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Reduction of multiple pregnancies in the advanced maternal age population after implementation of an elective single embryo transfer policy coupled with enhanced embryo selection: pre- and post-intervention study

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STUDY QUESTION: Is an elective single-embryo transfer (eSET) policy an efficient approach for women aged >35 years when embryo selection is enhanced via blastocyst culture and preimplantation genetic screening (PGS)?

SUMMARY ANSWER: Elective SET coupled with enhanced embryo selection using PGS in women older than 35 years reduced the multiple pregnancy rates while maintaining the cumulative success rate of the IVF programme.

WHAT IS KNOWN ALREADY: Multiple pregnancies mean an increased risk of premature birth and perinatal death and occur mainly in older patients when multiple embryos are transferred to increase the chance of pregnancy. A SET policy is usually recommended in cases of good prognosis patients, but no general consensus has been reached for SET application in the advanced maternal age (AMA) population, defined as women older than 35 years. Our objective was to evaluate the results in terms of efficacy, efficiency and safety of an eSET policy coupled with increased application of blastocyst culture and PGS for this population of patients in our IVF programme.

STUDY DESIGN, SIZE, DURATION: In January 2013, a multidisciplinary intervention involving optimization of embryo selection procedure and introduction of an eSET policy in an AMA population of women was implemented. This is a retrospective 4-year (January 2010–December 2013) pre- and post-intervention analysis, including 1161 and 499 patients in the pre- and post-intervention period, respectively. The primary outcome measures were the cumulative delivery rate (DR) per oocyte retrieval cycle and multiple DR.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Surplus oocytes and/or embryos were vitrified during the entire study period. In the post-intervention period, all couples with good quality embryos and less than two previous implantation failures were offered eSET. Embryo selection was enhanced by blastocyst culture and PGS (blastocyst stage biopsy and 24-chromosomal screening). Elective SET was also applied in cryopreservation cycles.

MAIN RESULTS AND THE ROLE OF CHANCE: Patient and cycle characteristics were similar in the pre- and post-intervention groups [mean (SD) female age: 39.6 ± 2.1 and 39.4 ± 2.2 years; range 36–44] as assessed by logistic regression. A total of 1609 versus 574 oocyte retrievals, 937 versus 350 embryo warming and 138 versus 27 oocyte warming cycles were performed in the pre- and post-intervention periods, respectively, resulting in 1854 and 508 embryo transfers, respectively. In the post-intervention period, 289 cycles were blastocyst stage with ($n = 182$) or without PGS ($n = 107$). A mean (SD) number of 2.9 ± 1.1 (range 1–4) and 1.4 ± 0.8 (range 1–3) embryos were

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transferred pre- and post-intervention, respectively ($P < 0.01$) and similar cumulative clinical pregnancy rates per transfer and per cycle were obtained: 26.8, 30.9% and 29.7, 26.3%, respectively. The total DR per oocyte retrieval cycle (21.0 and 20.4% pre- and post-intervention, respectively) defined as efficacy was not affected by the intervention [odds ratio (OR) = 0.8, 95% confidence interval (CI) = 0.7–1.1; $P = 0.23$]. However, a significantly increased live birth rate per transferred embryo (defined as efficiency) was observed in the post-intervention group 17.0 versus 10.6% ($P < 0.01$). Multiple DRs decreased from 21.0 in the preintervention to 6.8% in the post-intervention group (OR = 0.3. 95% CI = 0.1–0.7; $P < 0.01$).

LIMITATIONS, REASONS FOR CAUTION: In this study, the suitability of SET was assessed in individual women on the basis of both clinical and embryological prognostic factors and was not standardized. For the described eSET strategy coupled with an enhanced embryo selection policy, an optimized culture system, cryopreservation and aneuploidy screening programme is necessary.

WIDER IMPLICATIONS OF THE FINDINGS: Owing to the increased maternal morbidity and perinatal complications related to multiple pregnancies, it is recommended to extend the eSET policy to the AMA population. As shown in this study, enhanced embryo selection procedures might allow a reduction in the number of embryos transferred and the number of transfers to be performed without affecting the total efficacy of the treatment but increasing efficiency and safety.

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Key words: elective single embryo transfer / advanced maternal age / comprehensive chromosome screening / vitrification / preimplantation genetic screening

Introduction

Multiple pregnancies mean an increased risk of premature birth and perinatal death and are considered as adverse outcome after assisted reproductive interventions (Pinborg, 2005). To reduce the frequency of this complication, a limitation on the number of transferred embryos has been advocated for good prognosis patients (Thurin *et al.*, 2004). Elective single-embryo transfer (eSET) for this population has become mandatory in some countries and generally used in others during the past decade (Magli *et al.*, 2007). Although eSET in a fresh cycle may significantly decrease pregnancy rates, it can be compensated by performing blastocyst transfer, appropriate selection of the embryo transferred, using an efficient vitrification method for cryopreservation and obtaining pregnancies from the first warming cycle (Gardner *et al.*, 2004; Lukassen *et al.*, 2005; Pandian *et al.*, 2005; Papanikolaou *et al.*, 2006; Stoop *et al.*, 2011; Esinler *et al.*, 2014).

Owing to the restrictive law implemented in 2004 in Italy (40/2004, 'Norms on the matter of medically assisted procreation') the situation has been different. Embryo cryopreservation and destruction were forbidden, the rights of specialists to select and apply the optimal treatment were seriously compromised and eSET was considered illegal (Ragni *et al.*, 2005; La Sala *et al.*, 2008, 2009; Levi Setti *et al.*, 2008). Accordingly, very high multiple pregnancy rate have been reported by the National Registry (Data from Italian ART Register 2013 from Istituto Superiore di Sanità). In 2009, the Italian Constitutional Court ruled that part of the law is unconstitutional. Although the creation of a new law is the authority of the Parliament, and in the transitional period certain things remain unresolved, some critical restrictions were clearly eliminated including the limit of a maximum three oocytes to be fertilized and the need to transfer all the embryos that have been created. The strict ban of embryo cryopreservation was also alleviated (Molinelli *et al.*, 2012). However, to date, no policy regarding the number of embryos transferred has been implemented. Consequently, the multiple pregnancy rates have always been higher in Italy than in most European countries, making a possible advancement in this field especially important in our country.

Policies and guidelines usually suggest application of eSET for good prognosis patients focusing on the female population under 35–38 years of age (Cutting *et al.*, 2008; Kresowik *et al.*, 2011; Gianaroli *et al.*, 2012). However, very high multiple pregnancy rates were also observed in Italy in the advanced maternal age (AMA) patient populations in 2011: 19.2 and 12.0% for females aged 35–39 years and 40–42 years, respectively. Similarly, we recorded in our centre a 21.0% multiple pregnancy rate in women aged more than 35 years in the years 2010–2012.

In general, limited information about the application of eSET in the AMA population is available (Davis *et al.*, 2008; Niinimäki *et al.*, 2013; Schoolcraft and Katz-Jaffe, 2013).

The aim of this study was to analyse the clinical outcomes after the introduction of an eSET policy for AMA patients. Selection of the best embryo for transfer was promoted by blastocyst culture and, in selected cases, by trophectoderm biopsy and preimplantation genetic screening (PGS). The cumulative results of fresh and warmed embryo transfers after vitrification were also measured.

Materials and Methods

Study population

All consecutives oocyte retrievals in patients aged >35 years performed between January 2010 and December 2013 and subsequent cryopreservation cycles were included. Cycles involving PGD for monogenic disease or chromosomal abnormalities were excluded. Oocyte retrieval cycles were divided into two groups according to the embryo selection and transfer strategy used. In January 2013, a multidisciplinary intervention involving optimization of the embryo selection procedure and the introduction of an eSET policy in this population was implemented. Preintervention (January 2010–December 2012) and post-intervention (January 2013–December 2013) groups included 1609 and 574 oocytes retrieval cycles, respectively, and no major changes in the clinical and laboratory practices were introduced in the post-intervention period, in addition to those described (Supplementary data, Table SI). A total of 461 and 138 versus 231 and 27 subsequent embryo and oocyte cryopreservation cycles in the pre- and in the post-

intervention periods were analysed, respectively. One year of observation was included in the analysis, starting from the oocyte retrieval day, on a per patient basis. The embryo transfer policy was agreed with patients before oocyte retrieval and applied also for subsequent frozen cycles. Each couple gave written informed consent to the treatment, where the post-intervention embryo transfer policy was described and clearly explained to the patients before starting the IVF cycle. The Institutional Review Board of the Clinic approved the study.

Ovarian stimulation

Two protocols were used for ovarian stimulation: GnRH agonist long protocol and GnRH antagonist protocol as described previously (Ubaldi et al., 2010).

Criteria for agonist induction and/or freeze all in case of risk of hyperstimulation

To reduce the risk of ovarian hyperstimulation syndrome, the final oocyte maturation with GnRH agonist triggering (a single bolus of 0.5 mg of busere-lin) and/or freeze-all approach in a GnRH antagonist protocol has been performed in all patients with a serum estradiol level ≥ 3.600 pg/ml (Chen et al., 1997) and when >15 oocytes are retrieved in fresh autologous IVF cycles (Steward et al., 2014).

Oocyte insemination

The laboratory procedures used in this study have been described previously (Rienzi et al., 2010). Briefly, after oocyte retrieval, cumulus—oocyte complexes were exposed to 40 IU/ml hyaluronidase solution in fertilization medium (Sage In-Vitro Fertilization, Cooper Surgical Inc., Trumbull, CT, USA), and the corona radiata was removed mechanically with plastic pipettes of defined diameters (denuding pipettes; COOK Ireland Ltd, Limerick, Ireland) in a controlled CO_2 and temperature environment (Unica, IVF Tech, Denmark). Insemination of oocytes by ICSI was carried out immediately after denudation.

Embryo culture and evaluation

Each inseminated oocyte was placed in 25 μl of culture media (Quinn's Advantage®, Cleavage Medium, Cooper Surgical) covered by pre-equilibrated mineral oil and incubated in 6% CO_2 and 5% O_2 tension. When blastocyst culture was performed, the medium was changed on Day 3 after fertilization (Quinn's Advantage®, Blastocyst Medium, Cooper Surgical). Day 3 embryos were considered of good quality when more than 6 cells, <30% fragmentation a no multinucleations were observed (Rienzi et al., 2005). Blastocysts were evaluated according to the degree of expansion, quality of the inner cell mass and of the trophectoderm cells (Gardner and Sakkas, 2003). The inner cell mass was evaluated according to the number of cells and the degree of compaction, whereas trophectoderm cells were evaluated according to the number and dimension of the cells and the appearance of the epithelium (cohesive or loose). Good quality blastocysts consisted of a visible and compacted inner cell mass with a cohesive trophectoderm.

Preimplantation genetic screening

Selected blastocysts underwent trophectoderm biopsy 120—160 h post-insemination, as previously described (Capalbo et al., 2014). In brief, all biopsy procedures were conducted on a heated stage in 10 μl of HEPES-buffered medium (Quinn's Advantage®, Cooper Surgical) overlaid with pre-equilibrated mineral oil. A diode laser (Research Instruments, Cornwall TR11 4TA, UK) was used to assist the opening of a 10—20 μm hole in the zona pellucida. Five to 10 trophectoderm cells were then aspirated into the trophectoderm biopsy pipette (Research Instruments) followed by laser-assisted removal of the target cells from the body of the embryo. All blastocysts were vitrified immediately after biopsy. Trophectoderm biopsies were then sent to our genetic reference laboratory for the chromosome analysis (GENETYX srl, Marostica, Italy). Samples were all processed for comprehensive chromosomal screening by placing them in lysis buffer and performing real-time PCR using a previously described and internally validated protocol (Cobo et al., 2008; Treff et al., 2012; Capalbo et al., 2015). In brief, multiplex amplification of 96 loci was carried out, and a method of relative quantification (Scotland et al., 2011) was applied to predict the copy number status of each chromosome. A karyotype prediction was made for each embryo by a certified cytogeneticist. Euploid blastocyst transfers were all performed in the course of a warming cycle as described below.

Embryo transfer policy

In the preintervention group, up to four embryos were transferred without a specific policy.

In the post-intervention group, eSET was offered independently from ovarian reserve and male factor infertility according to the following inclusion criteria:

(i) Less than two previous implantation failures.
(ii) Presence of good quality embryos/blastocysts or screened euploid blastocysts.

The suitability of eSET was thus individually assessed on the basis of clinical and embryological prognostic factors and after having examined the possibility with the patients. Where clinical data and/or the cohort of embryos did not satisfy these criteria, the transfer of up to three embryos was performed. The same policy was also used in cryopreservation cycles.

PGS was offered to patients with a high risk of producing aneuploidy embryos, when female age was over 39 years, patients experiencing pregnancy loss (>2 consecutive miscarriages) and multiple implantation failures (>3 good quality embryos transferred).

Oocyte, embryo and blastocyst vitrification and warming procedures

The vitrification and warming procedures used for our study have been described by Cobo et al. (2008). Vitrification was performed by using the Cryotop device and solutions (Kitazato BioPharma Co., Japan). The first equilibration was carried out in 7.5% ethylene glycol and 7.5% dimethyl-sulphoxide at room temperature for 12—15 min. Subsequently, oocytes and embryos were transferred into 15% ethylene glycol, 15% dimethyl-sulphoxide and 0.5 M sucrose for 1 min, then placed on the film strip of the Cryotop in a single small drop. The excess solution was removed to leave just a thin layer around each embryo, and the Cryotop was submerged into liquid nitrogen. The strip was covered with the cap and the sample was stored submerged in liquid nitrogen. At warming, the cap was removed under liquid nitrogen and the film strip of Cryotop was quickly submerged into 1 ml of 37°C warming solution containing 1.0 M sucrose for 1 min, then oocytes and embryos were transferred to a room temperature solution containing 0.5 M sucrose and incubated for 3 min. After two subsequent washings in basic medium at room temperature for 6 min each, the oocyte or embryo was placed into 1 ml culture medium (Cleavage medium, Sage). Oocytes were inseminated by ICSI 2 h after warming as described previously (Rienzi et al., 2010). Embryo/blastocysts transfers were performed during a natural cycle.

Embryo transfer supplementation

The luteal phase was supported by vaginal micronized progesterone, 400 mg/day (Progeffik 200 mg, Effik, Cinisello Balsamo, Milan, Italy), starting on the day of oocyte retrieval or on the day of warming until 8 weeks of gestation if pregnancy occurred.

Outcome measures

The primary outcome measures were the cumulative delivery rate (DR) per oocyte retrieval cycle and multiple DR. Secondary outcomes measures were live birth rate (LBR) per transferred embryo and miscarriage rate per clinical pregnancy (CP). Clinical pregnancy rate (CPR) was determined by ultrasound demonstration of a gestational sac at 7 weeks. The DR was calculated as the number of deliveries per oocyte retrieval cycle or per transfer. LBR was calculated as the number of new-borns per transferred embryo. Miscarriage rate was classified as number of pregnancy loss per CP achieved. IVF treatment efficacy was defined as DR per egg retrieval cycle since in general epidemiology efficacy refers to the extent to which a specific intervention, procedure, regimen or service produces a desired result. IVF treatment efficiency was defined as LBR per transferred embryo, since efficiency in epidemiology refers to the effects or end results achieved in relation to the efforts expended in terms of resources, time and waste of material, as also previously used by Goldman *et al.* (2013) to describe the LBR per oocyte retrieved. Improved procedural safety was considered when lower IVF adverse outcomes were observed in terms of multiple pregnancies and miscarriages.

Statistical analysis

Cycle data were prospectively collected and saved in a relational database (FertilLab, Italy). Baseline characteristics [continuous data: female age, rank of trial, baseline FSH, total dose of gonadotrophins, days of stimulation, number of cumulus corona cell oocyte complexes retrieved, number of metaphase II (MII) oocytes obtained, number of embryos transferred, number of oocytes/embryos vitrified, number of oocytes/embryos warmed] are presented as absolute values, mean with SD and range. Categorical variables (oocyte/embryo survival, oocyte fertilization, embryo development and quality, clinical pregnancy, miscarriage, ectopic pregnancy, delivery, live birth) are presented as absolute and percentage frequency. Differences in frequencies were evaluated with Pearson's χ^2 test with Yates' continuity correction and Fisher's exact test.

To rule out the influence of potential confounding variables (female age, rank of trial, baseline FSH, stimulation protocol, the total dose of gonadotrophin, days of stimulation, number of MII oocytes obtained), the analyses on primary outcomes (cumulative and multiple pregnancy rates) were corrected using a logistic regression analysis. Data are presented as odds ratio (OR), 95% confidence interval (95% CI) and *P*-value. *P*-values lower than 0.05 were considered statistically significant. All statistical analyses were performed using R software version 2.8.0. (The R Foundation for Statistical Computing, Vienna, Austria.)

Results

During the study period, 2183 fresh oocyte retrieval cycles and 857 warming cycles were performed in 1660 women older >35 years. A total of 4590 fresh and cryopreserved embryos were replaced (mean of 1.9 ± 0.7 embryo per transfer). The cumulative DR was 20.8% (455/2183) and the multiple DR was 17.4% (79/455). Cycle characteristics were similar in the pre- and post-intervention groups (Table I). After 1 year of observation from the oocyte retrieval day, only 38 and 42 non-pregnant patients still have cryopreserved material unused from the pre- and post-intervention period, respectively

Oocyte retrieval cycles

In the preintervention period, 1609 oocytes retrieval cycles were performed, of which 1296 (78.9%) resulted in fresh embryo transfer (Table I). The embryo transfer cancellation rate because of absence of viable embryos was 7.6% (123/1296). In 190 cycles, all embryos and/or oocytes were cryopreserved for a subsequent warming cycle (freeze-all cycles) (11.8%). The main indications for the freeze-all approach were risk of hyperstimulation and PGS. A total of 365 CPs were obtained of which 105 resulted in miscarriages and 9 in ectopic pregnancies. DRs per cycle and per transfer were 15.6% (251/1609) and 19.4% (251/1296), respectively, and the multiple DR was 21.5% (54/251). Out of 2792 embryos transferred 305 resulted in live births (10.9%) (Table I).

In the post-intervention period, 574 oocytes retrieval cycles were performed, of which 280 (48.8%) resulted in fresh embryo transfer. An increased rate of blastocyst culture was recorded (50.3% versus 13.2%; *P* < 0.01). The transfer cancellation rate was also increased 27.2% (156/574) (*P* < 0.01). In 138 cycles all embryos and/or oocytes were cryopreserved for a subsequent warming cycle (24.0%) (*P* < 0.01). The increased rate of freeze-all cycles was mainly due to the increased application of PGS (32.1 versus 9.2% of cycles; *P* < 0.01). A total of 83 CP were obtained of which 19 resulted in miscarriages and 3 ectopic pregnancies. DRs per cycle and per transfer were 10.6% (61/574) and 21.8% (61/280), respectively, and the multiple DR was 11.5% (7/61). Out of 449 embryos transferred 68 resulted in live births (15.1%) (Table I).

In the preintervention period, 937 embryos and 756 oocytes were warmed in 599 cryopreservation cycles. In the post-intervention period, 350 embryos and 147 oocytes were warmed in 258 cryopreservation cycles. Laboratory and clinical outcomes for embryo and oocyte warming cycles in the two study periods are shown in Tables II and III, respectively.

Blastocyst culture and screening

The percentage of cycles having blastocyst culture increased from 13.2% (212/1609) in the preintervention period to 50.3% (289/574) in the post-intervention period.

The percentage of cycles having a PGS increased from 9.2% (148/1609) in preintervention period to 32.1% (184/574) in post-intervention period. All euploid blastocyst transfers performed in the post-intervention group were vitrified SET. Clinical outcomes of cycles with or without PGS performed during the whole study period are compared in Table IV.

eSET policy

The mean number of embryos replaced per transfer (including cryopreserved cycles) decreased after the introduction of the new embryo transfer policy, from 2.9 ± 1.1 (range 1–4) and 1.4 ± 0.8 (range 1–3) (Table V). The percentage of cycles having SET increased from 21.9% per embryo transfer (407/1854) in the preintervention period to 55.9% (284/508) per transfer in post-intervention period (*P* < 0.01) (Fig. 1). As a result of transferring fewer embryos, the proportion of cycles where surplus oocytes or embryos were cryopreserved improved significantly from 42% (677/1609) to 57% (325/574), respectively (*P* < 0.01).

Cumulative delivery, multiple pregnancy and miscarriage rates

Similar cumulative CPRs per transfer and per cycle (including fresh and cryopreserved oocytes and embryos) were obtained in the pre- and

Table I Oocyte retrieval cycle characteristics before and after the introduction of the elective eSET policy.

	Preintervention group	Post-intervention group	P-value
Number of patients	1161	499	
Number of oocyte retrieval cycles (mean/patient)	1609 (1.4)	574 (1.1)	
Mean female age at oocyte retrieval cycle (SD)	39.6 (2.1)	39.4 (2.2)	NS
Number of CCOCs (mean/oocyte retrieval cycle)	12 717 (7.9)	4564 (7.9)	NS
Number of MII (mean/oocyte retrieval cycle)	9588 (6.0)	3369 (5.9)	NS
Number of inseminated oocytes (mean/oocyte retrieval cycle)	8535 (5.3)	3076 (5.4)	NS
Number of oocytes cryopreserved (mean/oocyte retrieval cycle)	928	302	
Number of oocyte cryopreservation cycles (% oocyte retrieval cycles)	147 (9.1)	38 (6.6)	NS
Number of Day 3 embryos (mean/oocyte retrieval cycle)	5431 (3.4)	2195 (3.8)	NS
Number of good quality Day 3 embryos (mean/oocyte retrieval cycle)	3289 (2.0)	1238 (2.2)	NS
Number of embryos transferred fresh (mean/ transfer cycle)	2792 (2.1)	449 (1.6)	<0.01
Number of embryo fresh transfer (% oocyte retrieval cycle; 95% CI)	1296 (78.9; 76.8−80.8)	280 (48.8; 44.6−52.9)	<0.01
Number of Day 3 culture rate (% per oocyte retrieval cycle; 95% CI)	1397 (86.8; 85.1−88.4)	285 (49.6; 45.5−53.8)	<0.01
Number of Day 5 culture rate (% per oocyte retrieval cycle; 95% CI)	212 (13.2; 11.6−14.9)	289 (50.3; 46.2−54.5)	<0.01
Number of embryos cryopreserved (mean/oocyte retrieval cycle)	1435 (1.2)	831 (1.7)	<0.01
Embryo cryopreservation rate (% oocyte retrieval cycle; 95% CI)	530 (32.9; 30.6−35.3)	287 (50.0; 45.8−54.1)	<0.01
Freeze-all cycles (% oocyte retrieval cycle; 95% CI)	190 (11.8; 10.3−13.5)	138 (24.0; 20.6−27.7)	<0.01
Fresh transfer cancellation (% oocyte retrieval cycle; 95% CI)	123 (7.6; 6.4−9.0)	156 (27.2; 23.6−31.0)	<0.01
PGS cycles (% oocyte retrieval cycle; 95% CI)	146 (9.2; 7.8−10.7)	182 (32.1; 28.2−36.3)	<0.01
Number of clinical pregnancy (% embryo transfer; 95% CI)	365 (28.2; 25.7−30.7)	83 (29.6; 24.4−35.4)	NS
Number of miscarriages (% clinical pregnancy; 95% CI)	105 (28.8; 24.2−33.7)	19 (22.9; 14.4−33.4)	NS
Number of ectopic pregnancy (% clinical pregnancy; 95% CI)	9 (2.5; 1.1−4.6)	3 (3.6; 0.7−10.2)	NS
Number of delivery (% embryo transfer; 95% CI)	251 (19.4; 17.2−21.6)	61 (21.8; 17.1−27.1)	NS
Number of multiple pregnancy (% delivery; 95% CI)	54 (21.5; 16.6−27.1)	7 (11.5; 4.7−22.2)	NS
Number of live births (% per transferred embryos; 95% CI)	305 (10.9; 9.80−12.1)	68 (15.1; 12.0−18.8)	NS

MII, metaphase II oocyte; CI, confidence interval; PGS, preimplantation genetic screening; CCOC, cumulus corona oocyte complex.
Differences in frequencies were evaluated with Pearson's χ^2 test with Yates' continuity correction and Fisher's exact test. Student's t-test was used to compare continuous variables.
P-values lower than 0.05 were considered statistically significant.

Table II Cryopreserved embryo cycle characteristics before and after introduction of the eSET policy.

	Preintervention group	Post-intervention group	P-value
Number of cryopreserved embryo transfer cycles (% per oocyte retrieval cycle; 95% CI)	461 (28.6; 26.4−30.9)	231 (46.3; 36.2−44.4)	0.01
Number of warmed embryos (mean/embryo warming cycle)	937 (2.0)	350 (1.5)	0.01
Number of survived embryos (% warmed embryo; 95% CI)	896 (92.7; 90.9−94.3)	337 (96.3; 93.7−98.0)	NS
Number of transferred embryos (mean/embryo transfer cycle)	831 (1.9)	263 (1.2)	0.01
Number of embryo transfers (% embryo warming cycles; 95% CI)	437 (94.8; 92.3−96.6)	215 (93.1; 89.0−96.0)	NS
Number of clinical pregnancy (% embryo transfer cycles; 95% CI)	98 (22.4; 18.6−26.6)	64 (29.8; 23.7−36.4)	NS
Number of miscarriages (% clinical pregnancy; 95% CI)	31 (31.6; 22.6−41.8)	12 (18.7; 10.1−30.5)	NS
Number of ectopic pregnancy (% clinical pregnancy)	2 (2.0; 0.2−7.21)	0 (−)	NS
Number of delivery (% embryo transfer cycles; 95% CI)	65 (14.9; 11.7−18.6)	52 (24.2; 18.6−30.5)	NS
Number of multiple pregnancy (% delivery; 95% CI)	13 (20.0; 11.1−31.8)	1 (1.9; 0.1−10.3)	0.01
Number of live birth (% per transferred embryos; 95% CI)	78 (9.4; 7.5−11.6)	53 (20.1; 15.5−25.5)	0.01

Differences in frequencies were evaluated with Pearson's χ^2 test with Yates' continuity correction and Fisher's exact test. Student's t-test was used to compare continuous variables.
P-values lower than 0.05 were considered statistically significant.

Table III Cryopreserved oocyte cycle characteristics before and after introduction of the eSET policy.

	Preintervention group	Post-intervention group	*P*-value
Number of cryopreserved oocytes transfer cycles (% per oocyte retrieval cycle; 95% CI)	138 (8.6; 7.2−10.0)	27 (4.7; 3.1−6.8)	NS
Number of warmed oocytes (mean/oocyte warming cycle)	756 (5.5)	147 (5.4)	NS
Number of survived oocytes (% warmed oocytes; 95% CI)	630 (83.3; 80.5−85.9)	134 (91.2; 85.3−95.2)	NS
Number of transferred embryos (mean per cycle)	233 (1.9)	22 (1.70)	0.01
Number of embryo transfers (% warming cycle; 95% CI)	121 (87.7; 81.0−92.7)	13 (48.1; 28.7−68.0)	0.01
Number of clinical pregnancy (% embryo transfer; 95% CI)	34 (28.1; 20.3−37.0)	4 (30.8; 9.1−61.4)	NS
Number of miscarriages (% clinical pregnancy; 95% CI)	10 (29.4; 15.1−47.5)	0 (−)	NS
Number of ectopic pregnancy (% clinical pregnancy; 95% CI)	2 (5.9; 0.7−19.7)	0 (−)	NS
Number of delivery (% embryo transfer cycle; 95% CI)	22 (18.2; 11.8−26.2)	4 (30.8; 9.1−61.4)	NS
Number of multiple pregnancy (% delivery; 95% CI)	3 (13.6; 2.9−34.9)	0 (−)	NS
Number of live birth (% transferred embryos; 95% CI)	25 (10.7; 7.1−15.4)	4 (18.2; 5.2−40.3)	NS

Differences in frequencies were evaluated with Pearson's χ^2 test with Yates' continuity correction and Fisher's exact test. Student's *t*-test was used to compare continuous variables. *P*-values lower than 0.05 were considered statistically significant.

Table IV Clinical outcomes of IVF cycles with and without PGS during the whole study period.

	IVF without PGS	PGS	*P*-value
Number of cycles (% per total oocyte retrieval cycle; 95% CI)	1855 (95.0%; 83.4−86.4)	328 (15.0%; 13.5−16.6)	
Mean female age ($\pm$ SD)	39.5 (2.2)	39.6 (2.2)	NS
Number of cycles with euploid blastocysts (95% CI)	—	143 (43.6%; 38.2−49.1)	
Number of transfer cycles	2163	172	
Number of embryo transferred	4410	180	
Number of clinical pregnancy (% embryo transfer cycles; 95% CI)	558 (25.8; 24.0−27.7)	88 (51.2; 43.4−58.8)	0.01
Number of miscarriages (% clinical pregnancy; 95% CI)	169 (30.3; 26.5−34.3)	8 (9.1; 4.0−17.1)	0.01
Number of ectopic pregnancies (% clinical pregnancy)	16 (2.8; 1.6−4.5)	0 (−)	0.01
Number of deliveries (% embryo transfer cycles; 95% CI)	388 (18.0; 16.3−19.6)	80 (46.5; 41.0−55.8)	NS
Cumulative DR per oocyte retrieval cycle (95% CI)	20.9 (19.1−22.8)	24.4 (19.8−29.4)	NS
Number of multiple pregnancies (% delivery; 95% CI)	77 (19.8; 15.9−24.1)	1 (1.2; 0.1−6.8)	0.01
Number of life birth (% per transferred embryos; 95% CI)	465 (10.5; 9.6−11.5)	81 (45.0; 37.6−52.6)	0.01

Differences in frequencies were evaluated with Pearson's χ^2 test with Yates' continuity correction and Fisher's exact test. Student's *t*-test was used to compare continuous variables. *P*-values lower than 0.05 were considered statistically significant.

post-intervention group: 26.8 and 30.9% versus 29.7 and 26.3%, respectively. Relatively fewer transfers per oocyte retrieval cycle were performed in the post-intervention group (0.9 versus 1.1, $P < 0.01$, Table V). The DR per oocyte retrieval cycle was similar in the two groups as confirmed by logistic regression analysis adjusted for confounding factors: 21.1 and 20.4%, respectively (OR for group: 0.8; 95% CI = 0.7−1.1, Supplementary data, Table SI). As expected, female age and number of MII retrieved were significantly associated with DR per oocyte retrieval cycle (OR = 0.8; 95% CI = 0.7−0.8 and OR = 1.1, 95% CI = 1.1−1.2, respectively, Supplementary data, Table SI). Owing to improved embryo selection in the post-intervention group, a significantly increased LBR per transferred embryo was observed: 17.0 versus 10.6%, respectively ($P < 0.001$). The cumulative multiple DR decreased from 21.0 to 6.8% after the introduction of the eSET policy and enhanced application of blastocyst culture and PGS ($P < 0.001$)

(Table V). As shown by the logistic regression model, patients in the post-intervention group experienced a 60% reduction of their chance of multiple pregnancy (OR = 0.3; 95% CI = 0.2−0.7, Supplementary data, Table SII). When evaluating PGS cycles performed in the study period from 2010, a significant reduction in miscarriage rate was observed compared with standard IVF cycles (30.9 versus 9.1% for IVF and PGS, respectively, $P < 0.01$, Supplementary data, Tables SIII and SIV) with a similar DR per oocyte collection cycle (20.9 versus 24.4% for IVF cycles with or without PGS, respectively, OR = 1.1; 95% CI = 0.8−1.4, P = NS, Table IV and Supplementary data, Table SIV). In particular, patients with aneuploidy screening and transfer of euploid blastocysts only experienced a 65% reduction of their chance for miscarriage (OR = 0.3; 95% CI = 0.2−0.7, Supplementary data, Table SIII) compared with patients who underwent an IVF cycle without PGS (Table IV).

Table V Cumulative clinical outcomes before and after introduction of the eSET policy.

	Preintervention group	Post-intervention Group	P-value
Number of oocyte retrieval cycles	1609	574	
Number of embryo warming cycles (mean/oocyte retrieval cycle)	937 (0.6)	350 (0.6)	NS
Number of oocytes warming cycles (mean/oocyte retrieval cycle)	138 (0.1)	27 (0.1)	NS
Total number of cycles (mean/oocyte retrieval cycle)	2684 (1.7)	951 (1.7)	NS
Total number of embryo transferred (mean/oocyte retrieval cycle)	3856 (2.9)	734 (1.4)	<0.01
Total number of embryo transfers (mean/oocyte retrieval cycle)	1854 (1.1)	508 (0.9)	<0.01
Total number of clinical pregnancy (% per oocyte retrieval cycle; 95% CI)	497 (30.9; 28.6–33.2)	151 (26.3; 22.7–30.1)	NS
Total number of miscarriages (% per clinical pregnancy; 95% CI)	146 (29.4; 25.4–33.6)	31 (20.5; 14.4–27.9)	NS
Total number of ectopic pregnancy (% per clinical pregnancy; 95% CI)	13 (2.6; 1.4–4.4)	3 (2.0; 0.4–5.7)	NS
Total number of delivery (% per oocyte retrieval cycle; 95% CI)	338 (21.0; 19.0–23.1)	117 (20.4; 17.2–23.9)	NS
Total number of multiple pregnancy (% delivery; 95% CI)	71 (21.0; 16.8–25.7)	8 (6.8; 3.0–13.0)	<0.01
Total number of live birth (% per transferred embryos; 95% CI)	409 (10.6; 9.6–11.6)	125 (17.0; 14.4–19.9)	<0.01

Differences in frequencies were evaluated with Pearson's χ^2 test with Yates' continuity correction and Fisher's exact test. Student's *t*-test was used to compare continuous variables. *P*-values lower than 0.05 were considered statistically significant.
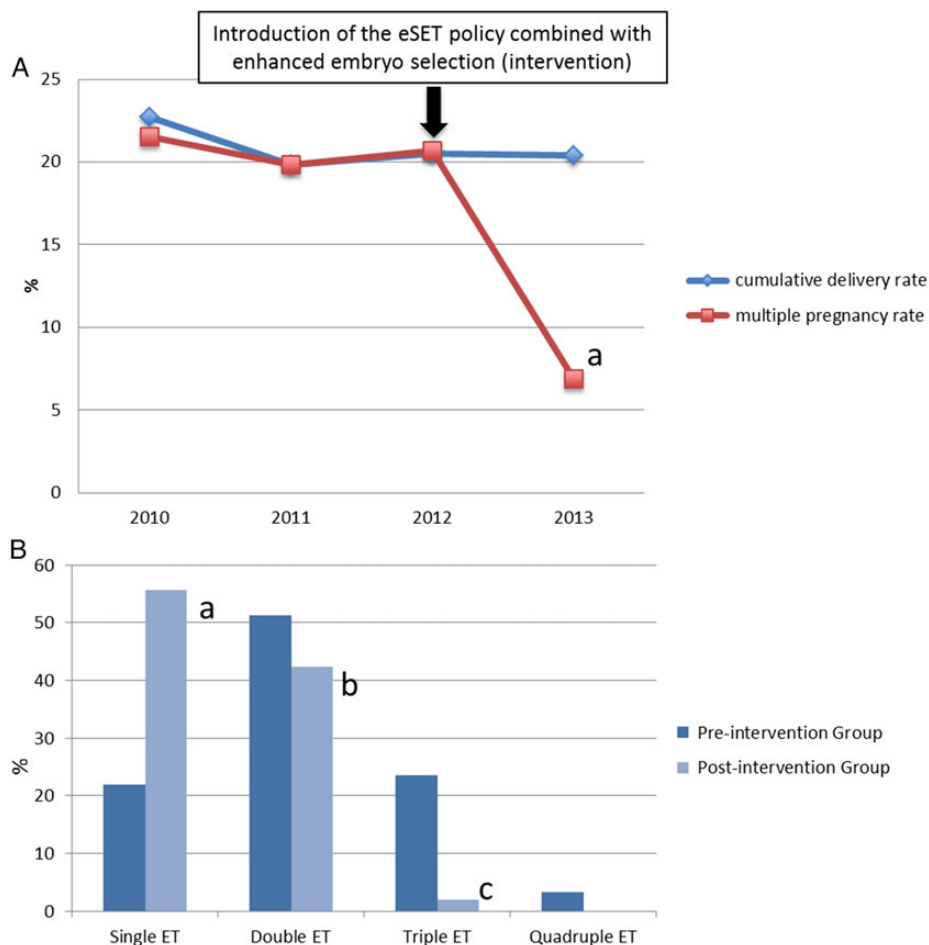

Figure 1 Graphical representation of the results obtained after the introduction of the eSET policy. (**A**) Impact of the eSET policy in cumulative DR and multiple pregnancy rate (MPR) over the 4 years. While no differences are observed in cumulative DR, the MPR is significantly reduced after the intervention (*P* < 0.01) as assessed by logistic regression analysis adjusted for confounding factors. (**B**) Distribution of single, double, triple and quadruple embryo transfers (ET) before and after the intervention (*P* < 0.01). Differences were evaluated with Pearson's χ^2 test.

Discussion

Our study indicated that eSET could be successfully applied for IVF cycles of AMA patients with appropriate clinical and embryological characteristics.

Compared with the 'good prognosis' population where criteria include young maternal age only, a few publications deal with eSET over 35 years. However, this patient population has an increased perinatal risk due to age alone and may become particularly vulnerable to multiple pregnancy (Davis et al., 2008).

In Italy, more than 15 000 cycles/year of IVF are performed in couples where the female partner is older than 39 years old, representing more than 30% of the total number of treatments (Data from Italian ART Register 2014 from Istituto Superiore di Sanità).

The first report investigating the feasibility of elective fresh single blastocyst transfer in AMA patients was published in 2005 by Davis et al. (2008). Retrospective analysis performed on 45 patients over 35 years has shown a 51% rate of ongoing pregnancies and no twins. Although the case number was low and the retrospective nature of the study resulted in some uncertainties, results were promising. Scotland et al. (2011) have found an ∼10-fold cumulative increase in twin LBRs when double embryo transfer (DET) versus eSET was applied in up to three fresh treatment cycles in in women over 35 years of age.

In a population of 40–44 years old women the retrospective cohort study of Niinimäki et al. (2013) did not find a significant difference in pregnancy, birth and twin rates between fresh eSET and DET. The subsequent frozen cycle (SET or DET depending on embryo availability and the opinion of the couple) resulted in a cumulative birth rate of 22.7 and 13.2%, respectively, with no significant difference in twin rates. Niinimäki et al. (2013) acknowledged that the two groups were not comparable due to the selection of eSET patients with good prognosis.

Blastocyst biopsy and comprehensive chromosome screening (CCS) has been shown to be a reliable procedure to detect chromosomally normal embryos for transfer (Capalbo et al., 2013a,b)

A recent publication of Schoolcraft and Katz-Jaffe (2013) has compared morphology-based selection for fresh and vitrified blastocyst transfer with CCS of vitrified embryos biopsied at the blastocyst stage. The ongoing pregnancy rate in the CCS group was significantly higher, proving the value of PGS for eSET in AMA patients. In accordance with this finding the multicentre retrospective study of Harton et al. (2013) indicated that implantation and ongoing pregnancy rates did not decrease in patients 35–42 years old when SET based on PGS was used. The value of trophectoderm biopsy, vitrification and CCS for SET in AMA patients was also emphasized in a recent review of Wu et al. (2014).

Our retrospective analysis based on a total of 2183 oocyte retrieval cycles and 1452 subsequent warming cycles performed in a single clinic in a 4-year period in a population with maternal age over 35 year supports this opinion. Although the embryo transfer policy was substantially modified, similar CPR and DR were obtained in the two populations of patients that were controlled for all possible confounding factors. Moreover, an increased live birth, decreased multiple delivery and miscarriage rates were observed after the systematic introduction of blastocyst culture and PGS.

Improved laboratory procedures play thus a decisive role in the success of eSET policy especially in a patient population with compromised prognosis including those with AMA. Culture to the blastocyst stage requires, however, an appropriate laboratory setting to avoid losses during the in vitro period (Gardner et al., 1998; Ly et al., 2011). The outcome certainly justifies the efforts, as eSET was found to be more efficient with blastocyst than with cleavage stage embryos or morulae (Papanikolaou et al., 2006; Kang et al., 2011, 2012). Blastocyst culture increases also both the proportion of euploid embryos (Adler et al., 2014) and the efficiency of morphological selection (Thompson et al., 2013).

A robust blastocyst cryopreservation method is also required to fully exploit the benefits of blastocyst transfers and PGS. Although traditional slow-rate freezing was also reported to be applicable for eSET (El-Toukhy et al., 2009; Grifo et al., 2013), the introduction of ultrarapid vitrification methods has offered a safe and efficient solution for this problem (Kuwayama et al., 2005; Cobo et al., 2012), and the approach was used successfully for eSET combined with warming cycles (Stoop et al., 2011; Schoolcraft and Katz-Jaffe, 2013; Roy et al., 2014).

Selection of the best embryo for transfer is crucial for the eSET. During the past 15 years, various approaches were suggested, including morphological classification, proteomic and metabolomic investigations and time lapse follow-up (Gardner and Sakkas, 2003; Nel-Themaat and Nagy, 2011; Stoop et al., 2011; Kirkegaard et al., 2012; Thompson et al., 2013). The increasing incidence of aneuploidy with AMA requires a reliable diagnostic method for chromosome aneuploidies, and recent reports indicate that none of the non-invasive approaches are reliable enough for this purpose (Yang et al., 2012; Kramer et al., 2014; Rienzi et al., 2015). The only reliable procedure is embryo biopsy, preferably at the blastocyst stage (Scott et al., 2013a) and accompanied by CCS (Treff et al., 2012; Schoolcraft and Katz-Jaffe, 2013; Scott et al., 2013a; Forman et al., 2014; Wu et al., 2014). Since aneuploidy is a very well-known factor associated with implantation failure, embryo selection based on euploidy is believed to significantly improve implantation rates. In this study, similar to the most recent literature (Scott et al., 2013b; Forman et al., 2014; Werner et al., 2014), the ongoing implantation rate transferring euploid blastocysts was around 50% across all female ages, suggesting that SET has to be considered mandatory in PGS cycles. In our analysis, indeed, all vitrified SETs have been performed in PGS cycles, resulting in only one monozygotic twin pregnancy. Furthermore, aneuploidies are also the single most important factor involved with spontaneous miscarriages. The analysis of PGS cycles provided preliminary evidence that miscarriage rate can be significantly reduced by 65% following the transfer of euploid blastocysts in a selected population at risk of producing aneuploid embryos. The increased application of such a technology holds the potential to reduce one of the main adverse events of IVF cycles, that is miscarriage rate, especially in countries where the application of IVF is mainly performed in women of advanced reproductive age. Obviously, being a screening technology, the application of PGS is not able to enhance gamete or embryo quality and thus the cumulative DR per stimulation cycle. Importantly, in this study we report preliminary evidence that similar cumulative DRs can be obtained following the application of blastocyst transfer combined with biopsy, trophectoderm aneuploidy screening and vitrification/warming compared with transferring embryos not subjected to all these additional procedures. However, it should be stressed that such procedures have to be gradually implemented and carefully monitored when introduced in IVF laboratories.

Even though randomized trials are needed to corroborate this evidence in a more controlled fashion, our results show that the application of eSET policy, combined with enhanced laboratory procedures,

including systematic application of blastocyst culture, PGS and vitrification, in the unselected AMA population, helps to increase safety. Decreased multiple pregnancies, and unsuccessful transfers were in fact obtained while maintaining the same DR per oocyte retrieval. Since the new embryo transfer policy was gradually implemented and mainly applied to blastocyst transfers, cleavage stage embryo transfers were still associated in the post-intervention period with a relative high multiple pregnancy rate (13.5%). From January 2014, extended culture and PGS has been routinely offered to all infertile patients where the female partner was older than 35 years. Cumulative DR, and a further reduction of multiple pregnancy and miscarriage rates associated with our eSET policy will be the main focus of our next analysis toward the continuous improvement of safety and efficiency of IVF treatments in our setting.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

F.M.U. and L.R. designed the study and took the lead in writing the manuscript. L.R. and A.C. developed and finalized the data set and performed the data analysis. A.C. provided a critical discussion of the manuscript as well as aneuploidy screening analysis. S.C., S.F., F.S., M.G., E.G. and A.V. performed the patient consultation and the clinical procedures. R.M. and D.C. performed the laboratory procedures. G.V. provided a critical revision of the manuscript.

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Conflict of interest

None declared.

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