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ORIGINAL ARTICLE *Embryology*

Definition of a clinical strategy to enhance the efficacy, efficiency and safety of egg donation cycles with imported vitrified oocytes

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STUDY QUESTION: Which is the most suitable clinical strategy in egg donation IVF cycles conducted with imported donated vitrified oocytes?

SUMMARY ANSWER: The importation, and allocation, of at least eight vitrified eggs per couple during an egg donation cycle is associated with a high cumulative live birth delivery rate per cycle, as well as the confident adoption of a single blastocyst transfer strategy to minimize the risk of multiple pregnancies.

WHAT IS KNOWN ALREADY: IVF using donor eggs is commonly used worldwide to treat women who are unable to conceive with their own oocytes. In 2014, the Constitutional Court (n.162/2014) gave permission for gamete donation to be allowed for ART in Italy. Initially recommended as a therapeutic approach for premature ovarian insufficiency, the use of donated oocytes has become more and more common. In countries such as Italy, fresh oocyte donation is theoretically possible, but practically impossible due to the lack of donors. In fact, the Italian law does not allow reimbursement to the young women, who can only voluntarily donate their eggs. Therefore, Italian IVF centers have established several collaborations with international oocyte cryo-banks. The most popular workflow involves the importation of donated oocytes that have been vitrified. However, recent evidence has questioned the overall efficacy of such an approach. This is because detrimental effects arising from oocyte vitrification and warming might reduce the number of eggs available for insemination, with a consequential reduction in the achievable live birth rate per cycle.

STUDY DESIGN, SIZE, DURATION: This was a longitudinal cohort study, conducted between October 2015 and December 2018 at two private IVF centers. Overall, 273 couples were treated (mean maternal age: 42.5 ± 3.5 years, range: 31–50 years; mean donor age: 25.7 ± 4.2, 20–35 years) with oocytes purchased from three different Spanish egg banks.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We performed an overall analysis, as well as several sub-analyses clustering the data according to the year of treatment (2015–2016, 2017 or 2018), the number of warmed (6, 7, 8 or 9) and surviving oocytes (≤4, 5, 6, 7, 8 or 9) and the cycle strategy adopted (cleavage stage embryo transfer and vitrification, cleavage stage embryo transfer and blastocyst vitrification, blastocyst stage embryo transfer and vitrification). This study aimed to create a workflow to maximize IVF efficacy, efficiency, and safety, during egg donation cycles with imported vitrified oocytes. The primary outcome was the cumulative live birth delivery rate among completed cycles (i.e. cycles where at least a delivery of a live birth was achieved, or no embryo was produced/left to transfer). All cycles, along with their embryological, obstetric and neonatal outcomes, were registered and inspected.

MAIN RESULTS AND THE ROLE OF CHANCE: The survival rate after warming was 86 ± 16%. When 6, 7, 8 and 9 oocytes were warmed, 94, 100, 72 and 70% of cycles were completed, resulting in 35, 44, 69 and 59% cumulative live birth delivery rates per completed cycle, respectively. When ≤4, 5, 6, 7, 8 and 9 oocytes survived, 98, 94, 85, 84, 66 and 68% of cycles were completed, resulting in 16, 46, 50,

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61, 76 and 60% cumulative live birth delivery rates per completed cycle, respectively. When correcting for donor age, and oocyte bank, in a multivariate logistic regression analysis, warming eight to nine oocytes resulted in an odds ratio (OR) of 2.5 (95% CI: 1.07–6.03, $P = 0.03$) for the cumulative live birth delivery rate per completed cycle with respect to six to seven oocytes. Similarly, when seven to nine oocytes survived warming, the OR was 2.7 (95% CI: 1.28–5.71, $P < 0.01$) with respect to ≤6 oocytes. When cleavage stage embryos were transferred, a single embryo transfer strategy was adopted in 17% of cases ($N = 28/162$); the live birth delivery rate per transfer was 26% ($n = 43/162$), but among the pregnancies to term, 28% involved twins ($n = 12/43$). Conversely, when blastocysts were transferred, a single embryo transfer strategy was adopted in 96% of cases ($n = 224/234$) with a 30% live birth delivery rate per transfer ($N = 70/234$), and the pregnancies to term were all singleton ($n = 70/70$). During the study period, 125 babies were born from 113 patients. When comparing the obstetric outcomes for the cleavage and blastocyst stage transfer strategies, the only significant difference was the prevalence of low birthweight: 34 *versus* 5%, respectively ($P < 0.01$). However, several significant differences were identified when comparing singleton with twin pregnancies; in fact, the latter resulted in a generally lower birthweight (mean ± SD: 3048 ± 566 g *versus* 2271 ± 247 g, $P < 0.01$), a significantly shorter gestation (38 ± 2 *versus* 36 ± 2 weeks, $P < 0.01$), solely Caesarean sections (72 *versus* 100%, $P = 0.02$), a higher prevalence of low birthweight (8 *versus* 86%, $P < 0.01$), small newborns for gestational age (24 *versus* 57%, $P = 0.02$) and preterm births (25 *versus* 86%, $P < 0.01$).

LIMITATIONS, REASONS FOR CAUTION: This retrospective study should now be confirmed across several IVF centers and with a greater sample size in order to improve the accuracy of the sub-analyses.

WIDER IMPLICATIONS OF THE FINDINGS: Single blastocyst transfer is the most suitable approach to achieve high success rates per procedure, thereby also limiting the obstetric complications that arise from twin pregnancies in oocyte donation programs. In this regard, the larger the cohort of imported donated vitrified oocytes, the more efficient the management of each cycle.

STUDY FUNDING/COMPETING INTEREST(S): None.

TRIAL REGISTRATION NUMBER: None.

Key words: oocyte donation / imported donated vitrified oocytes / blastocyst / cryo-bank / single embryo transfer

Introduction

IVF with donor eggs is a commonly used global strategy to treat women unable to conceive with their own oocytes (Imrie and Golombok 2018). The prevalence of this condition is increasing for a number of reasons, including longer life expectancy, the changing role of women in society, the absence of supportive family policies and a general economic uncertainty (Mills *et al.,* 2011). Our first experience of oocyte donation dates back to 1983, when it was used to solve a case of primary ovarian failure (Trounson *et al.,* 1983). Since then, oocyte donation has been considered an extraordinary technique that is used to manage and solve irreversible forms of female infertility. Over time, the use of donated oocytes has become more and more common, particularly for the treatment of age-related fertility decline, or for repeated IVF failure and other indications, such as the risk of transmitting a genetic disorder (Barri *et al.,* 1992; Borini, *et al.,* 1995a; Borini *et al.,* 1995b; Kawwass *et al.,* 2013; Ubaldi *et al.,* 2019).

In 2014, the Constitutional Court (n.162/2014) in Italy changed their legislative scenario (Law 40/2004) by affirming that the restraint of access to gamete donation for ART was unconstitutional. In Italy, the use of donor gametes is currently legal for all heterosexual couples, married or partners, and those who cannot rely on their own reproductively competent gametes. Even though both oocyte and sperm donation are allowed, the former predominates in Italy according to the latest data collection: 76% of IVF cycles with donated gametes were performed with donor oocytes, 19% with donor sperm and 5% with both (www.iss.it/pma). Oocyte donation involves retrieving eggs from a donor woman, insemination with sperm from the recipient's partner, culture *in vitro* and the transfer of cleavage, or blastocyst, stage embryos into the recipient's uterus. However, there are limitations to the supply of donor oocytes, such as the need to synchronize the donor and the recipient, the inability to quarantine oocytes and the added burden of a potential cancellation of a cycle due to a poor response to ovarian stimulation. Moreover, in countries such as Italy, the donation of fresh oocytes is theoretically possible, but practically impossible due to the lack of donors. In fact, Italian law does not allow a reimbursement to the young women who can voluntarily donate their eggs. Nonetheless, the high reliability of oocyte cryopreservation performed via vitrification permits the creation of donor egg banks and the use of vitrified-warmed donor oocytes. This allows us to improve the management strategies underlying donor programs by overcoming the limitations imposed by fresh eggs (Rienzi *et al.,* 2017). Although there is a remarkable body of evidence showing that there are no significant differences between fresh and vitrified oocytes in egg donation programs (Domingues *et al.,* 2017), only limited data exist relating to egg donation cycles performed after egg banking. Vitrified metaphase II (MII) oocytes can perform in a similar manner to fresh ones and be efficiently transported between IVF centers, provided that suitable precautionary measures are adopted to avoid physical shock during shipping and to preserve oocyte viability and intrinsic competence (Rienzi *et al.,* 2010; Cobo *et al.,* 2016; Alikani and Parmegiani 2018). Italian IVF centers have established several collaborations with international oocyte cryo-banks in order to meet the huge demand for oocyte donation treatments over the last few years. According to the last report of the Italian IVF registry, the number of couples who gained access to treatment involving donated gametes increased from 133 in 2014, to 2838 in 2017 (www.iss.it/pma). Cross-border oocyte donation treatments are carried out using two strategies. The first approach is based on the export of cryopreserved sperm from an Italian IVF center, which is then used in a foreign IVF center to inseminate fresh donor oocytes and produce embryos that are then vitrified and shipped back to the Italian IVF center to conduct vitrified-warmed embryo transfer (La Marca *et al.,* 2019). The second, and more popular approach, involves the importation of donated vitrified

oocytes, which are then warmed to undergo ICSI with fresh sperm obtained from the male partner. This is then followed by embryo transfer and the cryopreservation of supernumerary viable embryos. However, recent evidence has questioned the overall efficacy of the latter approach, suggesting that the detrimental damage arising from oocyte vitrification and warming might reduce the number of oocytes available for insemination and therefore reduce the achievable live birth delivery rate per cycle (Kushnir *et al.*, 2018).

In this paper, we describe how we established and manage our oocyte donation program based on the importation of donated vitrified oocytes. We also describe the number of vitrified oocytes, and the cycle strategy, required to enhance cycle efficacy, efficiency and safety.

Materials and Methods

Study design

This was a longitudinal cohort study, conducted between October 2015 and December 2018. All consecutive oocyte donation cycles conducted at Clinica Valle Giulia, GENERA Center for Reproductive Medicine of Rome ($n = 211$), and at G.EN.E.R.A. Veneto, G.EN.E.R.A. Center for Reproductive Medicine of Marostica ($n = 63$), were included. All oocytes were previously vitrified at three different egg banks (Dexeus, ES0599, Barcelona, Spain = Bank 1; Ceifer Biobanco Sevilla SI, ES006261, Sevilla, Spain = Bank 2; Clinicas Ginemed, SLP ES006263, Sevilla, Spain = Bank 3) from Caucasian women. These were shipped to our center and warmed to undergo ICSI with fresh sperm obtained from the male partner. All patient, cycle and clinical outcomes were registered in a relational database (FertiLab Manager, Italy) and retrospectively analyzed. We performed an overall analysis, as well as several sub-analyses clustering the data according to the year of treatment (2015–2016, 2017 or 2018), the number of warmed (6, 7, 8 or 9) and surviving oocytes ($\leq$4, 5, 6, 7, 8 or 9) and the cycle strategy adopted (cleavage stage embryo transfer and vitrification, cleavage stage embryo transfer and blastocyst vitrification, blastocyst stage embryo transfer and vitrification). In only nine IVF cycles did less than four oocytes survive warming; these cycles were therefore clustered together with the 35 IVF cycles in which four oocytes survived warming. Table I shows how the cycles were distributed across the previously listed sub-groups, while Supplementary Table SI shows the similar distribution of the sperm factor (as previously defined by (Mazzilli *et al.*, 2017)) in the four groups, according to the number of warmed oocytes. This study aimed to describe a clinical and embryological workflow (including the number of vitrified oocytes allocated, the stage of embryo development at transfer and the number of embryos transferred) to maximize IVF efficacy and efficiency and minimize the risk of multiple pregnancy during egg donation cycles with imported vitrified oocytes. The primary outcome measure was the cumulative live birth delivery rate among completed cycles (i.e. a live birth obtained, or no embryos left to transfer). All cycle, embryological, obstetric and neonatal outcomes, were registered and inspected. We then calculated the oocyte-to-baby rate as the proportion of warmed and survived oocytes that resulted in a live birth, as well as the number of oocytes needed to achieve a live birth by dividing the number of warmed and survived oocytes for the number of babies born (as performed by (Cobo *et al.*, 2015)). The same calculation was conducted to estimate the oocyte-to-delivery rate, and the number of oocytes needed to achieve a delivery. These estimates were conducted overall, and only among cycles that were completed during the study period.

Patients' work-up

Before entering an egg donation program, all patients received a general assessment of their health status, including peripheral blood tests and Rhesus (RH) factors, thyroid, coagulation screening and immunological screening, cardiological evaluation and gynecological evaluation, breast examination and infectious disease tests. The work up for the male partner included semen analysis, karyotyping, hemoglobin electrophoresis and tests for glucose-6-phosphate dehydrogenase and infectious diseases. Moreover, each male partner underwent an expanded carrier screening for single gene disorders to estimate genetic risk for the couple (Reprogene, Igenomix, Italy). Lastly, psychological counseling was strongly recommended to each couple. All patients signed an informed consent, and the study was approved from the Institutional Review Board (IRB) of Clinica Valle Giulia.

Oocyte donor selection

In order to be recruited and screened, all oocyte donors (aged 20–35 years) must fulfill the selection criteria outlined by the Italian and European regulations and match with the infertile couples seeking oocyte donation. Besides the screening for infectious and genetic diseases required by Law, our internal clinical protocol also demands that we perform carrier screening for the donors, in order to evaluate their carrier status and ultimately estimate and quantify, the residual risk for the newborn. After donor selection and matching, oocyte retrieval was performed, and mature oocytes were cryopreserved by vitrification. The chosen protocol involved a combination of 15% dimethyl sulfoxide (DMSO), 15% ethylene glycol, and 0.5 M sucrose (Kitazato, Japan); for this, we used a Cryotop device (Kitazato) (Kuwayama *et al.*, 2005). Two different strategies were adopted to ship the gametes from Spain to Italy: vapor-phase nitrogen shipping performed by an elected IVF courier as carry-on baggage (Bank 1), or by a conventional courier as hold baggage (Bank 2 and 3). In both cases, we used an electronic detector to ensure that temperature was continuously monitored.

Oocyte warming and insemination

Donor oocytes were warmed in accordance with the Kitazato protocol, as previously described (Rienzi *et al.*, 2010). In brief, for warming, each Cryotop was quickly plunged in 1 ml of 37°C prewarmed thawing solution (TS) containing 1.0 M sucrose for 1 min; the oocytes were then gently removed from the filmstrip. Afterwards, the oocytes were transferred to a dilution solution (DS) containing 0.5 M sucrose and were then incubated for 3 min at room temperature. After two consecutive steps in washing solution (WS), for 5 min each, the oocytes were transferred into culture medium (Continuous Single Step Medium, CSCM, Irvine Scientific) and incubated in a controlled atmosphere (6% CO_2 and 5% O_2) for 1.5–2 h prior to insemination. Surviving oocytes were placed in HEPES-buffered medium (Irvine Scientific, USA) supplemented with 5% human serum albumin (HSA, Irvine Scientific) and injected with processed sperm obtained from the male partner. After ICSI, the embryos were cultured in separate 30-µl drops (CSCM, Irvine Scientific) up to the blastocyst stage (Days 5–7) in a controlled atmo-

sphere in either a standard benchtop incubator (MINC, Cook Medical, USA, or K-system, Cooper Surgical, USA), or a time-lapse incubation system (EmbryoScope, Vitrolife, Sweden, or Geri, Merck, Germany).

Embryo evaluation and transfer

Embryo morphological assessment was performed as previously described (Rienzi *et al.*, 2010). In brief, cleaving embryos were assessed according to the number of blastomeres, symmetry, extent of fragmentation and the presence or absence of multinucleation (Alpha and ESHRE 2011). The quality of blastocyst expansion, trophectoderm and the inner cell mass was evaluated as previously described (Capalbo *et al.*, 2014). Single or double embryo transfer was performed at either the cleavage or the blastocyst stage, as previously described (Vaiarelli *et al.*, 2018). Supernumerary embryos, at either the cleavage or blastocyst stage, were vitrified, regardless of their morphological quality, as previously described (Ubaldi *et al.*, 2015; Cimadomo *et al.*, 2018). Embryo transfer was performed in the course of hormone replacement therapy (Glujovsky *et al.*, 2010). Prior to therapy commencing, all patients underwent basal transvaginal gynecological ultrasound evaluation of the endometrial thickness and of the basal state of the ovaries. Following the scan, 4 mg of oral estradiol valerate (Progynova, Bayer, Germany) was administered twice a day from the second or third day of the cycle for 5 days, then 6 mg every day. When the endometrial thickness reached at least 7–8 mm, and the aspect was trilaminar, we planned oocyte warming and sperm collection (3–5 days of abstinence). On the same day, the recipients commenced vaginal micronized progesterone administration (Progeffik, Effik, Italy): 400 mg on the first day, and 600 mg/day from the second day to support the luteal phase until the 10th week of gestation, in case of pregnancy. Embryo transfers were performed after 4 or 6 days of progesterone supplementation according to whether they underwent cleavage or blastocyst stage embryo transfer, respectively. Beta-hCG level was measured after either 14 or 11 days depending on the stage of preimplantation development.

Biochemical pregnancy loss was defined as serum beta-hCG levels $\geq$50 IU/l, which was required to show an increase by the second examination 2–4 days later, but without being associated with ultrasonographical evidence of intrauterine or extrauterine pregnancy 20–25 days after transfer, as previously described (Vaiarelli *et al.*, 2018)). Clinical pregnancy was defined as the presence of a gestational sac with a fetal heartbeat per warming cycle. A pregnancy loss earlier than the 20th gestational week was considered as a miscarriage. A clinical pregnancy that resulted in at least one live birth was defined as a 'live birth delivery'. Positive pregnancy tests, and the live birth delivery rates, were calculated using the number of transfers performed. The biochemical pregnancy loss rate was calculated using the number of positive pregnancy tests. The miscarriage rate was calculated using the number of clinical pregnancies. Finally, the cumulative live birth delivery rate was defined as the number of deliveries that resulted in at least one live birth per warming cycle, including all embryos obtained from one cohort of allocated oocytes per couple (Zegers-Hochschild *et al.*, 2017a, 2017b). This was calculated across all cycles included in the study and across only those cycles that had been completed (i.e. a delivery of at least a live birth obtained, or no embryos left to transfer).

The oocyte-to-baby, and oocyte-to-delivery, rates were calculated as the total number of warmed oocytes included in the study divided by the total number of live births and deliveries achieved, respectively. The mean number of oocytes required per live birth, and per delivery, was calculated as the total number of live births and deliveries achieved divided by the total number of warmed oocytes included in the study, respectively. All of these outcomes were reported for all cycles, and only for completed cycles.

Obstetric and neonatal outcomes (gestational weeks, birthweight and fetal growth) were evaluated according to the World Health Organization (Kiserud *et al.*, 2017; Kiserud *et al.*, 2018).

Statistical analysis

Categorical data are shown as a ratio with 95% confidence intervals. Statistically significant differences were defined by chi-squared or Fisher's exact tests. Continuous variables are shown as means, with standard deviation and range. Statistically significant differences were defined by ANOVA or by *t* tests. The main variables under investigation were the number of warmed and surviving oocytes. The results were corrected for confounders by logistic regression analyses. To this end, all study variables were tested for their association with the primary outcome under investigation (donor age, oocyte bank, IVF center, recipient age, paternal age, previous conceptions, duration of infertility, sperm factor, cycle strategy, type of incubator) and eventually used in multivariate logistic regression analyses.

Results

A total of 273 patients (mean maternal age: 42.5 ± 3.5, 31–50 years) underwent an IVF cycle with imported donated vitrified oocytes (mean donor age: 25.7 ± 4.2, 20–35 years), purchased from three different egg banks. Table I outlines the main patient and cycle characteristics.

Table I Main couples' and cycles' characteristics.

Patients, *n*	**273**
IVF center, *n*,%	
1	210, 76.9%
2	63, 23.1%
Recipient age, mean ± SD, min–max	42.5 ± 3.5, 31–50 years
Donor age, mean ± SD, min–max	25.7 ± 4.2, 20–35 years
Paternal age, mean ± SD, min–max	43.6 ± 5.8, 28–60 years
Previous conception(s), *n*, %	
No	191, 70.0%
Yes	82, 30.0%
Duration of infertility, mean ± SD, min–max	4.4 ± 2.6, 1–16 years
Oocyte bank, *n*, %:	
1	150, 54.7%
2	63, 23.0%
3	61, 22.3%
Incubator, *n*, %:	
Standard	182, 66.7%
Undisturbed	91, 33.3%

IVF center 1, GENERA Rome; IVF center 2, GENERA Veneto; oocyte bank 1, Dexeus; oocyte bank 2, Ceifer Biobanco Sevilla; oocyte bank 3, Clinicas Ginemed.

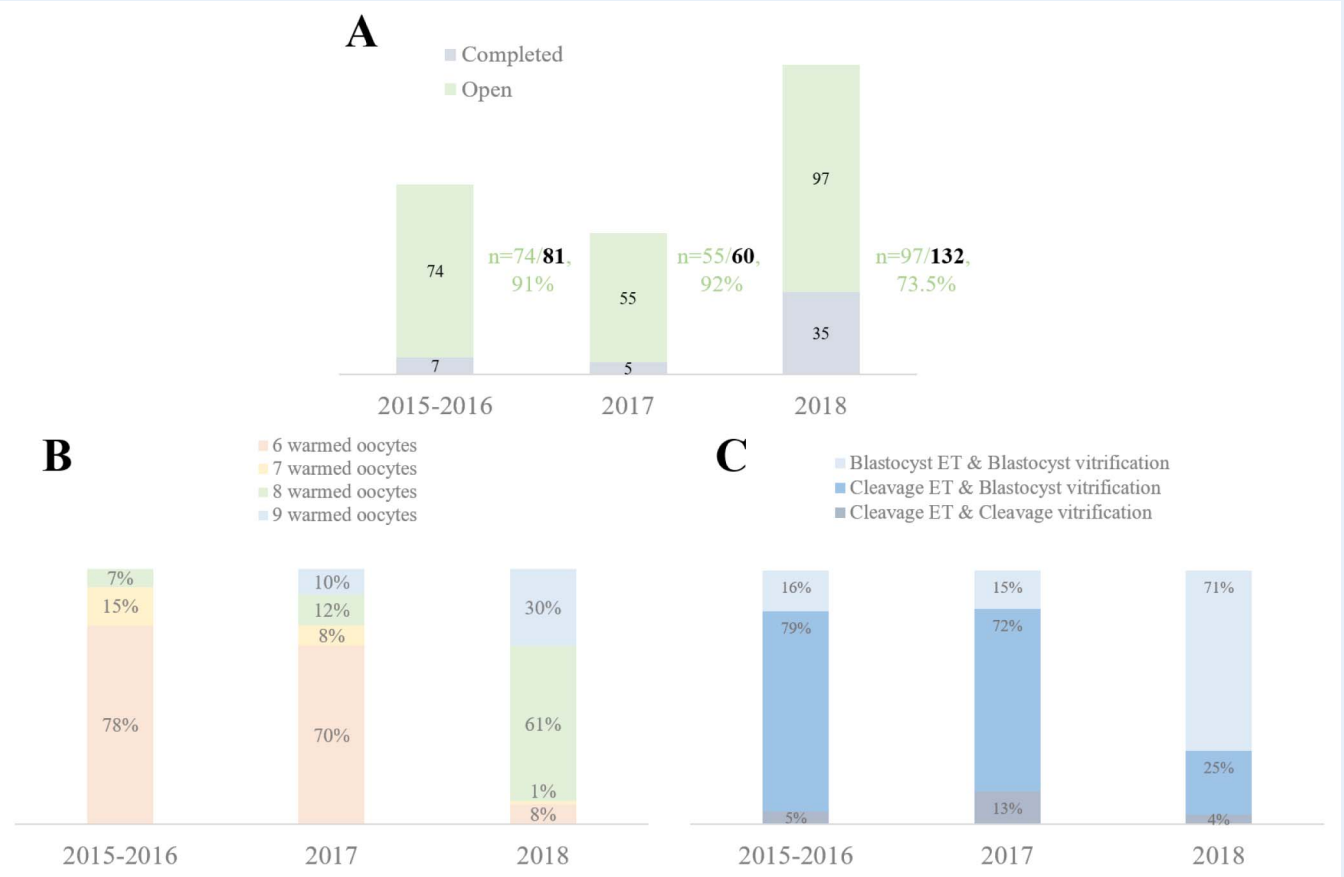


Figure 1 Distribution of cycles according to year. (**A**) Rate of completed cycle; (**B**) number of warmed oocytes; (**C**) cycle strategy. ET, embryo transfer.

The study embraced a period of 3 years and 3 months, from October 2015 until December 2018. Overall, 81 patients underwent IVF in 2015–2016, 60 patients in 2017, and 132 in 2018 (Fig. 1A). Across the years, the policy at our centers has changed in terms of the number of imported donated vitrified oocytes: in 2015–2016, six to seven oocytes were assigned per patient in 93% of cases, while in 2018, only in 9% of cases (Fig. 1B). This countertendency resulted in a contemporary change in the cycle strategy; in particular, the 'cleavage stage embryo transfer and blastocyst vitrification' strategy showed a sharp reduction, from 79% of the treatments performed in 2015–2016, to 25% of treatments performed in 2018. In parallel, the 'blastocyst embryo transfer and blastocyst vitrification' strategy increased from 16% to 71% (Fig. 1C). Table II shows the embryological data from the entire study period, for (i) all cycles (including the cycles for which embryo culture was conducted only up to the cleavage stage), (ii) the cycles in which only the supernumerary embryos were cultured to blastocyst (after cleavage stage embryo transfer) and (iii) the cycles planned for blastocyst culture. Of note, the survival rate after warming was generally good (86% ± 16%); in fact, all patients had at least one oocyte to inseminate. Table III clusters the 273 cycles according to the number of imported donated vitrified oocytes that were warmed, and the corresponding number of oocytes that survived, and therefore inseminated per patient. Overall, only three patients had no zygote; consequently, 98.9% of the cycles had at least one fertilized oocyte (Table II). All the latter cycles, except for one, obtained at least one cleavage stage embryo (98.5%) (Table II). Of the 140 cycles in which embryo culture was extended to the blastocyst stage after a cleavage stage embryo transfer, 75 patients (53.6%) obtained at least one supernumerary blastocyst to vitrify (mean: 1.9 ± 1.0) (Table II). Of the 116 cycles in which *in vitro* culture was extended to the blastocyst stage for the whole cohort of zygotes, 112 obtained at least one embryo to transfer (95.6%), and 92 also had a supernumerary blastocyst to vitrify (a mean of 3.3 ± 1.7 blastocysts were obtained per cycle) (Table II).

Overall, the positive pregnancy test rate per transfer was 44.2% ($n = 175/396$), the biochemical pregnancy loss rate per positive pregnancy test was 17.7% ($n = 31/175$), the miscarriage rate per clinical pregnancy was 21.5% ($n = 31/144$), and the live birth delivery rate per transfer was 28.5% ($n = 113/396$). There were no significant differences in terms of biochemical pregnancy loss and miscarriage rates with respect to the transfer strategy adopted (Supplementary Table SII). Figure 2 shows clinical outcomes according to the stage of transfer (cleavage or blastocyst). When cleavage stage embryos were transferred, a single embryo transfer strategy was adopted in 17% of cases ($n = 28/162$); the live birth delivery rate per transfer was 26% ($n = 43/162$), but among the pregnancies to term, 28% involved twins ($n = 12/43$). Conversely, when blastocysts were transferred, a single embryo transfer strategy was adopted in 96% of

Table II Embryological and cycle data.

Data related to all cycle strategies (*N* = 273)	
Warmed oocytes, *mean ± SD (min–max)*	7.3 ± 1.2 (6–9)
Surviving and injected oocytes per cycle, *mean ± SD (min–max)*	6.3 ± 1.7 (1–9)
Survival rate per cycle, *mean ± SD (min–max)*	86% ± 16% (17–100)
Cycles with survived oocytes, *n*, %	273/273, 100%
Fertilized oocytes, *mean ± SD (min–max)*	4.8 ± 1.8 (0–9)
Fertilization rate per cycle, *mean ± SD (min–max)*	76% ± 19% (0–100)
Cycles with fertilized oocytes, *n*, %	270/273, 98.9%
Cleavage stage viable embryos, *mean ± SD (min–max)*	4.6 ± 1.8 (0–9)
Rate of cleavage stage viable embryos per injected oocyte per cycle, *mean ± SD (min–max)*	72% ± 21% (0–100)
Rate of cleavage stage viable embryos per fertilized oocytes per cycle, *mean ± SD (min–max)*	95% ± 13% (0–100)
Cycles with cleavage stage embryos, n, %	269/273, 98.5%
Data related to cleavage fresh ET & blastocyst vitrification cycle strategy (*N* = 140)	
Supernumerary blastocysts cryopreserved, *mean ± SD (min–max)*	1.0 ± 1.2 (0–5)
Blastulation rate per supernumerary cleavage stage embryo, *mean ± SD (min–max)*	38% ± 36% (0–100)
Cycles with cryopreserved blastocysts, *n*, %	75/140, 53.6%
Supernumerary vitrified blastocysts, *mean ± SD (min–max)*	1.9 ± 1.0 (1–5)
Data related to blastocyst fresh ET & blastocyst vitrification cycle strategy (*N* = 116)	
Blastocysts, *mean ± SD (min–max)*	3.3 ± 1.7 (0–7)
Blastulation rate per fertilized oocytes, *mean ± SD (min–max)*	57% ± 24% (0–100)
Cycles with blastocysts, *n*, %	112/116, 95.6%
Cycles with supernumerary blastocysts to vitrify, *n*, %	92/116, 79.3%
Supernumerary vitrified blastocysts, *mean ± SD (min–max)*	2.9 ± 1.4 (1–6)

Table III Number of surviving oocytes in each warming cycle according to number of warmed oocytes.

	6 MII oocytes warmed	7 MII oocytes warmed	8 MII oocytes warmed	9 MII oocytes warmed	TOT
≤4 oocytes surviving and injected, *n*, %	39, 33.9%	1, 5.6%	4, 4.3%	0, 0%	**44**
5 oocytes surviving and injected, *n*, %	37, 32.2%	5, 27.8%	3, 3.2%	2, 4.3%	**47**
6 oocytes surviving and injected, *n*, %	39, 33.9%	4, 22.2%	16, 17.0%	2, 4.3%	**61**
7 oocytes surviving and injected, *n*, %	-	8, 44.4%	22, 23.4%	7, 15.2%	**37**
8 oocytes surviving and injected, *n*, %	-	-	49, 52.1%	13, 28.3%	**62**
9 oocytes surviving and injected, *n*, %	-	-	-	22, 47.8%	**22**
TOT	**115**	**18**	**94**	**46**	**273**

cases (*n* = 224/234) with a 30% live birth delivery rate (*n* = 70/234), and the pregnancies to term were all singletons (*n* = 70/70).

During the study period, 125 babies were born from 113 patients (Supplementary Table SIII). The mean birthweight was 2920 ± 601 g, the mean gestational age was 37 ± 2 weeks and mostly Caesarean sections were performed (77%). The newborns showed a low birthweight (<2500 g) in 21% of cases and a very low birth weight (<1500 g) in 2% of cases. Preterm births (<37th week) accounted for 35% of cases, and the 29% of newborns were small for gestational age; 5% of newborns were large for gestational age. Overall, 7% of women showed obstetric complications, and 4% of newborns showed neonatal issues. There was no significant difference between embryo transfers involving fresh embryo(s) from vitrified-warmed oocyte(s) and vitrified-warmed embryo(s) from vitrified-warmed oocyte(s). When comparing cleavage and blastocyst stage transfer strategies, a significant difference was reported only for the prevalence of low birthweight: 34 *versus* 5%, respectively ($P < 0.01$). The main significant differences were reported when comparing singleton and twin pregnancies; the latter resulted in a generally lower birthweight (3048 ± 566 *versus* 2271 ± 247 g, $P < 0.01$), a significantly shorter gestation (38 ± 2 *versus* 36 ± 2 weeks, $P < 0.01$), solely Caesarean sections (72 *versus* 100%, $P = 0.02$), a higher prevalence of low birthweight (8 *versus* 86%, $P < 0.01$), small newborns for gestational age (24 *versus* 57%, $P = 0.02$) and preterm births (25 *versus* 86%, $P < 0.01$).

Figures 3 and 4 show the cumulative live birth delivery rate according to the number of warmed and surviving oocytes among all cycles, and

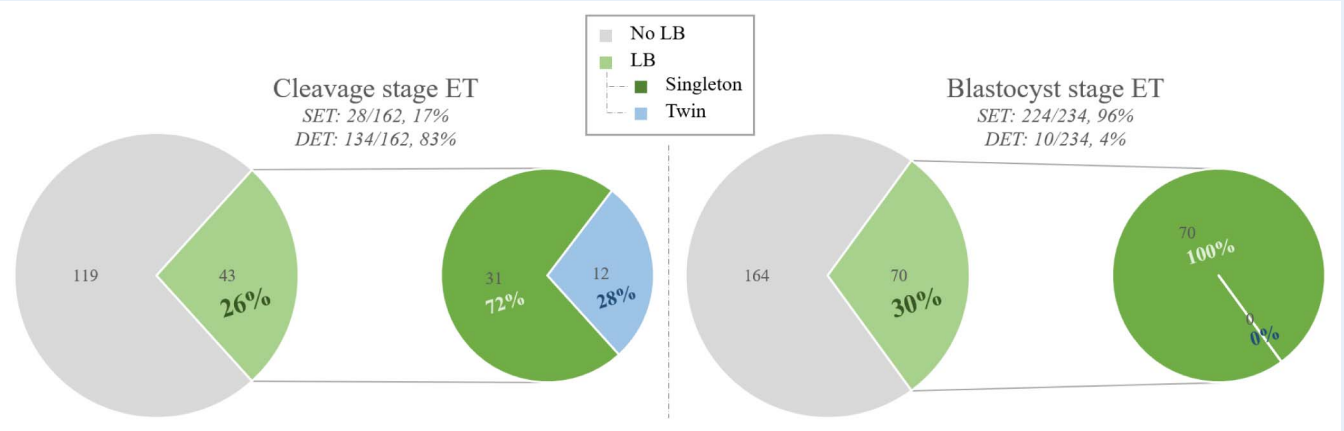


Figure 2 Live birth (LB) rate per embryo transfer (ET). SET, single ET; DET, double ET.

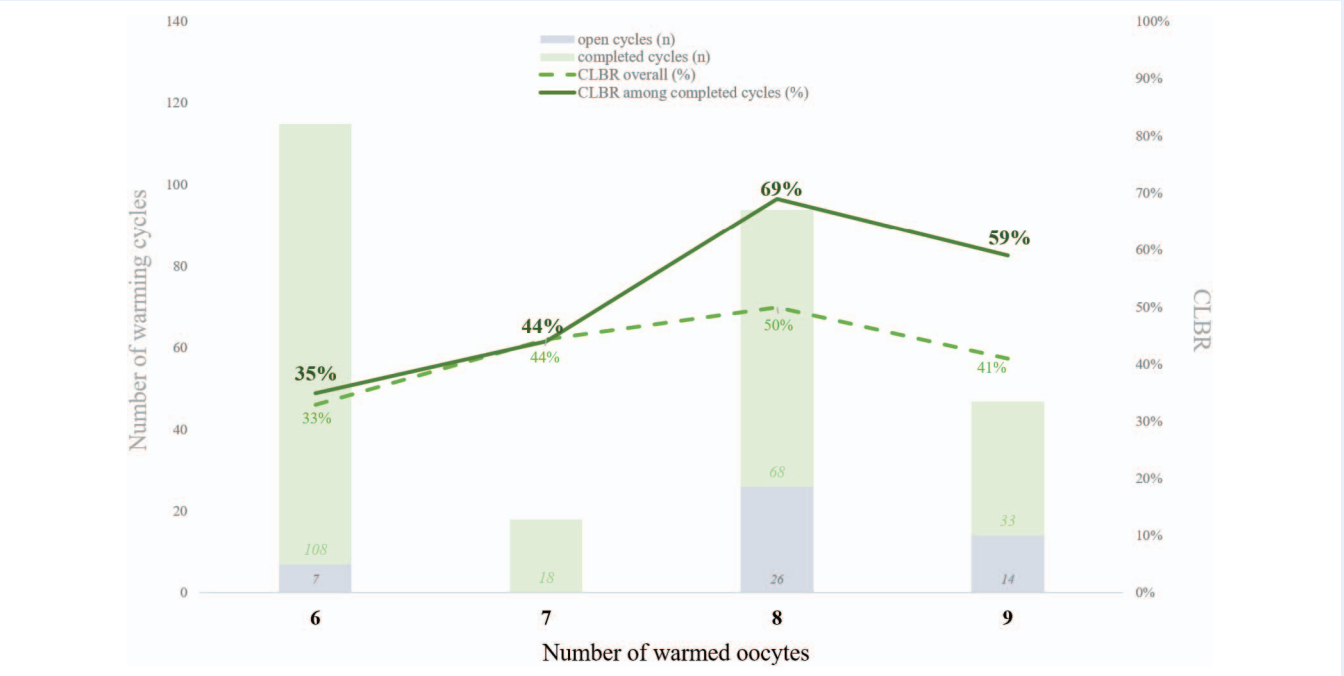


Figure 3 Cumulative live birth delivery rate (CLBR) among all, and only completed cycles, according to the number of warmed oocytes.

only completed cycles (i.e. cycles in which at least one live birth was achieved, or no embryos were produced/left to transfer; $n = 227/273$, 83%), respectively. Among the open and completed cycles, 2.2 ± 1.3 (1–6) *versus* 0.9 ± 1.4 (0–6) embryos were left to transfer. When six, seven, eight and nine oocytes were warmed, 94, 100, 72 and 70% of cycles were completed, resulting in 35, 44, 69 and 59% cumulative live birth delivery rates per completed cycle, respectively (Fig. 3). When $\leq$4, 5, 6, 7, 8 and 9 oocytes survived, 98, 94, 85, 84, 66 and 68% of cycles were completed, resulting in 16, 46, 50, 61, 76 and 60% cumulative live birth delivery rates per completed cycle, respectively (Fig. 4).

Tables IV and V show the results arising from multivariate logistic analyses, in which the outcome was the cumulative live birth delivery rate per completed cycle. After correcting for donor age and oocyte bank, warming eight to nine oocytes resulted in an odds ratio (OR) 2.5 (95% CI: 1.07–6.03, $P = 0.03$) with respect to warming six to seven oocytes. Similarly, when seven to nine oocytes survived warming, the OR was 2.7 (95% CI: 1.28–5.71, $P < 0.01$) with respect to the cycles in which $\leq$6 oocytes survived. When comparing cycles in which nine oocytes were warmed, rather than eight, we found that all outcomes were similar, apart from the mean number of surviving eggs (8.1 ± 1.1 *versus* 7.1 ± 1.2, $P < 0.01$).

During the study period, a total of 1982 oocytes were warmed, and 125 babies were born. These numbers correspond to a 6.3% warmed oocyte-to-baby rate and to an estimate of 15.8 oocytes required

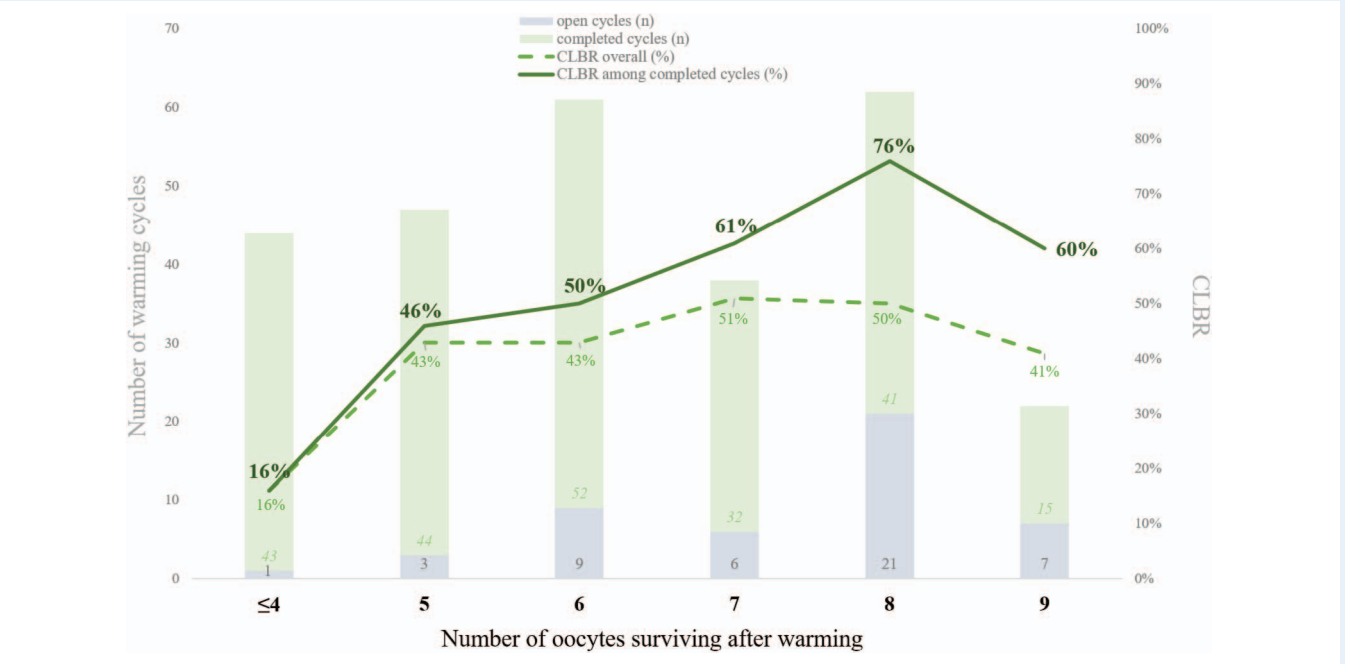


Figure 4 **Cumulative live birth delivery rate (CLBR) among all, and only completed cycles, according to the number of oocytes surviving after warming.**

Table IV **Multivariate logistic regression analysis (1).**

Variable	OR, 95% CI, *P* value
Number of warmed oocytes: 6–7	-
8–9	2.5, 1.07–6.03, $P = 0.03$
Donor age	0.94, 0.88–1.01, P = NS
Oocyte bank	1.1, 0.65–2.00, P = NS

The outcome under investigation was the cumulative live birth delivery rate among completed cycles. *OR, odds ratio*.

Table V **Multivariate logistic regression analysis (2).**

Variable	OR, 95% CI, *P* value
Number of survived oocytes: ≤6	-
7–9	2.7, 1.28–5.71, $P < 0.01$
Donor age	0.93, 0.87–1.92, P = NS
Oocyte bank	1.2, 0.73–2.00, P = NS

The outcome under investigation was the cumulative live birth delivery rate among completed cycles. *OR, odds ratio*.

per live birth. If counting only completed cycles, 1606 oocytes were warmed. Therefore, the warmed oocyte-to-baby rate was 7.8%, and 12.8 oocytes were required per live birth. On the other hand, the warmed oocyte-to-delivery rate was 5.7%, and 17.5 oocytes were required per delivery. For completed cycles, the same data were 7.0% and 14.2.

Discussion

We have experienced a constant increase in the application of oocyte donation in Italy in the last 4 years since its legalization. This study clearly outlines a training period, along which we grew expertise with a specific workflow that entails the use of donated vitrified oocytes that were imported from foreign countries. Specifically, we realized how critical it is to increase the number of oocytes that must be allocated to each couple in order to counteract the risks of degeneration after warming, fertilization failure and reduced developmental competence. Clearly, a higher number of oocytes warmed per cycle involved a more confident adoption of the 'blastocyst transfer and vitrification' strategy. This strategy also allowed the more confident adoption of a single embryo transfer policy (in 96% of cases when blastocysts were transferred *versus* 17% of cases when cleavage stage embryos were transferred), without affecting the live birth delivery rate per transfer, with obvious positive consequences on obstetric and neonatal outcomes (100% of singleton deliveries when blastocysts were transferred *versus* 72% with cleavage stage embryos).

Our main focus was to define the number of imported donated vitrified oocytes that should be allocated to each couple and warmed, thus aiming to maximize the clinical outcome. Clearly, this form of analysis requires that both the cryo-bank and the IVF center concur in guaranteeing high survival rates following warming. Furthermore, the latter must respect the high standards defined by international societies (e.g. the Vienna Consensus by the European Society of Human Reproduction and Embryology [ESHRE] and ALPHA scientists in reproductive medicine (ESHRE and Alpha 2017)), at least in terms of fertilization, cleavage and blastocyst rates. In our setting, the use of eight to nine imported donated vitrified oocytes was associated

with a cumulative live birth delivery rate of >50% per warming cycle. Furthermore, this number of oocytes was associated with an OR of 2.5 towards the cumulative live birth delivery rate per completed cycle with respect to six to seven oocytes; these relationships were independent of donor age and the cryo-bank from which the eggs were purchased. This outcome is mainly attributable to the number of surviving oocytes that could be inseminated. Specifically, when at least seven oocytes survived after warming, the OR towards the cumulative live birth delivery rate per completed cycle was 2.7 with respect to IVF cycles, with <7 oocytes surviving warming. Notably, we found that eight to nine oocytes should be allocated per patient, and they do not need to be warmed and inseminated all in a single round, thereby avoiding the production of several supernumerary embryos in case of a positive embryological outcome. This is an important aspect to consider in future studies, particularly since 78 out of the 113 patients who achieved a live birth (69%) still had, on average, 2.5 ± 1.3 (range: 1–6) supernumerary vitrified embryos. It is possible that these embryos will never be transferred for a second pregnancy considering the already advanced maternal age of these women at the moment of their first delivery. A previous study by Cobo *et al.,* investigated the clinical results of vitrified non-imported donor oocytes and showed similar lines of evidences as our present study: the oocyte-to-baby rate was 6.5%, and 15.4 oocytes were required to achieve a delivery (Cobo *et al.,* 2015). Here, we reported a 6.3% warmed oocyte-to-baby rate, a percentage that increased to 7.8% when we only considered completed cycles. Similarly, 15.8 oocytes needed to be warmed to achieve a live birth, an estimate that decreases to 12.8 among completed cycles. In other words, the ideal number of vitrified oocytes to allocate per patient lies between 13 and 16, but in our experience, we have never warmed more than nine imported donated eggs. The reason for this is mainly related to the cost-effectiveness of the treatment. In fact, by increasing the number of warmed oocytes from six to seven to eight to nine, we could still (i) maintain the same price per patient, (ii) adopt a 'blastocyst transfer and vitrification' strategy, but also (iii) retain a limited number of supernumerary vitrified embryos. This form of management would not be feasible if allocating 13–16 oocytes per couple. Therefore, a cumulative live birth delivery rate per warming cycle higher than 50%, as allowed by the use of eight to nine imported donated vitrified oocytes, is a reasonably good result with regards to balancing the costs of the treatment with a thoughtful cycle management plan. Clearly, a second attempt is always an option if the first fails. Finally, it is easier to collect eight to nine MII oocytes than 13–16 MII oocytes after a single ovarian stimulation; consequently, a higher number of donors might need to be enrolled.

In the present study, the distribution of the sperm factor (i.e. normozoospermia, moderate male factor, oligoasthenoteratozoospermia and azoospermia) was similar when clustered according to the number of imported donated vitrified oocytes. However, since severe male factor infertility has an important impact on both the fertilization and the blastulation rates (Mazzilli *et al.,* 2017), future studies should outline the ideal number of oocytes that should be allocated per couple in the presence of conditions such as oligoasthenoteratozoospermia or azoospermia.

The data published by La Marca *et al*., who adopted a workflow entailing frozen sperm transport, fresh donor oocyte insemination and vitrified embryo importation, led to an inseminated oocyte-to-baby rate of 7.1%, and 14.0 oocytes were required to achieve a live birth (La Marca *et al.,* 2019). However, this calculation is affected by the impact of multiple pregnancies (19% in the study by La Marca, and 11% in the present study). In fact, the number of patients delivering at least one baby per allocated oocyte may better describe the efficiency of the workflow. From this perspective, in the study by La Marca *et al.,*, the oocyte-to-delivery rate was 6.0%, and the number of oocytes required to achieve a delivery was 16.7; in the present study, these figures were 5.7% and 17.5, respectively, in the total population, and 7.0% and 14.2 among completed cycles, respectively. In other words, the two workflows appear similar in their efficiency, suggesting either could be adopted for the implementation of oocyte donation programs. However, to ensure safety, in both cases, a single blastocyst transfer is strongly recommended in order to reduce the incidence of multiple pregnancy.

Interestingly, all of the clinical outcomes of our study, including the miscarriage rate, are in line with those expected on the basis of egg donor age. Only the biochemical pregnancy loss rate (18%) was higher than the expected rate that is generally reported after IVF (Zeadna *et al.,* 2015; Vaiarelli *et al.,* 2018). This evidence incites future deeper investigations to unveil any putative underlying reason, particularly since this negative event was not associated with any of the embryo transfer characteristics. In a recent paper, Al Mamari *et al*. stated that the Day-16 beta-hCG serum cut-off level adopted to define a positive pregnancy test should be revised, since the lower the value, the higher the risk for a false positive result (Al Mamari *et al.,* 2019). In the present study, as in Vaiarelli's previous report in a conventional IVF setting, we adopted a beta-hCG serum 50 IU/l cut-off value for the first test, which must have increased by the time of second examination in order for a pregnancy test to be considered positive (Vaiarelli *et al.,* 2018). Further and deeper studies are certainly required in future to generate a consensus on this issue.

Obstetric outcomes are a critical aspect when dealing with oocyte donation programs since they are generally worse than the ones used for autologous IVF, particularly in terms of preterm delivery, birthweight, intrauterine growth restriction, preeclampsia and Caesarean section (Pados *et al.,* 1994; Tarlatzi *et al.,* 2017; Quaas and Pennings 2018; Schwarze *et al.,* 2018). Clearly this is predominantly a consequence of (i) advanced maternal age which, although not representing a constraint in terms of endometrial receptivity, is instead critical in terms of gestation, and (ii) the whole allogenic nature of the embryo with respect to the mother (Salha *et al.,* 1999). Indeed, maternal antibodies could disrupt the process of trophoblast invasion (Keegan *et al.,* 2007). It is therefore preferable to avoid the establishment of multiple pregnancies. In this regard, we have shown that single blastocyst transfer represents a more efficient and suitable strategy during oocyte donation cycles. When it comes to neonatal complications instead, we found no increased risk for morbidity and malformations, as confirmed by previous reports (Stoop *et al.,* 2012; Tarlatzi *et al.,* 2017).

Conclusion

The import of donated vitrified oocytes is a suitable and safe strategy with which to manage egg donation cycles in high-quality IVF laboratories. According to our experience, when at least eight oocytes were allocated per couple, we achieved a cumulative live birth delivery rate in excess of 50%. On average, this number of oocytes resulted

in seven surviving eggs and allowed the embryologists to confidently extend embryo culture to the blastocyst stage and adopt a single embryo transfer approach. This, in turn, increased the safety of the treatment by preventing the establishment of multiple pregnancies. In fact, multiple gestations must be avoided, particularly after oocyte donation cycles since they could only worsen obstetric and neonatal outcomes, which are already worrisome *per se*.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

L.R., D.C., R.M. and F.M.U. designed the study. D.C., R.M. and A.V. analyzed the data and drafted the manuscript. All authors contributed to the collection, interpretation and discussion of the data.

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Conflict of interest

None of the authors have any conflicts of interest to declare.

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