild stimulation or natural cycle IVF (Nargund et al., 2007). Hence, the current study is unable to comment on a potential difference between euploidy rates in mild stimulation or natural cycles versus standard stimulation cycles. It is well known that the number of embryos generated in an assisted reproduction cycle is directly proportional to the number of oocytes collected; however, it should be noted that the number of oocytes collected was not available in the database. Furthermore, there is little evidence that fertilization patterns and cleavage-stage patterns before

genome activation are related to maternal age. Therefore, embryo number is a good estimator of egg number and the current findings indirectly corroborate that women with high ovarian reserve should be more likely to achieve a pregnancy and live birth as they are more likely to generate more embryos and have at least one euploid embryo.

The median number of biopsied embryos in this study was 7, which is comparatively higher than reported in Europe, e.g. the median number of embryos generated in the UK between 1991 and 2008 is 5, for over 400,000 cycles (Sunkara et al., 2011). However, this may not be the case for the USA where stimulation protocols are more aggressive in general. Unfortunately, the Society of Assisted Reproductive Technology does not report number of embryos produced so it is difficult to demonstrate this point. Even if this study's population could have had more embryos than the average patient, for example due to cancellation of the PGS cycle when only few embryos are available by some physicians, any bias due to a higher number of embryos or women's age (which could also be higher than the general patient on average) is eliminated by stratifying the data according to embryo number and maternal age and by the use of regression modelling.

Given the fact that generation of more embryos is not associated with an increased aneuploidy rate in a conventional stimulation cycle, a reasonable number to aim for can be around 15 metaphase-II oocytes, as suggested by other studies (Garrido et al., 2011; Sunkara et al., 2011). However, this study does not argue in favour or against mild or conventional stimulation protocols since there might be repercussions to that in endometrial receptivity, and it is outside the scope of this study to link euploidy, hormonal stimulation regimens and implantation rates (Baart et al., 2007; Shapiro et al., 2011). Moreover, the reader should consider whether the number of embryos generated per cycle in the current sample is comparable to their own patient population.

The aim of assisted reproduction treatment is to achieve healthy live birth, preferably a singleton, in the shortest time by using the safest approach and the lowest level of intervention. Where the transfer of multiple embryos is permitted, one also needs to take into account the risk of a multiple pregnancy. Therefore, the decision to undertake array CGH will depend on the number of embryos available for transfer, the anticipated incidence of euploid embryos and the legal or voluntary limitations on the number of embryos that can be transferred. It is difficult to draw strict guidelines for the use of PGS with array CGH.

When there are strict limitations on the number of embryos transferred, array CGH can help to achieve a viable singleton pregnancy in shorter time with less transfer attempts for all women. Even in women younger than 35 years, the aneuploidy rate seems to exceed 50% among cleavage-stage embryos and reaches 30–40% in blastocysts (**Tables 1 and 2**). With advancing female age, it becomes more likely to transfer an aneuploid embryo that is not capable of leading to a live birth. In theory the selection of a euploid embryo with array CGH could increase the chances of achieving a clinical pregnancy, decrease the risk of miscarriage and eventually increase the likelihood of a live birth. The alternative could be transferring one or two embryos, depending on the restrictions, selected according to conventional morphological criteria, coupled with cryopreservation of any surplus embryos followed by sequential transfer in thaw cycles if pregnancy or live birth is not achieved. The latter approach would prevent any damage caused by the biopsy procedure (especially for day-3 embryos) and cut the cost of genetic testing while having the potential of providing good cumulative pregnancy and live birth rates. This not only requires the presence of a competent cryopreservation programme but such an approach will also cause a substantial proportion of women to undergo repeated embryo transfers requiring medication for endometrial preparation, monitoring scans and repeat visits to the treatment centre. Moreover, the transfer of aneuploid embryos to some women would result in miscarriages, sometimes requiring interventions such as curettage. In addition to the emotional stress of repeat transfer cycles, the direct and indirect costs, including loss of working days, that are associated with repeat cycles and the possible miscarriages could possibly exceed the cost of genetic testing and could be avoided with embryo selection with array CGH. Under strict restrictions on number of embryos transferred, even young women with good prognosis may benefit from the selection of a euploid embryo for the first transfer cycle. Perhaps the only group who would not benefit could be reproductively older women, aged $\geq$40 years, who have just a few cleavage-stage embryos. These women would possibly be better served using multiple-embryo transfer and the low incidence of euploid embryos ($\sim$6–17%, **Table 1**) would render multiple pregnancy a rare event. Women older than 42 years can also be counselled regarding the low incidence of euploid embryos and the $\sim$75% risk of not having any euploid embryos for transfer (**Table 1**). This would enable them to make a well-informed decision for proceeding with their own oocytes or donor oocytes. However, even women in this age group can benefit from array CGH if they can produce multiple blastocysts with relatively higher incidence of euploidy (**Table 2**), which can lead to a multiple pregnancy in case of multiple-embryo transfer.

In the context of multiple-embryo transfers, i.e. absence of legal restrictions, it is likely that the transfer cohort will include at least one euploid embryo for most women younger than 35 years of age. In case of failure, a second cohort chosen from cryopreserved embryos of the index cycle would most likely include euploid embryos. Therefore, embryo selection with array CGH may not be beneficial in decreasing the number of transfer cycles to a substantial extent for these women. However, such an approach would undoubtedly lead to a high rate of multiple pregnancies and complications and costs associated with them. It is prudent to limit the number of embryos transferred for these women even in the absence of legal restrictions. Women aged 35–42 years would be more likely to benefit from array CGH if they have numerous cleavage-stage embryos from which to select for transfer. On the other hand, almost half of the blastocysts from women aged 35–39 years and approximately one-third of the blastocysts from women aged 40–42 years are euploid and transfer of multiple blastocysts would undoubtedly lead to a high incidence of multiple pregnancies. Therefore, it would also be prudent to limit the number of blastocysts transferred in this age group. When the number of embryos transferred is limited, array CGH can be expected to improve overall treatment

outcome in all women, with the exception of women aged 40–42 years who have less than five cleavage-stage embryos and women aged ≥ 43 years undergoing cleavage-stage embryo transfer. However, for such patients in whom most embryos are abnormal after PGS, the transition to egg donation may be more acceptable.

Advances in genetic testing technology undoubtedly increase the scope and efficiency of genetic testing of human embryos. The current technology allows assessment of the whole chromosomal component while former FISH technology enabled testing for only a number of chromosomes. This is expected to improve clinical outcome over that achieved with FISH. However, the above-mentioned assumptions need to be tested in appropriately designed randomized trials to prove efficiency beyond doubt. The data presented in this study can also serve to inform design of such trials. Once efficiency is proved, decision-making and cost-effectiveness studies can determine relative advantages of implementation of array CGH into assisted reproduction practice.

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