Analysis of a preimplantation genetic test for aneuploidies in 893 screened blastocysts using KaryoLite BoBs: a single-centre experience

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Abstract

Introduction: Does euploidy of trophectoderm (TE) biopsies correlate with conventional blastocyst morphological, maternal age and implantation potential?

Methods: This is a one-centre, retrospective, observational study.

Results: Eight hundred and ninety-three blastocysts were biopsied; 57.73% were euploid. The euploidy rate was found to be significantly higher for the embryos with good morphology of inner cell mass (ICM) and TE. Between ICM and TE morphology variables, TE was more predictive of the euploidy rate. When broken down into different age groups, the percentage of good morphology embryos remained similar across all age groups, while the percentage of euploid embryos dropped with increasing age. These results suggest that the correlation between blastocyst morphology and ploidy status was present but poor. Faster growing day 5 blastocysts showed a significantly higher euploidy rate than slower growing day 6 or 7 blastocysts. The number of good-quality blastocysts per cycle, euploid blastocysts per cycle and the euploidy rate were strongly associated with maternal age. A trend towards an increased implantation rate was found with euploid embryo transfers compared to the control group without preimplantation genetic test for aneuploidies (PGT-A).

Conclusions: Blastocyst morphology, rate of development and maternal age were found to be significantly associated with euploidy rate. There is a trend that suggests PGT-A may help to improve the pregnancy rate, but it is not statistically different, and therefore, PGT-A remains an unproven hypothesis. Due to the limitation of a small size of the control group, further studies with more data are needed.

Keywords: Blastocyst biopsy, blastocyst morphology, KaryoLite BoBs, maternal age, PGT-A

INTRODUCTION

Assisted reproductive technology (ART) aims to identify embryos with good implantation potential that, in turn, will result in a single healthy live birth. As such, the embryo quality predicts the success of implantation and subsequent pregnancy. Morphological evaluation of embryos has always been and still is the preferred approach for selecting the embryo with the best quality.

Embryo aneuploidy is the most common factor that causes reproductive failure[1] and implantation failures in ART.[2] Genetically normal embryos can be identified using preimplantation genetic test for aneuploidies (PGT-A),[3] suggesting that improved implantation rates, reduced miscarriage rates and the subsequent increase in live birth rates can be achieved by removing aneuploid embryos before transfer.[4]

Performing day 3 biopsy and using fluorescence *in situ* hybridisation (FISH) for genetic analysis constituted the initial

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version of PGT-A in the 1990s. However, several randomised controlled trials conducted during that period concluded that this approach did not increase the live birth rates but reduced it in some cases.[5]

A new generation of preimplantation genetic testing was introduced in the last decade to overcome the limitations of FISH. Trophectoderm (TE) biopsy allowed aneuploidy assessment of all chromosome pairs instead of a limited set of chromosomes.[6] Some retrospective studies demonstrated the benefit of using preimplantation genetic screening to evaluate 23 chromosome pairs from the TE stage.[7-9] Patients over the age of 37 who had euploid embryos for transfer benefitted from it, but no benefit was demonstrated when all age groups were included in the study.[2] However, a retrospective study cautioned against the routine use of PGT-A in fresh *in vitro* fertilisation (IVF) cycles until its safety and effectiveness are established.[10] When miscarriage rates and ongoing pregnancy rates were reviewed, another study suggested that evaluating embryos by PGT-A may not improve the IVF outcome.[11] As PGT-A has remained an unproven hypothesis,[12] it should not be routinely used for all IVF cycles. Based on current evidence, PGT-A should not be utilised indiscriminately but should only be used when there is an increased risk of aneuploidy.[13] The American Society for Reproductive Medicine (ASRM) Practice Committee released the following opinion in 2018: 'The value of PGT-A as a screening test for *in vitro* fertilisation (IVF) patients has yet to be determined'.

Increasing maternal age results in an increase of aneuploid preimplantation embryos,[14-17] and euploidy rate is linked to blastocyst morphology.[11,18,19] This finding, however, differs from another study that did not establish an association between embryo genetic status and morphological parameters. Interestingly, aneuploidy rate was not associated with maternal age.[20]

Currently, platforms such as comparative genomic hybridization (CGH) microarrays, microarrays including single-nucleotide polymorphisms, quantitative polymerase chain reaction (PCR)/real-time PCR and next generation sequencing are commonly utilised for PGT-A genetic diagnostic technologies. However, there are no differences in identifying whole chromosome aneuploidy when validated properly.[21] The differentiating point is that some genetic technologies can additionally identify segmental chromosome deletions or duplications.[22]

In 2011, Gross *et al*. demonstrated that applying bacterial artificial chromosomes (BACs)-on-Beads (BoBs) to detect prenatal aneuploidies and microdeletions is rapid and reliable.[23] All 46 chromosomes can be screened using KaryoLite BoBs® (Perkin Elmer, Waltham, MA, USA), thus enabling identification of chromosomal abnormalities and arm-specific (segmental) aneuploidies.[24] The superiority of KaryoLite BoBs also included reduced hands-on time and lower consumables and reagent cost per sample, thus making it a cost-effective screening tool.[24]

Therefore, the aim of this study is to determine the euploidy of TE biopsies assessed by KaryoLite BoBs and correlate it with conventional blastocyst morphological evaluation, maternal age and implantation potential.

METHODS

Patients and cycles

This is a retrospective analysis of variations in chromosomal copy number identified in blastocysts of couples undergoing intracytoplasmic sperm injection (ICSI) between November 2017 and February 2020. Of the total 893 blastocysts included in this study, there were 599 embryos from 124 couples without any gamete donation and 294 embryos from 33 couples with donated oocytes. This retrospective study was approved by the Gleneagles Medini Hospital Ethics Committee. All patients were counselled by trained members of the team regarding ICSI and PGT-A, which included the possible advantages, risks of misdiagnosis and reported success rates. Signed consent forms were obtained from couples included in the present study.

Laboratory protocols

The oocytes were retrieved between 35 h and 36 h after human chorionic gonadotrophin (hCG) administration. The cumulus cells of oocytes were stripped about 4 h after retrieval, and ICSI was performed at 40–42 h post-hCG administration. Half an hour after ICSI, all the injected oocytes were cultured in a time-lapse incubator (EmbryoScope+; Vitrolife Sweden AB, Västra Frölunda, Sweden) capturing images every 7 min in ten focal planes with one-step universal medium (GTL; Vitrolife Sweden AB) till the blastocyst stage without changing the medium, at 37°C with 5% O_2 and 6% CO_2. Biopsy of the fully expanded blastocysts (day 5, 6 or 7) with differentiated inner cell mass (ICM) and TE was followed by vitrification about 1 h post biopsy.

Blastocyst grading

For verification and consistency, two embryologists assessed blastocysts' quality right before performing TE biopsy and graded them according to the criteria defined by Gardner and Schoolcraft, whereby AA, AB and BA were categorised as good-quality blastocysts. ICM and TE scores were recorded individually.[25] The TE and ICM were assigned one of the following grades: Grade A: TE is composed of many cells organised in the epithelium and ICM is composed numerous tightly packed cells; Grade B: TE is composed of several cells organised in loose epithelium and ICM is composed of several loosely packed cells; Grade C: TE is composed of a few large cells and ICM is composed of very few cells. Day 5 biopsied blastocysts were defined as 'faster growing' embryos and day 6 or 7 biopsied blastocysts as 'slower growing' embryos.

Blastocyst biopsy procedure

Only full blastocysts of expansion grade 4 or higher were selected from day 5 to day 7 for TE biopsy. All biopsies were done on the

heated stage of an Olympus IX-73 microscope equipped with Narishige (Tokyo, Japan) micromanipulation tools, in dishes prepared with droplets of 10 µL GMops-buffered handling medium (Vitrolife, Sweden) overlaid with pre-equilibrated mineral oil. One to two noncontact diode laser pulses (Lykos; Hamilton Thorne, Beverly, MA, USA) were applied in the zona pellucida of the blastocyst, opposite to the ICM. After zona drilling, the biopsy pipette was gently pushed beyond the hole in the zona and about four to six cells were gently aspirated into the biopsy pipette. Two or three laser pulses were directed at the junctions between the TE cells at the thinnest part of the aspirated cells before mechanical separation was performed. The biopsied embryos were then immediately washed and placed back into individual 20 µL droplets of culture media under oil for traceability. The biopsied cell samples were rinsed in several drops to remove the cell fragments and then pipetted into individual PCR tubes under a laminar flow in strictly aseptic conditions to avoid contamination, and the samples were then sent for PGT-A.

PGT-A procedure using KaryoLite BoBs

All procedures were done in accordance with the manufacturer's protocol. PicoPLEX WGA kit (Perkin Elmer) was used to perform whole genome amplification (WGA). The success of amplification was confirmed by assessing the amplified products. Briefly, 2 µL of TE cells' WGA products were amplified with biotin-labelled deoxy-ribonucleoside triphosphate (dNTP) mix for 60–90 min. The samples were hybridised to the KaryoLite BoBs bead set at 52°C in a shaking incubator (800 rpm) for 16 h after removing unbound biotin-labelled dNTPs. Luminex® 200™ instrument was used for signal detection. Initial data processing was performed using xPONENT® software version 3.1.971 (Luminex Corp., Austin, TX, USA). The cvs data file generated was then imported to BoBsoft® v2.1 (Perkin Elmer) for aneuploidy detection.

Frozen-thawed embryo transfer

Both morphological score and PGT-A results were used to select blastocysts for transfer. PGT-A result is the primary choice to choose embryo, and the secondary choice is based on morphology from good to poor. The selected vitrified blastocyst was warmed and cultured at 37°C (6% CO_2 and 5% O_2) until transfer was performed. Only frozen-thawed embryo transfer (FET) cycles with single embryo transfer (SET) or double embryo transfer (DET) were included. Blood β-hCG test was defined positive when serum hCG value was >25 IU/L on day 14 and double the value 48 h later. Definitions used were according to those of Farquharson *et al*., where 'biochemical pregnancy loss' is defined as the absence of an identifiable pregnancy on ultrasound examination, clinical pregnancy is the presence of gestational sac in Week 7 after transfer, implantation rate is the number of foetuses with heart activity per transferred embryo and ongoing pregnancy is ≥ 20 completed weeks of gestation.[26]

Statistical analysis

The chi-square test was used to evaluate the significance of differences between proportions (in percent). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Genetic analysis results

From November 2017 to February 2020, a total of 893 blastocysts from 157 stimulation cycles were biopsied for PGT-A, of which 33 cycles were oocytes from a donor programme and 124 cycles were from patient's own oocytes. Thirteen biopsies that yielded no result (1.46%) due to poor-quality results or failed amplification were excluded from the analysis. Among the 880 diagnosed blastocysts, 508 (57.73%) were euploidy and 372 (42.27%) were aneuploidy; 218 embryos (24.77%) had one affected chromosome, 115 embryos (13.07%) showed multiple affected chromosomes and only 39 embryos (4.43%) were segmental imbalances (partial losses or gains of chromosomal material), a consequence of chromosome breakage. Among all the 880 diagnosed blastocysts, 431 embryos (48.98%) were XX, 430 (48.86%) were XY, 10 (1.14%) were XXY and 9 (1.02%) were XO (monosomy X). The sex ratio was one perfectly [Table 1]. Chromosomal abnormalities totalling 501 were detected in the 372 aneuploid embryos [Figure 1]. Chromosomal aneuploidies were most frequent in chromosome 16 (10.98%), followed by chromosome 22 (9.38%), 21 (7.58%), 19 (5.99%), 15 (5.59%), 14 (4.99%), and 18 and 20 (4.79%).

Blastocyst morphology and euploidy status

There was a clear correlation between blastocyst morphology and euploidy rate [Table 2]. The euploidy rates of blastocysts with good, average and poor morphology were 69.95%, 56.89% and 40.07%, respectively ($P < 0.0001$). Individually,

Table 1. Demographic variables according to the aneuploidy type and sex chromosome.

Variable	*n* (%)
Blastocysts biopsied	893 (100)
From cycles with patient's oocytes	599 (67.08)
From cycles with donor oocytes	294 (32.92)
No diagnosis	13 (1.46)
Diagnosed	880 (98.54)
Chromosome euploidy and aneuploidy	
Euploidy	508 (57.73)
Single chromosome aneuploid	218 (24.77)
Multiple chromosome aneuploid	115 (13.07)
Segmental aneuploid	39 (4.43)
Sex chromosome	
XX	431 (48.98)
XY	430 (48.86)
XXY	10 (1.14)
XO	9 (1.02)

both the morphology grades of ICM and TE showed a strong association with euploidy rate. A significantly higher euploidy rate was noted in embryos with good morphology of ICM and/or TE compared to those with poor morphology (ICM scores A, B and C: 70.03%, 55.56% and 41.56%, respectively; $P < 0.01$; TE scores A, B and C: 71.83%, 57.54% and 35.18%, respectively; $P < 0.01$). Between the variables of ICM and TE morphology scores, TE score was more predictive of the euploidy rate [Table 2]. When the TE score was A, the euploidy rates for ICM scores A, B and C were 74.18%, 64.29% and 66.67%, respectively ($P = 0.25$). When the TE score was B, the euploidy rates for ICM scores A, B and C were 64.29%, 57.82% and 49.49%, respectively ($P = 0.095$); When the TE score was C, the euploidy rates for ICM scores A, B and C were 36.36%, 37.74% and 34.07%, respectively ($P = 0.99$).

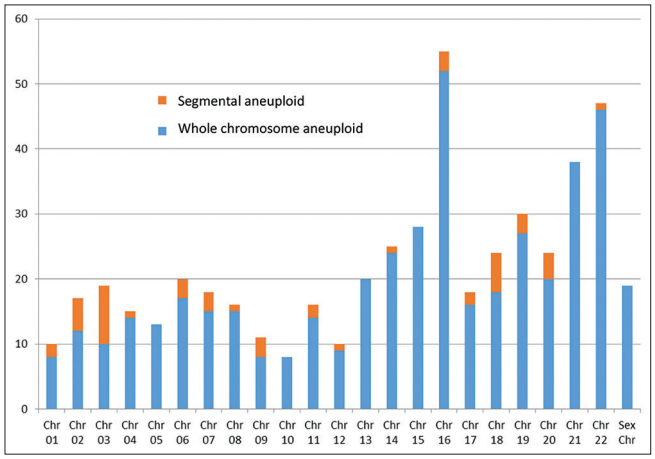

Figure 1: Graph shows the number of whole chromosome and segmental aneuploidies affecting each chromosome. Data are from 880 blastocysts.

Table 2. Euploidy blastocyst rate according to blastocyst morphology score.

Blastocyst morphology score		Euploid blastocyst rate	
ICM	**TE**	***n* (%)**	***P***
A	–	257/367 (70.03)	<0.00001
B	–	150/270 (55.56)	
C	–	101/243 (41.56)	
–	A	232/323 (71.83)	<0.00001
–	B	206/358 (57.54)	
–	C	70/199 (35.18)	
A	A	181/244 (74.18)	0.2524
B	A	45/70 (64.29)	
C	A	6/9 (66.67)	
A	B	72/112 (64.29)	0.0947
B	B	85/147 (57.82)	
C	B	49/99 (49.49)	
A	C	4/11 (36.36)	0.9919
B	C	20/53 (37.74)	
C	C	46/135 (34.07)	
Good	AA, AB, BA	298/426 (69.95)	<0.00001
Average	BB, CA, AC	95/167 (56.89)	
Poor	BC, CB, CC	115/287 (40.07)	

ICM: inner cell mass, TE: trophectoderm

Among the 372 aneuploid blastocysts, the percentages of aneuploidy embryos that were assessed to have good, average and poor morphology were 34.41% (128/372), 19.35% (72/372) and 46.24% (172/372), respectively.

The day of biopsy and embryonic euploidy status

Of the 893 biopsies, 387 (43.34%) were from day 5 blastocysts, 495 (55.43%) were from day 6 blastocysts and 11 (1.23%) were from day 7 blastocysts. The sex ratio results showed that faster growing embryos were more likely to be male. There were more XY in day 5 blastocysts (55.00%, 202/380) and more XX in day 6 or 7 blastocysts (54.05%, 260/481), but there was no significant difference ($P > 0.05$). Table 3 details the relationship between the day of biopsy and the genetic status of embryo. Day 5 blastocysts that grew faster showed a significantly higher euploidy rate (69.90%, 267/382) compared to slower growing day 6 (49.18%, 240/488) and day 7 blastocysts (10.00%, 1/10). Evaluations were performed according to the oocytes' source to eliminate the effect of maternal age. A strong correlation [Figure 2] was found between the embryo ploidy status and both maternal age and the day the blastocysts were biopsied ($P < 0.01$).

Maternal age and embryonic euploidy status

A total of 599 blastocysts from 124 stimulation cycles with patient's own oocytes were biopsied. The mean maternal age was 35.60 ± 4.86 (22–45) years, and 48.25% (289/599) of blastocysts were of good quality according to the Gardner blastocyst grading scale score (AA, AB, BA) and the euploidy rate was 50.09% (294/587). There was an average of 4.83 blastocysts per cycle for biopsy, 2.33 had good morphology, and 2.37 were euploid. The results for different age groups are detailed in Table 4.

Only the percentage of good morphology blastocysts did not show any significant difference among all age

Table 3. Correlation between euploidy rate and day of biopsy in different maternal age groups.

Oocyte source/ age group (yr)	*n* (%)		
	Day 5	**Day 6**	**Day 7**
Donor oocyte			
23.24±2.91	110/135 (81.48)	103/155 (66.45)	1/3 (33.33)
Patient's own oocyte			
≤31	65/87 (74.71)	48/87 (55.17)	0
32-35	61/84 (72.62)	40/89 (44.94)	0/3 (0)
36-39	20/44 (45.45)	41/111 (36.94)	0/2 (0)
≥40	11/32 (34.38)	8/46 (17.39)	0/2 (0)
Total	267/382 (69.90)*	240/488 (49.18)*	1/10 (10.00)*

*P<0.01 when comparing between day 5 and day 6 euploids, day 6 and day 7 euploids, and day 5 and day 7 euploids.

groups (P = 0.77). The number of good-quality blastocysts per cycle, euploid blastocysts per cycle and the euploidy rate were strongly associated with patient age [Figures 3 and 4].

Table 4 and Figure 3 are instructive, as they show that a correlation between good morphology embryos and euploidy was observed only when all embryos from all age groups were used in the correlation. When broken down into different age groups, however, the percentage of good morphology embryos remained similar across all age groups (from 54.72% to 42.11%), while the percentage of euploid embryos dropped with increasing age (from 65.31% to 11.11%). Clearly, the percentage of good morphology embryos did not track the declining percentage of euploid embryos with increasing maternal age.

Women younger than 33 years old had the highest percentage of euploid embryos. There was no difference observed in the euploidy rate among three age groups: <30 years, 65.31%; 30–31 years, 64.47%; and 32–33 years, 64.15% (P = 0.98). Euploidy rates declined with increasing maternal age after the age of 33. This is demonstrated by the close fit between the dashed regression line and the data points [Figure 5]. Average euploidy rates in women aged 33–45 years can be interpolated from the equation for the line based on the strong linear correlation (R^2 = 0.9716)

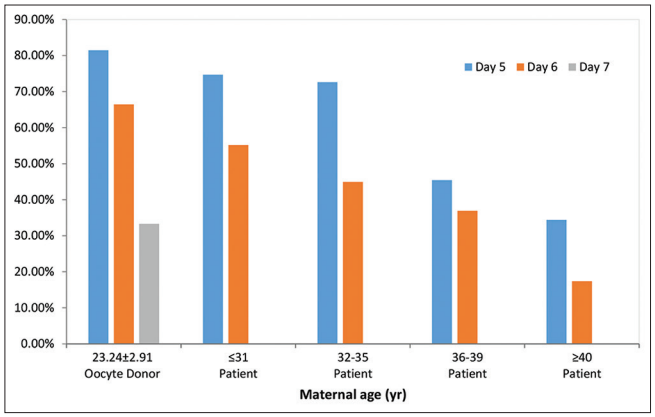

Figure 2: Graph shows the association between euploidy rate and day of embryo biopsy in different maternal age groups. Data are from 880 blastocysts.

Paternal age and euploidy in oocytes donor programme

A total of 294 blastocysts from 33 donor oocytes were biopsied. The mean oocyte donor age was 23.24 ± 2.91 (20–32) years; 48.30% (142/294) of blastocysts were of good quality according to the morphology score (AA, AB, BA) and the euploidy rate was 73.04% (214/293). There was an average of 8.91 blastocysts per cycle for biopsy; 4.30 had good morphology and 6.48 were euploid [Table 5]. The results for different paternal age groups showed that there was no association between paternal age and aneuploidy rate (P = 0.89) and no association between paternal age and the number of good-quality/euploid blasts per cycle.

Cycle outcome after FET

From December 2017 to February 2020, a total of 111 embryo transfers were performed; 15 of them with frozen-thawed blastocysts without PGT-A (six SET and nine DET used as the control group during the same period) and 96 embryo transfers done after PGT-A (90 with euploid blastocysts [44 SET and 46 DET], five with segmental aneuploid blastocysts [five SET] and one transfer with whole chromosome aneuploid blastocyst [patient requested and proper consent was taken from the patient as there were no euploid blastocysts available]). One hundred and seventy-three blastocysts were thawed, of which 168 (97.11%) survived and were transferred. A positive hCG test was detected in 70 (63.06%) patients; 52 (46.85%)

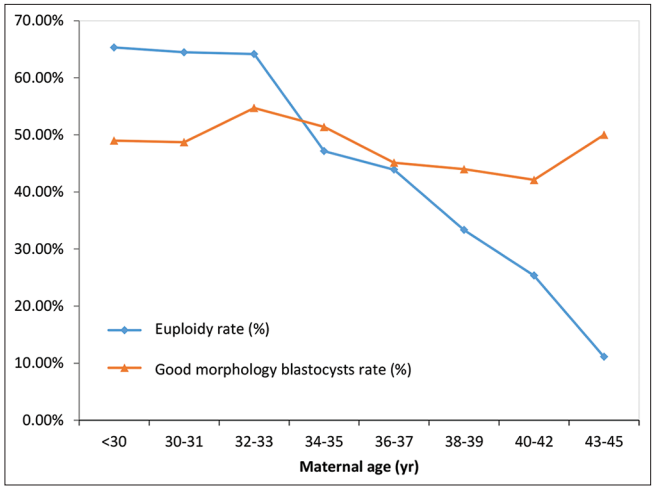

Figure 3: Graph shows the association of maternal age with euploidy rate and good quality blastocyst rate. Data were from 599 blastocysts from 124 cycles using patients' own oocytes.

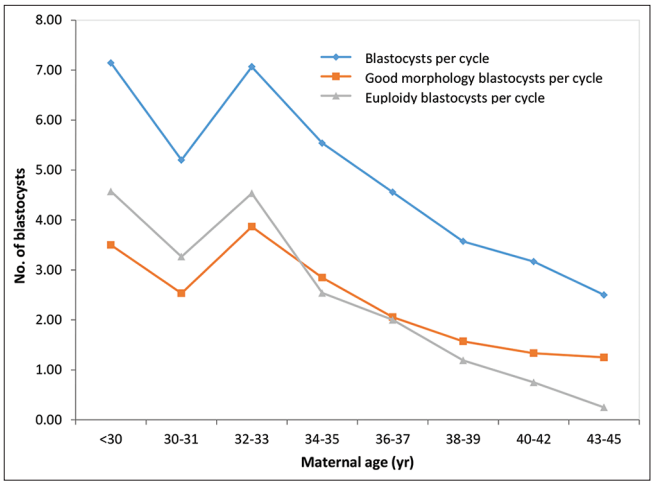

Figure 4: Graph shows the association of maternal age with number of blastocysts per cycle, good morphology blastocysts per cycle and euploid blastocysts per cycle. Data were from 599 blastocysts from 124 cycles using patients' own oocytes.

Table 4. Correlation between maternal age and euploidy rate.

Patient's age group (yr)	No. of cycles	No. of blastocysts biopsied	No. of blastocysts per cycle	Blastocyst with good morphology (AA, AB, BA)		No. of blastocysts diagnosed	Euploid blastocysts	
				n (%)	Per cycle		*n* (%)	Per cycle
<30	14	100	7.14	49 (49.00)	3.50	98	64 (65.31)	4.57
30–31	15	78	5.20	38 (48.72)	2.53	76	49 (64.47)	3.27
32–33	15	106	7.07	58 (54.72)	3.87	106	68 (64.15)	4.53
34–35	13	72	5.54	37 (51.39)	2.85	70	33 (47.14)	2.54
36–37	18	82	4.56	37 (45.12)	2.06	82	36 (43.90)	2.00
38–39	21	75	3.57	33 (44.00)	1.57	75	25 (33.33)	1.19
40–42	24	76	3.17	32 (42.11)	1.33	71	18 (25.35)	0.75
43–45	4	10	2.50	5 (50.00)	1.25	9	1 (11.11)	0.25
Total	124	599	4.83	289 (48.25)	2.33	587	294 (50.09)	2.37

Table 5. Correlation between paternal age and euploidy rate in oocytes donor programme.[a]

Donor paternal age (yr)	No. of cycles	No. of blastocysts biopsied	No. of blastocysts per cycle	Blastocyst with good morphology (AA, AB, BA)		No. of blastocysts diagnosed	Euploid blastocysts	
				n (%)	Per cycle		*n* (%)	Per cycle
<40	13	103	7.92	46 (44.66)	3.54	103	76 (73.79)	5.85
40–48	14	137	9.79	70 (51.09)	5.00	136	100 (73.53)	7.14
>50	6	54	9.00	26 (48.15)	4.33	54	38 (70.37)	6.33
Total	33	294	8.91	142 (48.30)	4.30	293	214 (73.04)	6.48

[a]No significant difference in all groups, $P > 0.05$

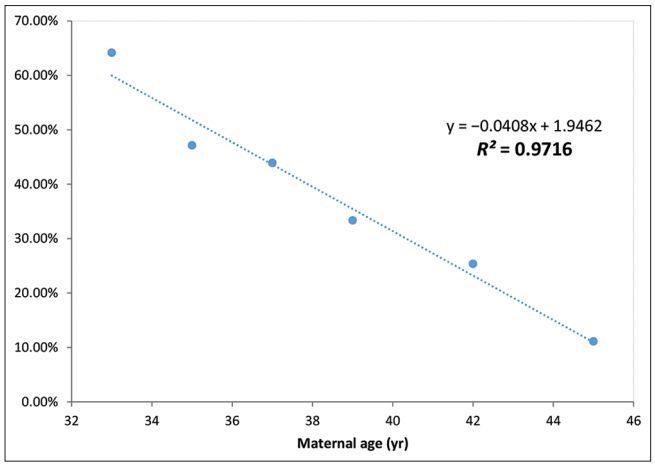

Figure 5: Graphs shows a decline in mean euploidy rates with increasing maternal age (after age 33), as shown by the close fit between the data points and the dashed regression line (strong linear correlation: $R^2 = 0.9716$).

had clinical pregnancies and the other 18 had biochemical pregnancies. At the time of writing this article, 50 (45.05%) were live births ($n = 31$) or ongoing pregnancy ($n = 19$).

There was no significant difference in biochemical pregnancy rate (15.56% vs. 20.00%, $P > 0.05$). A trend towards an increased hCG positive rate (64.44% vs. 53.33%, $P > 0.05$), clinical pregnancy rate (48.89% vs. 33.33%, $P > 0.05$), ongoing pregnancy rate (47.78% vs. 33.33%, $P > 0.05$) and implantation rate (41.91% [57/136] vs. 24.00% [6/25], $P = 0.09$) was found with euploid embryos transfer compared to the embryos without PGT-A (control group), but these were not statistically significant [Table 6].

Significantly higher pregnancy rates and live birth/ongoing pregnancy rates were obtained when two euploid blastocysts were transferred compared to transfer of one euploid blastocyst [Table 7]. Implantation rate was also significantly higher with double blastocyst transfer compared to single blastocyst transfer (47.83% [44/92] vs. 29.55% [13/44], $P = 0.043$). However, there was no difference in biochemical pregnancy rate (15.22% vs. 15.91%, $P > 0.05$). Multiple pregnancy did not occur in single euploid blastocyst transfer, but a very high multiple pregnancy rate (43.33%, 13/30) was observed when double euploid blastocysts were transferred, with one occurrence of triplets. Due to the higher multiple pregnancy rate for DET, SET was recommended. No correlations were observed between maternal age and clinical outcome (pregnancy rate, implantation rate and ongoing pregnancy rate) with euploid embryo transfer [Table 8].

DISCUSSION

Numerical chromosomal abnormalities usually cause implantation failure and early miscarriage. To achieve higher implantation and pregnancy rates as well as reduced miscarriage rates, euploid embryos are selected using PGT-A before transfer. The increasing demands for successful IVF cycles cannot be met by

Table 6. Clinical outcome after FET.[a]

Blastocyst genetic status	No. of FET	Patient age Mean±SD (yr)	No. of blastocysts transferred per cycle	*n* (%)			
				hCG-positive test	Biochemical pregnancies	Clinical pregnancies	Live birth ongoing
Non-PGT-A (control)	15	37.86±7.72	1.67	8 (53.33)	3 (20.00)	5 (33.33)	5 (33.33)
Euploidy	90	36.57±6.78	1.51	58 (64.44)	14 (15.56)	44 (48.89)	43 (47.78)
Segmental aneuploidy	5	36.4±3.36	1	3 (60.00)	1 (20.00)	2 (40.00)	2 (40.00)
Whole chromosome aneuploidy	1	40	2	1 (100.00)	0 (0.0)	1 (100.00)	0 (0.00)
Total	111	36.76±6.73	1.51	70 (63.06)	18 (16.22)	5 (4.50)	50 (45.05)

[a]No significant difference in all groups, P >0.05. FET: frozen-thawed embryo transfer, PGT-A: preimplantation genetic test for aneuploidies, SD: standard deviation

Table 7. Clinical outcome of single and double euploid embryo transfers.

Group	No. of FET	Patient age Mean±SD (yr)	*n* (%)				
			hCG-positive test	Biochemical pregnancies	Clinical pregnancies	Live birth ongoing	Twin
SET	44	36.59±5.61	20 (45.45)*	7 (15.91)	13 (29.55)*	13 (29.55)*	0 (0.0)*
DET	46	36.61±7.74	38 (82.61)*	7 (15.22)	31 (67.39)*	30 (65.22)*	13 (43.33)*
Total	90	36.6±6.74	58 (64.44)	14 (15.56)	44 (48.89)	43 (47.78)	13 (30.23)

*P <0.01. FET: frozen-thawed embryo transfer, SD: standard deviation

Table 8. Clinical outcome of different age groups after euploid embryos transfer.[a]

Age group (yr)	No. of FET	No. of blastocysts transferred per cycle	Total no. of blastocysts transferred	*n* (%)				
				hCG-positive test	Biochemical pregnancies	Clinical pregnancies	Foetal hearts[b]	Live birth ongoing
≤30	18	1.67	30	14 (77.78)	3 (16.67)	11 (61.11)	16 (53.33)	11 (61.11)
31–35	22	1.50	33	14 (63.64)	4 (18.18)	10 (45.45)	13 (39.39)	10 (45.45)
36–39	25	1.40	35	15 (60.00)	4 (16.00)	11 (44.00)	16 (45.71)	11 (44.00)
≥40	25	1.52	38	15 (60.00)	3 (12.00)	12 (48.00)	15 (39.47)	11 (44.00)
Total	90	1.51	136	58 (64.44)	14 (15.56)	44 (48.89)	60 (44.12)	43 (47.78)

[a]No significant difference in all groups, P>0.05. [b]Implantation rate. FET: frozen-thawed embryo transfer

the approaches developed towards 24-chromosomal analysis, as these methods are expensive and difficult to scale up.[24] Compared to the conventional 24 Sure arrayCGH, KaryoLite BoBs was validated as a robustly accurate method for PGT-A, with a resolution down to chromosome arm level.[24] In this study, as there was no euploid embryo, upon the patient's request and with proper consent, two aneuploid blastocysts were transferred in one DET cycle. Even though foetal heart was observed, subsequently, the non-invasive prenatal test results verified the PGT-A report and the pregnancy was aborted. Segmental aneuploid embryos were transferred for five FET cycles; one cycle was a biochemical pregnancy and two cycles resulted in live births with normal babies [Table 6]. This may be due to embryonic mosaicism or a false-positive diagnosis.[27] For KaryoLite BoBs technique with only four probes on each chromosome, there was a detection limitation for segmental aneuploidy compared to other PGT-A platforms, and it was difficult to identify mosaicism. This suggested the need for caution in interpreting arm/segmental aneuploidy when using KaryoLite BoBs for PGT-A. The segmental aneuploidy rate (4.43%) in this study was similar to those of other reports (5.5%–6%).[28,29]

With regards to chromosome abnormalities, errors did not equally affect all chromosomes in this study [Figure 1]. The chromosome mainly affected was 16 (10.98%), followed by 22 (9.38%), 21 (7.58%), 19 (5.99%), 15 (5.59%), 14 (4.99%), 18 (4.79%) and 20 (4.79%). These results are similar to previous reports where the majority of anomalies occurred in chromosomes 22, 16, 21, 15 and 19;[28] 16, 22, 21 and 15;[15] 22, 21, 16 and 15;[30] and 16, 22 and 21.[31] Both nondisjunction and unbalanced chromatid predivision

tended to affect smaller chromosomes in metaphase II oocytes and corresponding polar bodies.[32] The results from a large survey of TE biopsies also supported the detection of disproportionate maternal age effects on aneuploidies of chromosomes 13, 15, 16, 18, 19, 21 and 22.[14] Likewise, our findings are in agreement with previous findings that chromosomes with a small structure had the highest aneuploidy.[14,15]

In this study, the sex ratio results showed that in total, 880 diagnosed blastocysts had a perfect 1 to 1 ratio. However, faster growing embryos were more likely to be male, with more XY in day 5 blastocysts (55.00%, 202/380) and more XX in day 6 or 7 blastocysts (54.05%, 260/481), but both were not significantly different and gave an expected ratio of 1 to 1 ($P > 0.05$). Differences in gender ratios in relation to different IVF procedures have been studied.[33,34] The findings from analysing 208 embryos in intracytoplasmic morphologically selected sperm injection (IMSI) cycles compared to 322 embryos ICSI cycles showed a significantly higher incidence of female embryos in IMSI cycles. This led the authors to conclude that "alterations in the Y chromosome may lead to morphological changes that would prevent spermatozoon selection under high magnification".[35] Another group analysed a larger number of babies born after IMSI (18,520) and ICSI (867). No statistically significant difference was found in the gender ratio.[36] We analysed our non-PGT ICSI cycle delivery data in the last 5 years (unpublished), there was no significant difference in sex ratio of babies born after day 5 blastocyst transfer in both fresh (56 male [41.79%] vs. 78 female [58.21%], $P = 0.1775$) and FET cycles (331 male [49.55%] vs. 337 female [50.45%], $P = 0.8696$). This is in agreement with a recent meta-analysis review which showed that there was no difference in the number of males between day 5 and day 6 embryo transfer.[37] Our findings suggest that the sex ratio is not significantly determined by selecting embryos on the basis of embryo developmental speed and embryo morphological characteristics and it is also not influenced by selecting the sperm by shape under a high power of magnification during ICSI. However, there is a need for more research in this area.

Our data showed a significant association between blastocyst morphology and euploidy rate [Table 2]. Any blastocyst that was of good morphology and having a high scoring ICM and TE had a greater chance of being euploid than those with poorer scoring. The higher implantation potential of good-quality embryos during conventional IVF cycles is associated with blastocyst morphology and aneuploidy status. Individually, the TE or ICM score was also positively associated with the euploidy status of the embryo. This finding is in agreement with previous reports that demonstrated an association between the blastocyst morphology and the euploidy rate[11,18,20] and that the morphology could predict PGT-A result.[17] However, these results differ from those of a previous study, which suggested otherwise.[38] A weak correlation between variables was found when embryo morphology was compared to the rate of aneuploidy, thus leading to the conclusion that establishing embryo quality does not ensure that the embryo is euploid.[39] In this study, we also found that 34.41% of the 372 aneuploid blastocysts had good morphology. Between the variables of ICM and TE morphology scores, we found that the TE score was more predictive of the euploidy rate [Table 2]. This observation is still contentious in the current reports. It is in agreement with a previous study[40] which demonstrated that for predicting live births, TE morphology is a more important parameter than ICM when selecting the best blastocyst for embryo replacement, but it is contrary to a study[41] which suggested that blastocyst morphological grading, particularly ICM grade, is a useful predictor of ongoing pregnancy per euploid embryo.

Our data showed that the rate of development of a blastocyst was highly indicative of euploidy [Table 3]. The embryos that had a slower rate of development and reached the blastocyst stage on day 6 or 7 had a significantly higher aneuploidy rate when compared to the faster growing embryos that achieved full expansion on day 5. This finding is in agreement with previous reports.[11,18] Fragouli *et al*. reported that the embryos that showed a faster progression to the fully expanded stage on day 5 tended to have a significantly higher euploidy rate compared to the early blastocysts on day 5.[42] Although morphological evaluation is used for choosing embryos to transfer, it is important to understand that attempting to select an euploid blastocyst based on the morphology is not failproof, as many aneuploid blastocysts are able to achieve top quality scores.[11] Our findings also show that morphological parameters are important but do not always predict euploid embryos, and a correlation between good morphology embryos and euploidy is observed only when all embryos from all age groups are used in the correlation. When broken down into different age groups, however, the percentage of good morphology embryos remains similar across all age groups and the percentage of euploid embryos drops with increasing age. Clearly, the percentage of good morphology embryos does not track the declining percentage of euploid embryos with increasing maternal age. These results would suggest that the correlation between blastocyst morphology and ploidy status is present but poor, and that blastocyst morphology is a weak predictor of ploidy status. What the blastocyst morphology does appear to reveal is that poorer morphology translates into higher chances that an embryo is aneuploid, while good morphology embryos have a higher chance of being euploid.

It is established that embryo aneuploidy rate increases in tandem with a woman's age. In this study, the euploidy rate, the number of good-quality blastocysts per cycle and the number of euploid blastocysts per cycle were strongly associated with maternal age. These results support previous reports.[11,14,29,42] Nondisjunction and premature separation of sister chromatids are examples of meiotic flaws during oogenesis that are

related to the woman's age.[16] After the age of 33, euploidy rates decline with increasing maternal age. The strong linear correlation ($R^2 = 0.9716$) observed in this study is in accordance with that previously reported ($R^2 = 0.981$).[43] We observed that the percentage of good morphology blastocysts did not show significant difference among all age groups [Figure 3]. This is likely because the aneuploid embryos are also capable of reaching high morphological scores.[11,38,40] Although older women have fewer euploid embryos, it has been reported that they were of comparable morphology and had similar development rates to the blastocyst stage when compared to those of younger women.[19] As the total embryo numbers decreased with increasing age, the number of embryos with good morphology also decreased, as reflected in a declining slope in Figure 4.

In this study, the proportion of euploid embryos from both egg donors and nondonors under 33 years of age was not statistically significantly different. We observed that paternal age did not affect the embryo quality and euploidy rate. This disagrees with previous reports showing that men ≥50 years have a higher percentage of sperm DNA fragmentation, which results in significant aneuploidy rates in embryos from donated oocytes.[44] However, the largest study thus far concluded that paternal age has no specific effects on the prevalence of embryo aneuploidy in embryo biopsies from egg donor cycles.[45] The incidences of autosomal disomy, sex chromosome disomy, total chromosome disomy, diploidy and total numerical abnormalities were reported to be not significantly different between younger and older men.[46]

In this paper, a trend towards an increased pregnancy rate and implantation rate was found with euploid embryo transfer compared to embryo transfer without PGT-A, but these were not statistically significant. As such, PGT-A does not appear to be clearly associated with increased pregnancy and implantation rates compared to morphological assessment alone, and therefore, PGT-A remains an unproven hypothesis. Significantly higher pregnancy rates and multiple pregnancy rates were obtained when double euploid blastocysts were transferred compared to single euploid embryo transfer. It is clear that transferring less embryos per embryo transfer reduces the chance of pregnancy.[47] In this paper, no correlations were observed between maternal age and clinical outcome with euploid embryo transfer. This result is in agreement with the results of previous studies which showed that although maternal age at retrieval influences the number of euploid embryos, it does not affect their implantation potential.[17,19]

Selection of euploid embryos is based on blastocyst morphology and speed of development. Even when all 23 chromosome pairs are evaluated during TE biopsy, pregnancy maintenance is dependent on a host of other factors such as an altered ratio of mitochondrial copy number to nuclear DNA, *de novo* clinically significant deletions or duplications, autoimmune factors, endometrial receptivity, anatomic abnormalities, endocrinological abnormalities and other factors that are not currently known.[48] Our results are in agreement with the following statement: PGT-A has remained an unproven hypothesis,[12] and it is controversial to adopt it in routine use for all IVF cycles.[13]

In conclusion, this retrospective study demonstrated the following: sex ratio was not influenced by ICSI; chromosomal aneuploidies were most frequent in chromosomes 16, 22 and 21; blastocyst morphology, the rate of development and maternal age were significantly associated with euploidy rate; paternal age did not affect euploidy rate in donor programme and double euploid embryo transfer increased the pregnancy and implantation rates. PGT-A may help to improve pregnancy rates, ongoing pregnancy and implantation rates, but it is not statistically different. The main limitation of this study is its retrospective nature and the small size of the control group when making comparisons about implantation rates. To get clearer conclusion, further studies with more data are needed in the future.

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Conflicts of interest

There are no conflicts of interest.

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