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Fresh *vs.* frozen embryo transfer in assisted reproductive techniques: a single center retrospective cohort study and ethical-legal implications

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Abstract. – OBJECTIVE: Several studies have shown higher pregnancy rates and better perinatal outcomes with frozen embryo transfers than with fresh techniques, with better results in patients with polycystic ovary syndrome (PCOS) but with a higher rate of pregnancy complications such as preeclampsia. This retrospective cohort study aims to compare the cumulative live birth rates, maternal and neonatal complications of fresh embryo transfers (ET) and frozen-embryo transfers (FET) in infertile women who underwent assisted reproduction techniques (ART) at the Azienda Ospedaliera Ospedali Riuniti (AOOR) Villa Sofia Cervello, Palermo, Italy. In addition, the authors have focused on the legislative and ethical complexities which such a procedure entails.

PATIENTS AND METHODS: Out of 475 women undergoing *in vitro* fertilization programs from January 2017 to January 2021, 128 were enrolled; 70 patients underwent ET, and 58 patients FET. The main outcome measure was live birth rates. Secondary outcomes were clinical pregnancy, ongoing pregnancy, pregnancy loss, low birth weight (LBW), ectopic pregnancy, and obstetrical and perinatal complications.

RESULTS: The cumulative live birth rates were similar between the fresh transfer (95.7%) and frozen transfer (93.1%). Biochemical pregnancy rates, clinical pregnancy, ongoing pregnancy, and pregnancy loss were similar between the groups.

CONCLUSIONS: Obstetrical outcomes were not statistically different between the two groups; a higher preterm delivery rate was reported in the FET group. ET birth weights were notably lower for singletons compared to the freeze-all strategy. ET patients also had higher LBW rates, with a 2.5-fold higher rate compared to FET. No significant differences were found in cumulative live birth rates between ET and FET, which is consistent with earlier studies. FET protocols are linked to higher neonatal birth weight and lower risk of LBW than fresh ET. The ethical and legal quandaries inherent in such techniques, as technology moves on and outpaces current legislative frameworks, cannot be discounted.

Key Words:
Frozen-embryo transfers (FET), Fresh embryo transfers (ET), Cryopreservation, Vitrification, neonatal outcomes, Legal/ethical implications.

Introduction

Infertility, a disease of the reproductive system, is a global public health issue. Both men and women who fail to achieve pregnancy after 12 or more months of unprotected intercourse are defined as having infertility issues[1]. Almost 48 million couples (one in six) and 168 million individuals worldwide reportedly have infertility issues, which also entail major social and psychological implications[2], and must be addressed through a gender-based comprehensive approach[3]. Almost 10% of couples worldwide are subfertile[4-7]. Since *In Vitro* Fertilization (IVF) was introduced in 1978, it has fast evolved. Currently, over 8 million babies worldwide have been deliv-

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ered by assisted reproduction techniques (ART)[8]. In ART, gametes and embryos are manipulated outside the human body by *in vitro* fertilization with or without intracytoplasmic sperm injection (ICSI). Controlled ovarian stimulation with gonadotropins, which increase the oocytes number retrieved, and cryopreservation of embryos, which makes it possible to preserve them, have improved cumulative live birth rates after IVF cycles. Despite such progress, implantation rates after single-embryo transfer are very low. Fresh and frozen embryo transfer can be performed after fertilization. In 2011 Devroey et al[9] developed the 'freeze-all' strategy in order to avoid ovarian hyperstimulation syndrome (OHSS), a potentially life-threatening condition due to the high doses of gonadotropins used for ovarian stimulation, causing a fluid shift from blood vessels to the abdominal cavity. This can result in abdominal bloating, a higher risk of thrombosis, and kidney and liver ischemia. Multiple pregnancies, after fresh embryo transfers, can lead to extra human chorionic gonadotropin (hCG) rise and consequently exacerbate already existing OHSS or induce late-onset OHSS. A delayed and scheduled transfer, which can be obtained by frozen-thawed embryos, is believed to potentially avoid the unfavorable condition for embryo implantation and placentation, due to the hormonal response to fertility drugs after ovarian stimulation. Gonadotropines used during ovarian stimulation lead to multiple follicles development and consequently elevated oestradiol and progesterone levels, hence a hormonal environment that may reduce endometrial receptivity for the implanting embryo[10-12]. Higher pregnancy rates and better perinatal outcomes with FET rather than fresh ET have been reported[13-17], particularly in patients with polycystic ovary syndrome (PCOS), but with a higher rate of pregnancy complications, such as preeclampsia[18]. A recent meta-analysis[19] included 15 studies with low to moderate quality evidence; however, similar results were seen among the strategies with a live birth rate of 58% following fresh ET and a cumulative live birth rate following the 'freeze-all' strategy between 57% and 63%. The OHSS rate was 3% following the fresh embryo transfer and 1% following the 'freeze-all' strategy. Few differences were found between the two strategies in the cumulative ongoing pregnancy rate with risk of hypertensive disorders of pregnancy, having a large-for-gestational-age baby, and higher birth weight for children born through the 'freeze-all' strategy. The study aims to compare the cumulative live birth rate, and maternal and neonatal complications between patients receiving fresh embryo transfer (ET) and patients receiving frozen-embryo transfer (FET) among infertile women with different ages and various ovarian reserves who underwent ART in our center with almost biochemical pregnancy confirmed.

Patients and Methods

In this single-center retrospective cohort study, 475 women undergoing *in vitro* fertilization programs were enrolled in an IVF program at AOOR Villa Sofia Cervello, Palermo – Italy, from January 2017 to January 2021, of whom 128 female patients were included in this study 70 patients underwent fresh ET and 58 patients FET.

The main outcome measure was live birth rates. Secondary outcomes were clinical pregnancy, ongoing pregnancy, pregnancy loss, low birth weight (LBW), ectopic pregnancy, and obstetrical and perinatal complications.

The main causes of infertility were tubal defects (including bilateral tubal occlusion), uni or bilateral salpingectomy, and male factors, including oligospermia, asthenospermia, or obstructive azoosperima. Data were collected from clinical records.

Eligibility, Inclusion and Exclusion Criteria

The eligible criteria were: ages under 43 years; normal menstrual cycles (defined as cycles>21 days and <35); good embryo quality according to the Istanbul consensus workshop[20] on embryo assessment; gonadotropin-realizing hormone (GnRh) antagonist protocol; GnRH agonist protocol that underwent a first cycle of fresh embryo transfer or freeze-all strategy and transfer in a subsequent cycle.

The exclusion criteria were: history of recurrent abortion, abnormal couple karyotype, and unpregnancy. A total of 475 cycles were performed, while 347 cycles met the exclusion criteria and were therefore left out.

In total, 128 women patients were included in this study, 70 of whom received fresh embryo transfer in group I and 58 received frozen embryo transfer in group II (Figure 1). The patients' baseline characteristics in the fresh ET and e-FET groups are shown in Table I. No significant differences were found between the frozen ET group of patients and the fresh ET group concerning the

number of oocytes retrieved, the number of mature oocytes, embryos transferred, and endometrial thickness on the trigger day. Also, in Table I, the baseline characteristics of both groups are outlined.

Protocols

Controlled ovarian stimulation (COS) was performed using gonadotropin-realising hormone (GnRh) agonist and antagonist protocols at the discretion of the reproductive physician. Women underwent basal serum levels testing for estradiol and progesterone, and if they were, respectively, <80 pg/ml and <1.5 ng/ml, the treating physician decided which exogenous gonadotropins should be used [recombinant follicle-stimulating hormone (FSH) or highly purified urinary human menopausal gonadotropin] according to their hormonal and clinical profiles.

The short-acting GnRH agonist protocol by subcutaneous injection of Triptoelin (Decapeptyl 0.1 mg/ml) was administered daily from day 1 or 2 of the menstrual cycle until the day of the hCG trigger. Ovarian stimulation with recombinant follicle-stimulating hormone or highly purified human menopausal gonadotrophins (HMG) (Gonal, Meriofert, Meropur) was carried out on day 3 of the menstrual cycle until the hCG trigger, with a dose of 75 to 300 UI per day, at the discretion of the reproductive specialist.

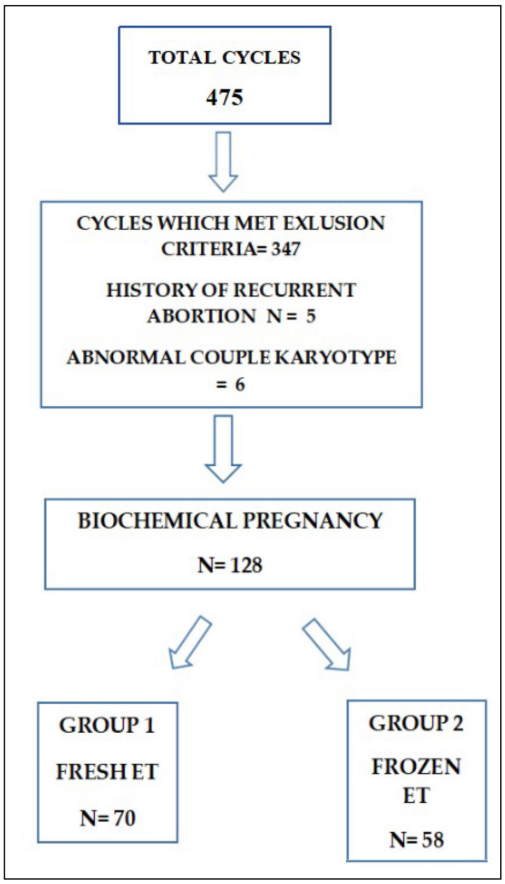

Figure 1. Number of patients included in the study.

Table I. Baseline characteristics of participants.

Characteristics	Fresh-Embryo Group n=70 (54.6%)	Frozen-Embryo Group n=58 (45.4%)	*p*-value
Age – year	34±4.2	33.2±4.3	0.13
Body mass index (kg/m^2)	23±3.1	22.8±3.0	0.47
Infertility duration (year)	3.4±1.1	2.8±1.7	>0.05
Infertility diagnosis			
Male Factor	31 (44.3%)	28 (48.3%)	
Tubal Factor	10 (14.3%)	7 (12%)	
Uterin factor	2 (2.8%)	2 (3.4%)	
Endometriosis	2 (2.8%)	4 (6.9%)	
Combined factor	10 (14.3%)	8 (13.8%)	
Unexplained	7 (10%)	3 (5.2%)	
Low ovarian reserve	4 (5.7%)	4 (6.9%)	
Others	4 (5.7%)	2 (3.4%)	
Endometrial thickness (mm)	6±2.4	6.2±2.1	
FSH IU/liter	6.6±1.5	6.7±1.6	
LH IU/liter	4.9±1.9	4.8±2.0	
Estradiol pg/ml	37.8±16.9	36.5±17.2	
PRL ng/ml	17.9±7.5	18.2±7.7	
Gestational week	38±5	39±1	
Oocyte retrived	7.1±4	7.3±3	
Number of Embryo transferred	1.8±0.3	1.9±0.2	

All data were obtained by Chi-square statistics with Yates correction. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), Prolactin hormone (PRL).

hCG triggering was performed with a dose of 4,000-10,000 IU (Gonasi HP) to induce the final maturation of oocytes.

Regarding the rare long-acting GnRH protocol, Triptorelin (Decapeptyl 0.1 mg/ml) was given daily by subcutaneous injection for 10-14 days, beginning in the mid-luteal phase of the menstrual cycle, then the ovarian stimulation with gonadotropin (Gn) started at a dose of 75 to 300 UI on day 3 (menstrual cycle) and continued until the hCG administration (dose 4,000-10,000 IU Gonasi HP) to induce the final oocytes maturation. As for the flexible GnRH antagonist protocol, treatment with recombinant follicle stimulating hormone or highly purified human menopausal gonadotrophins (HMG) (Gonal, Meriofert, Meropur) began on the second-third day of the menstrual cycle, and the antagonist (Cetrotide, 0.125-0.25 mg or Fyremadel 0.25 mg/0.5 ml) was administered when the dominant follicle diameter was 14 mm or more (supported by estradiol levels). The antagonist administration continued until the trigger day by human chorionic gonadotrophin (u-hCG-Gonasi) or Triptorelina (Decapeptyl 0.1 mg/ml) administration in order to induce the oocyte maturation[21]. The monitoring of ovarian response, gonadotropin dose adjustment, and timing of the final oocyte maturation trigger during ovarian stimulation were carried out according to the individual Estradiol levels (E2) and follicular growth assessed by ultrasound as previously reported[22].

The hCG triggering was administered 34 to 36 hours before the oocyte retrieval.

When fresh embryo transfer (ET) was performed, the luteal phase was supported by vaginal progesterone 600 mg/day, intramuscular progesterone 100 mg every three days, and continued until the day of serum hCG testing.

All embryos were frozen by vitrification[23,24]. Embryo quality was scored according to the morphologic criteria set at the Istanbul consensus workshop[20] on embryo assessment. Embryo transfer was performed on day 3 or 5 of development, using the most viable ones. Whether to transfer one or two embryos was decided by the clinician in agreement with the couple.

As support therapies, the following were recommended: prednisone 5 mg/day, aspirin 100 mg/day, folic acid 400 mcg/daily, vaginal progesterone 600 mg/day, and intramuscular progesterone 100 mg every three days until a serum quantitative hCG test was performed 14 days after ET. With a positive test result, vaginal progesterone was administered until a pregnancy ultrasonography confirmation.

When frozen embryo transfer was performed, no fresh luteal phase support was provided following oocyte retrieval.

All good-quality embryos were vitrified on day 2 or day 3, while the others were at the cleavage or blastocyst stage. As for the thawing blastocyst, embryo transfer was performed on the fifth day after starting the administration of vaginal micronized progesterone. In the next cycle after oocyte retrieval, endometrial preparation was implemented by Estradiol Valerate (Progynova) administration, 4 mg to 8 mg daily, starting on day 1 or 2 of the menstrual cycle. Vaginal progesterone 600 mg daily was added when the endometrium reached 7 mm or more at ultrasonography. A total of one or two frozen embryos were thawed and transferred three days after the start of progesterone. The post-thawed embryo transfer was supported in the same way as in ET cases. The endometrial preparation in the frozen embryo group was performed using Progynova 2 mg (Bayer Ltd, Isando, South Africa) three times daily from the second day of the menstrual cycle; vaginal support by micronized progesterone 200 mg three times per day was administered when a 7 mm endometrium thickness or more was detected by the vaginal scan. Two weeks later, ET, hCG serum levels were checked to confirm pregnancy. Clinical information regarding pregnancy outcomes and perinatal complications was obtained from obstetrical and neonatal medical records.

The subdivision into the two arms was made based on each patient's clinical characteristics, the ovarian response, and the ovarian stimulation protocol and, in any case, on the strict indication of the Reproductive Medicine doctor (IVF specialist) by selecting the most suitable technique on an individual basis, in order to lower the risks for the health and follow the principles of personalized medicine, especially for those cases more exposed to the risk of ovarian hyperstimulation (OHSS), for whom embryo/blastocyst freezing by vitrification (freeze-all technique) was used before proceeding with the next embryo transfer.

Results

Outcomes

The live birth rate was the main outcome following fresh- or frozen embryo transfer. Live birth was defined as the delivery of a viable newborn at 28 weeks or more of gestation. Secondary outcomes were clinical pregnancy, ongoing

pregnancy (defined as the number of pregnancies confirmed by ultrasound scan and continued for at least 21 weeks after embryo transfer), pregnancy loss, low birth weight (LBW), ectopic pregnancy, obstetrical and perinatal complications such as placental abruption, gestational diabetes, preterm delivery, gestational hypertension, placenta praevia, preeclampsia, neonatal hospitalization for >3 days, perinatal death and fetal abnormalities (chromosomopathy and congenital anomaly).

Statistical Analysis

All statistical analyses were performed with Social Science Statistical Package (SPSS Statistics Software version: 20.0, IBM Corp., Armonk, NY, USA), relying on Chi-square and Chi-square statistics with Yates correction. Both an odds ratio and a *p*-value were obtained. The level of significance was set at $p<0.05$. For continuous variables, mean, and standard deviation was used to summarize descriptive data on participant characteristics, while counts and proportions were used for the categorical variables. Differences in these variables between the treatment groups were assessed by χ^2 test to compare the continuous variables of different groups' covariance, or a Kruskal-Wallis test (also known as H test) was used, i.e. the nonparametric alternative to the one-way ANOVA test for unpaired data. The difference in the primary outcome (Cumulative live birth rate) and secondary outcome between the two treatment groups was analyzed by the Pearson χ^2 test. The odds ratio and 95% CIs were calculated.

Discussion

Patients included in this study underwent IVF treatment from January 2017 to January 2021. The overall biochemical pregnancy rate was 26.9%. A total of 70 patients who underwent fresh ET (54.6%) and 58 patients who underwent frozen-thawed ET (45.4%), were analyzed (Table II).

Patient age, BMI, infertility factor, ovarian stimulation, and IVF procedures were not significantly different between the fresh ET and frozen ET groups.

The cumulative live birth rates were similar between the fresh transfer (95.7%) and frozen transfer (93.1%) with odds ratio 0.62; 95% CI 0.6238 to 1.6943; $p<0.91$.

No notable differences were found between the frozen ET group of patients and the fresh ET group concerning the number of oocytes retrieved, the number of mature oocytes, embryos transferred, and endometrial thickness on the trigger day (Table I).

Similar embryo quality rates for e-FET and fresh ET were reported. Biochemical pregnancy rates, clinical pregnancy, ongoing pregnancy, and pregnancy loss, were similar between groups.

Fresh ET birth weights were notably lower, (3072 *vs.* 3295 *p*-value>0.029) for singletons compared to the freeze-all strategy. These patients (fresh group) also had higher LBW rates with a 2.5-fold increase probability compared to frozen ET, however, these findings were not significant (3 *vs.* 1; OR 2,5; CI 0.25 to 24.5; *p*-value<0.43). Similar embryo quality rates for e-FET and fresh ET were reported. Biochemical pregnancy rates, clinical pregnancy, ongoing pregnancy, and pregnancy loss, were similar between groups, as shown in Table III.

The perinatal and obstetric outcomes are shown in Table IV. The obstetrical outcomes were not statistically different between the two groups, although a higher preterm delivery rate was observed in the FET group (1 *vs.* 4; OR 0.2; CI 0.0225 to 1.9050; *p*-value<0.16).

Cumulative Live Birth Rate

In this single-center retrospective cohort study, no significant difference was found in cumulative live birth rates with frozen embryo transfer compared to fresh embryo transfer. These results seem to match those from studies[21,25] previously published.

Other studies[16,17,19,25] reported higher live birth rates in patients that underwent the 'freeze-all'

Table II. Patient statistical analysis breakdown. Day 3 embryo transfer *vs*. day 5 blastocyst transfers.

	Fresh	Frozen	Marginal Row Totals
Day 3	22 (22.97) [0.004]	20 (19.03) [0.05]	42
Blastocyst	48 (47.03) [0.02]	38 (38.97) [0.02]	86
Marginal Columns Total	70	58	128

The Chi-square statistic is 0.1342. The *p*-value is .714108. Not significant at $p<.05$. The Chi-square statistic with Yates correction is 0.0314. The *p*-value is .859301. Not significant at $p<.05$.

Table III. Outcomes after fresh and frozen embryo transfer of pregnancies ending in birth.

	Fresh-Embryo Transfer n=70 (54.6%)	Frozen-Embryo Transfer n=58 (45.4%)	Odds Ratio	95% CI	*p*-value
Livebirth	67 (95.7%)	54 (93.1%)	1.03	0.6238 to 1.6943	0.91
Singleton livebirth	64 (91.5%)	48 (82.8%)	1.07	0.6152 to 1.6426	0.78
Twin livebirth	3 (4.2%)	6 (10.3%)	0.4	0.0963 to 1.6865	0.2
Singleton Birthweight	3,072±399 SD	3,295±437 SD			<0.029
Biochemical pregnancy	70 (100%)	58 (100%)	1	0.6113 to 1.6359	1
Clinical pregnancy	70 (100%)	58 (100%)	1	0.6113 to 1.6359	1
Ongoing pregnancy	66 (94.3%)	54 (93.1%)	1.01	0.6139 to 1.6704	0.96

strategy focused on outcomes that were reported after the first transfer, while our result refers to the cumulative live birth rate per woman. This was in line with findings from a recent review[19]; differences between strategies could be time to pregnancy and possible differences in pregnancy and neonatal complications.

The live birth rate after the first transfer increased following the 'freeze-all' strategy (OR 1.17, 95% CI 1.06 to 1.28; 13 RCTs, 7,766 women) for all transfer stages.

However, the rate of live births herein evaluated should be interpreted as less relevant, since the very few cases where a second FET was performed have been disregarded in order to make the sample homogeneous and useful for statistical purposes. To make the sample even, only patients who received just one FET have, in fact, been accounted for.

Consequently, in light of the acquired data, it might be more appropriate to compare cumulative live birth rates between groups instead of live birth rates after the first transfer, as previously reported[26].

Recently, two systematic reviews and meta-analyses[27,28] reported a higher live birth rate after the first transfer using the 'freeze-all' strategy, especially in hyper-responders and in cycles with preimplantation genetic testing for aneuploidies compared to the fresh transfer technique. However, no significant difference was found in cumulative live birth rates between both groups. These results are in line with other studies[25,29-31].

Birth Weight

In our study, birth weights were significantly lower for fresh ET compared to the freeze-all strategy in singletons ($p<0.05$). Other studies[18] reported similar results with increased risk of higher

Table IV. Perinatal and obstetric outcomes.

	Fresh-Embryo Transfer n=70 (54.6%)	Frozen-Embryo Transfer n=58 (45.4%)	Odds Ratio	95% CI	*p*-value
Hypertensive disorders of pregnancy	0	2	0.16	0.0078 to 3.5258	0.2494
Gestational diabetes	2	2	0.8286	0.1132 to 6.0654	0.8531
Premature rupture of membrane	3	2	1.2429	0.2008 to 7.6919	0.8152
Preterm delivery <37 WG	1	4	0.2071	0.0225 to 1.9050	0.1643
Birthweight <2,500 gr	3	1	2.4857	0.2518 to 24.5417	0.4357
Miscarriage	5	4	1.0357	0.2658 to 4.0357	0.9597
Ectopic pregnancy	0	0	0.8298	0.0162 to 42.4663	0.9260
Congenital abnormalities in newborns	2	0	0.24	0.0111-5.154	0.19
Fetal congenital abnormalities	0	1	0.2766	0.0111 to 6.9184	0.4340
Neonatal hospitalization for >3 days	15	10	41.29.00	0.5194 to 2.9742	0.63
Perinatal death	0	0	138.18.00	0.0162 to 42.4663	0.9260

birth weights for singleton babies following the freeze-all strategies. This is probably due to the unfavorable uterine environment thought to be present in the fresh embryo transfer cycle.

Liu et al[32] observed that in early pregnancy, the risk of the small-for-gestational-age status of the neonate after the fresh-embryo transfer was inversely associated with the estradiol level. It is plausible that the uterine environment in a fresh-embryo transfer cycle could be less affected in ovulatory women with a normal ovarian response. In fact, ovulatory women usually have a lower stimulated level of estradiol compared to women that have polycystic ovary syndrome or high ovarian response.

Our findings also point out that FET may lead to a lower risk of LBW and higher neonatal birth weight. These results, despite not reaching statistical significance, are in line with the conclusions of many clinical studies[34-46].

Congenital Abnormalities

We found only two instances of congenital abnormalities in the fresh group and none in the freeze-all group (p=0.1945). The odd ratio was 0.24, therefore, a protective factor could be assigned to the frozen technique; however, this difference was not statistically significant.

Pelkonen et al[33] reported that the risk for at least one major congenital anomaly (CA) in the children born after FET was not increased compared with the children born after fresh ET. Other studies[19,28,32] reported congenital abnormalities, and based on such findings, it is still quite uncertain whether the two strategies differ in congenital abnormalities rate per live birth. Several studies[34-46] have shown the potential advantages of FET cycles over fresh ET regarding perinatal outcomes and offspring safety. From a biological point of view, controlled ovarian hyperstimulation (COH) in fresh ET cycles leads to a supraphysiological oestradiol environment. This type of environment is thought to affect embryo implantation due to lower endometrial receptiveness. That may explain the negative impacts of fresh transfers on neonatal safety[34-36].

Conversely, the absence of COH in FET cycles could explain the improved perinatal outcomes[37]. It has been reported by Pereira et al[38] that the chances of a full-term LBW were 6.1-7.9 times higher with E2 levels >2,500 pg/ml than with the E2 levels of the reference E2 group (E2<500-1,500 pg/ml). Suboptimal endometrial perfusion has been reported in fresh ET, probably due to hyperoestrogenic milieu.

Lee et al[39] demonstrated that fresh ET cycles develop advanced endometrial angiogenesis after gonadotrophin stimulation, in particular, the decrease in Ang-1 and increase in Ang-2 expression and consequential decrease in Ang-1/Ang-2 ratio after ovarian stimulation may increase endometrial perfusion, leading to an impaired endometrial vasculature.

These results may account for the increased edema found in these patients[40]. In fact, Ng et al[41] found that endometrial and subendometrial blood flow were considerably lower in stimulated cycles compared to natural cycles and that serum E2 levels had negative effects on endometrial blood flow in IVF cycles[41,42]. Gonadotrophin treatments could negatively affect placental development, embryo implantation and fetal growth, affecting the genes-associated expressions with transcriptional activity[34,35,43-46]. Kolibianakis et al[47] speculated that controlled ovarian stimulation (COS) is linked to advanced endometrial maturation, which results in the asynchrony of the endometrium and embryo, compromising the window for implantation.

Several studies[48-50], on the other hand, reported higher rates of large for gestational age (LGA) and macrosomia in FET than in fresh ET and natural cycles. A more pronounced genomic imprinting and interaction of the cryoprotectants used with the main enzyme involved in epigenetic programming has been considered in order to explain this, but only in animal models[51,52].

As we conducted our research, we discovered that FET was linked to a higher rate of preterm delivery compared to fresh ET, the reasons for which are still unclear. In order to clarify such correlations, advancements and innovations such as Artificial Intelligence (AI) applied to ART diagnostics and therapeutics[53-55], including gene therapies[53] and new avenues of embryo selection[56], will certainly play an increasingly valuable role in the years ahead[57]. Nonetheless, innovations will have to be regulated by effective and updated legislation and regulations, which need to be designed to reconcile scientific/technological progress and ethics codes meant to preserve our core values.

ART and Embryo Ethics: Unsolved Issues Still Lingered

Irrespective of whether ART techniques are carried out using frozen or fresh embryos, such procedures are bound to be ethically and morally controversial. That is due to the very status of hu-

man embryos, and how far lawmakers and regulators should go to protect them, even by holding back scientific advancements. Various definitions of embryo status have been articulated, largely based on ethical, moral, and social standards. Still, the very notion of the frozen human embryo has long raised major ethical concerns. Multiple definitions of embryo have been elaborated on by ethicists, jurists, and legislators[58]. When it comes to IVF, frozen embryos are considered as 'potential' human life forms. However, many people consider frozen embryos not only a "potential" human life form but human life in every respect. The fundamental objective of utilizing an embryo in any given medical procedure is affected by whether an embryo is to be considered human life or a definite human stem cell source. Nonetheless, it is worth noting that the array of definitions for embryo only applies to frozen embryos produced *via* IVF procedures, but not to fresh embryos, which acquire social identity immediately after the development. Frozen embryos, on the other hand, get it only once applied for fertility treatment or human embryonic stem cell research[59]. ART techniques are among the most valuable tools for addressing reproductive issues and enable couples (or even singles, where legal) to achieve parenthood[60,61], although its legislative governance has proven difficult and polarizing over the years[62], even more so as the pandemic disrupted healthcare services all over the worls[63-65]. ART relies on the stimulation of ovulation and other physiological processes in order to manipulate egg or sperm production. Donor oocytes or sperm can be used for fertilization in artificial insemination procedures such as intrauterine insemination (IUI), *in-vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Yet, such "life-giving" technologies give rise to multiple major ethical concerns, liable to create polarization and social division based on incompatible worldviews and principles[66]. As extensively proven[67,68], IVF using frozen embryos is a highly successful fertility treatment. In a typical IVF process, the embryo is produced in a Petri dish through the artificial fertilization of an oocyte with the sperm of her male partner or donor through multiple fertilization processes. Following fertilization, some of the embryos are implanted, while the remaining healthy embryos are cryopreserved for future use.

Nevertheless, the fate of these supernumerary embryos is controversial. A considerable amount of case law on embryo rights can be considered to try and shed light on such a highly controversial area of research and ethics. Certainly, embryo status and rights have been widely debated over the past two decades. That trend is likely due to the ever-broader use of ART and IVF techniques. Although an in-depth analysis of such case law is beyond the scope of this article, it is worth mentioning that different legislative frameworks have been enacted at the national level, and international/supranational bodies and institutions have issued numerous, mostly non-binding declarations, such as the 1999 Convention on Human Rights and Biomedicine[69], the so-called Oviedo Convention, which enshrines in Article 2 the interests of the human being as outweighing the interest of science, and states that creation of human embryos to be used for scientific research must be banned and, as pointed out by recent guidelines from the European Board and College of Obstetrics and Gynaecology[70] the highest ethical principles of confidentiality ought to be pursued by all scientists, whose actions need to meet the criteria as set out by the international society for stem cell research[71]. In a comparative analysis performed by the Council of Europe[72] among member states centered around the degrees of restrictions on research involving human embryos, it was reported that Belgium, Sweden, and the United Kingdom allow scientific research on human embryos, and embryos may even be legally created for such a purpose. Conversely, the Czech Republic, France, Greece, Hungary, the Netherlands, have banned the creation of embryos aimed at scientific research, although using surplus embryos may be legal, provided that such requirements are met. Lastly, human embryo research is unregulated in many nations, e.g., Russia, hence research is conducted without any clean-cut legislative framework. The European Court of Human Rights (ECtHR) has never issued specific decisions on embryo rights, but rather it has focused on the rights of parents, e.g., who should decide whether unimplanted frozen embryos should be used after the couple had separated. That was the case of Evans v. the United Kingdom decision[73], a 2007 ruling. In that decision, the ECtHR stated that the embryos created by the applicant and her partner did not have a right to life as enshrined in Article 2 of the ECHR, one of the most fundamental provisions in the Convention (ECHR), which upholds the fundamental right to life[74]. To buttress that point, it is certainly worth mentioning the Vo v. France ECtHR ruling[75], when the Court asserted that the embryo, though regarded as an unborn child, may not be viewed as a fully-fledged human

being, worthy of protection under Article 2 of the ECHR. It can therefore be inferred that the human embryo has no right to life under the ECHR. In other decisions, however, the Court has shown a sort of reluctance to tackle the fundamental question of what legal status ought to be ascribed to embryos (e.g., in the case of Parrillo v. Italy[76,77], when the ECtHR deemed it unnecessary to tackle the extremely controversial question of when human life begins under Article 2 of the ECHR). At the same time, the ECtHR has conceded the opposite principle, enunciated in paragraph 59 of the Costa and Pavan v. Italy decision[78-80]; in it, the Court seems to have taken a "middle position", labeling the embryo an "other" entity, a subject with a legal status itself, which can and should be "weighed against the legal status of the progenitors…". Hence, not all ECtHR judges agree with the Evans "anti-life" conclusion asserted in 2007. Nonetheless, the Court appears not yet ready to acknowledge the embryo's right to life[81,82]. That degree of ambiguity is reflected in the fact that even though it is not to be deemed "a person", the embryo cannot be viewed as a possession according to Article 1 of Protocol No. 1 of the Convention, which means that "parents" may not freely utilize their embryos. Given the margin of appreciation (i.e. legislative and regulatory autonomy) granted by ECtHR jurisprudence to member states, it is incumbent upon national governments and lawmakers to decide the fate of embryos that will never be implanted[83,84]. As ART technologies make further progress, such dilemmas are bound to become even more intricate, in medical fields such as obstetrics and gynecology, and among the specialties most at risk for litigation arising from alleged negligence-based malpractice[85,86]. In addition, professionals may invoke conscientious refusal, which already happens very often with controversial and polarizing procedures such as abortion[87]. Unsolved ethics quandaries are certainly raised by heterologous fertilization practices. Such issues include the issue of donor-conceived children, in terms of their right to know their biological origins as opposed to the right to donor anonymity. Practices such as inter-country surrogacy, i.e., couples traveling abroad to countries where the procedure is legal to have children, further compound ethical-legal conflicts and difficulties[88-90]. The issue of whether or not children ought to have a right to find out about their biological origins entails multiple ethical, moral, social, and legal implications[91-93]. The underlying question that needs to be answered is: which party ought to be considered more deserving of safeguards? The children, who should be allowed to know about their origins, or the donors, with their right to anonymity? Because of the extremely sensitive issues at stake, well-balanced solutions can only come from thoroughly assessed and widely shared legislative choices. The rights of newborn children must be prioritized within the broader discourse about ART procedures. In fact, their best interest often turns out to be neglected, because of a tendency to prioritize the parents' instead. The Italian National Bioethics Committee[94,95] has invoked the principle of equality and argued that it «does not view as legitimate, from an ethical and legal standpoint, to prevent those born through MAP from seeking information as to their biological origins». Similarly, adopting abandoned cryopreserved supernumerary embryos may also be deemed viable, when parents cannot, or for any reason, refuse to have them implanted. Adopting frozen embryos may even be viewed as a solidarity-based action, which cannot cause any rift between adoptive parents and effectively prevents embryo destruction. The core provisions of law 40/2004[96] can provide a tenable solution in terms of positively upholding the right of embryos arising from their status as unborn humans. Fundamental rights include the right to be born, which would be in agreement with recent recommendations from the Ethics Committee of the American Society for Reproductive Medicine[97]. Arguably, it would be preferable for embryos to be implanted and carried to term, even under adoption rules, rather than have them stored indefinitely[98].

Conclusions

Our results indicate that there are no significant differences in cumulative live birth rates with frozen embryo transfers compared with fresh-embryo transfers. Such findings are largely consistent with previous studies[16-46]. FET protocols are linked to higher neonatal birth weight and lower risk of LBW than fresh ET.

The retrospective nature of our study, and the limited number of cohorts, means that it was restricted, and thus, further investigations and prospective studies with a larger number of patients are needed to validate and verify our results. Another limitation is that only live birth rate per first transfer were taken into account. The subsequent embryo transfer was not a parameter that the authors chose to consider, since most patients did not

have other frozen embryos, hence it would have made the study no longer uniform. Furthermore, the study has only accounted for pregnancies ending in birth, thus excluding unpregnancy, as shown in Figure 1. This clearly reduces the sample under study, which, however, still maintains statistical significance. Current findings point to a statistically higher rate of higher-quality blastocysts in the fresh oocyte group than in the vitrification group. Such a difference did not affect clinical outcomes in any way other than a slight statistical reduction in biochemical pregnancies from vitrified oocytes compared to fresh oocytes.

Embryos from vitrified oocytes have been reported[99] to have a different pattern of early embryonic development than those derived from fresh oocytes. Furthermore, vitrified oocytes have been shown[100] to have cytoplasmic shortcomings that may not support fertilization and early embryonic development. Obviously, such findings could be useful in explaining the possible lower rate of blastocystic quality in embryos obtained from vitrified oocytes. If not, such a discrepancy could be deemed coincidental, due to the small sample, and would disappear in a larger-scale study or that morphology is not a reliable standard to assess blastocystic competence. By combining the lowest rate of high-quality blastocysts with the lowest biochemical pregnancy rate obtained in the vitrified group, it can be assumed that vitrification could be a useful filter to eliminate oocytes of dubious quality, capable to be fertilized and reaching the blastocyst stage but not implantation[101,102]. Hence, it is possible that oocyte vitrification could block the development of embryos, thus causing a biochemical pregnancy, although further prospective studies are needed to confirm this hypothesis.

The closed vitrification system can be deemed effective for the vitrification of oocytes under aseptic conditions, and biologically safe for the vitrified human tissue. Reliable and safe aseptic vitrification protocols are essential for the cryopreservation of human tissues, particularly after several studies by Bielański et al[103,104], which have fueled skepticism as to the reliability of open vitrification protocols[105]. The guidelines issued by the European Parliament and the Council of the European Union in 2004 and 2006[106-108] have prodded the search for solutions to keep vitrification in aseptic conditions, e.g., through liquid nitrogen sterilized by filtration[109], UV irradiation[110], or liquid nitrogen vapor storage[111], to address the problems of open vitrification systems. Although closed systems were considered potentially harmful to cells due to lower cooling rates, our results show closed vitrification systems are safe and can be used to collect biological samples without risk.

Nowadays, the indications for cryopreservation of oocytes are certainly increasing. Oocyte social freezing and egg donation banks can be used with good frequency. Vitrified oocytes, in fact, seem to have the same potential in terms of fertilization and implantation compared to fresh oocytes. Our main concern is safety in the cryopreservation of biological materials. A closed system device can, in fact, ensure proper isolation from any negative, low molecular weight toxic substances found in liquid nitrogen, whose duration of action is still unknown. Very promising results have been achieved by using the safest choice for storing oocytes in liquid nitrogen, and such success has encouraged studies in the field of human reproduction biotechnology. Furthermore, the introduction of new techniques using stem cells to improve implantation or foster the generation of gametes is currently being debated. Ultimately, the use of cryopreserved stem cells and embryo research must be governed by rigorous and safe regimes. To that end, updated legal, regulatory, and ethical frameworks need to take into account the complexities of embryo research, in order to effectively reconcile the values and needs of all parties involved.

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The Authors declare that they have no conflict of interests.

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Authors' Contributions
GG, GC, MEG, VC and AP conceived and designed the study; GB, SZ, and SM participated in the literature search; GG, GC, MEG, VC and AP collected and analyzed the da-

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