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Original Article

Embryology

Cumulative pregnancy rates of two strategies: Day 3 fresh embryo transfer followed by Day 3 or Day 5/6 vitrification and embryo transfer: a randomized controlled trial

A. Mengels [1,*], A. Van Muylder [2], K. Peeraer [1], J. Luyten [2], A. Laenen [3], C. Spiessens [1], and S. Debrock [1]

[1]Department of Obstetrics and Gynaecology, Leuven University Fertility Centre, University Hospital Leuven, Leuven, Belgium
[2]Department of Public Health and Primary Care, Leuven Institute for Healthcare Policy, KU Leuven, Leuven, Belgium
[3]Department of Public Health and Primary Care, Leuven Biostatistics and Statistical Bioinformatics Centre, KU Leuven, Leuven, Belgium

*Correspondence address. Department of Obstetrics and Gynaecology, Leuven University Fertility Centre, University Hospital Leuven, 3000 Leuven, Belgium.
E-mail: annemie.mengels@uzleuven.be https://orcid.org/0000-0002-9106-2762

ABSTRACT

STUDY QUESTION: Are cumulative pregnancy rates better if supernumerary embryos are vitrified on Day 5/6 instead of Day 3?

SUMMARY ANSWER: The results do not show a significant difference in cumulative pregnancy rates between the Day 3 and Day 5/6 vitrification groups.

WHAT IS KNOWN ALREADY: Pregnancy and live birth rates following IVF or ICSI treatment are higher after extended embryo culture and blastocyst transfer (Day 5/6) compared to cleavage-stage (Day 3) transfer. Cumulative pregnancy rates from one oocyte retrieval (OR) cycle show no significant difference after fresh and frozen embryo transfers, but only one study has used vitrification for the cryopreservation of supernumerary embryos while four studies have used a slow freezing protocol.

STUDY DESIGN, SIZE, DURATION: Our prospective randomized controlled trial was performed in an academic centre between January 2018 and August 2020. Patients were randomized into vitrification Day 3 (n = 80) or Day 5/6 (n = 81) groups. The primary outcome was the cumulative ongoing pregnancy rate (cOPR), considering only the first pregnancy for each couple. The power calculation revealed that 75 patients were required in each group, when assuming a 50% cOPR with four embryo transfers in the vitrification Day 3 group vs two transfers in the vitrification Day 5/6 group.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Patients <38 years undergoing their first or second OR cycles were randomized at the start of the first cycle. Up to two cycles were included in the analysis. A fresh embryo transfer was performed on Day 3. Supernumerary embryos (with ≥6 cells, <25% fragmentation, and equal blastomeres) or blastocysts (with expansion grade ≥2 with inner cell mass and trophectoderm score A/B) were vitrified on Day 3 or Day 5/6, respectively, and then transferred at a later date. A time-to-event analysis was performed with the patient's first ongoing pregnancy as the event of interest and the number of embryo transfers as the time component. The statistical comparison was performed by a Cox proportional hazards model. Cumulative costs of vitrification on Day 3 vs Day 5/6 were explored and compared using Mann–Whitney U tests.

MAIN RESULTS AND THE ROLE OF CHANCE: By December 2021, 233 transfers (96 fresh and 137 frozen) in 77 patients were performed in the vitrification Day 3 group and 201 transfers (88 fresh and 113 frozen) in 77 patients were performed in the vitrification Day 5/6 group. The time-to-event analysis did not show a difference between the two arms with regard to the patient's first ongoing pregnancy as the primary study outcome (hazard ratio [HR] 1.25, 95% CI 0.82; 1.92, $P = 0.30$). The cumulative ongoing pregnancy rate after eight transfers (from one or two ORs) was 57% in the vitrification Day 3 group vs 58% in the vitrification Day 5/6 group. The median number of embryo transfers until a pregnancy was achieved was five vs four, respectively, in the vitrification Day 3 group vs the Day 5/6 group. Similar results were found for the secondary study outcome, i.e. clinical pregnancy with foetal heart rate (HR 1.19, 95% CI 0.78; 1.80, $P = 0.41$). The cumulative clinical pregnancy rate (cCPR) after eight embryo transfers was 62% in the vitrification Day 3 group vs 59% in the vitrification Day 5/6 group. The median number of transfers until a pregnancy was achieved was four in both groups. The healthcare consumption pattern differed between the two groups and we observed higher costs for the vitrification Day 3 group compared to the vitrification Day 5/6 group, although these differences were not statistically significant.

LIMITATIONS, REASONS FOR CAUTION: Although our power calculation revealed that only 75 patients were needed in each study group ($\beta = 0.87$, $\alpha < 0.05$), the numbers were low. Also, different numbers of single and double embryo transfers were performed between the two groups, which may have affected the results. The cost analysis was performed on a subset of the patients and is therefore exploratory.

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WIDER IMPLICATIONS OF THE FINDINGS: Our study shows no difference in the cumulative pregnancy rate nor costs after fresh and frozen embryo transfers of at most two sequential OR cycles between the Day 3 and Day 5/6 vitrification groups; however, obstetric and perinatal outcomes should be taken into account to determine the best strategy.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded as an investigator-sponsored study of S.D. by Merck nv/sa Belgium, an affiliate of Merck KGaA, Darmstadt, Germany, and by Gedeon Richter Benelux (PA18-0162). The authors declare no conflict of interest related to this study.

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Introduction

Selecting the embryo with the highest implantation potential is crucial in an IVF laboratory (Paternot *et al.*, 2013) and is mostly based on developmental and morphological characteristics according to the Istanbul consensus (2011). Extended embryo culture is currently considered the best non-invasive option for embryo selection (Glujovsky *et al.*, 2022). Although it is used in routine clinical practice worldwide, there is an ongoing debate about the benefits of extended embryo cultures and blastocyst-stage transfer (Day 5/6) compared to cleavage-stage transfer (Day 3).

There are two main arguments in favour of extended embryo cultures. Transferring blastocysts may improve uterine and embryonic synchronicity resulting in higher implantation rates (Glujovsky *et al.*, 2022). Second, only the most viable embryos are expected to develop into blastocysts, which means that due to the process of self-selection, blastocysts have a higher implantation potential compared with cleavage-stage embryos (Blake *et al.*, 2007). On the other hand, this process of self-selection entails two important arguments against extended embryo cultures. Couples undergoing blastocyst culture have a higher incidence of embryo transfer cancellations due to failed embryo development (Karaki *et al.*, 2002; Levitas *et al.*, 2004) and a reduced embryo freezing rate (De Vos *et al.*, 2016) as not all embryos successfully develop to blastocyst. Furthermore, studies on obstetric and perinatal outcomes show contradictory results regarding preterm delivery when comparing cleavage-stage vs blastocyst-stage transfer (Maheshwari *et al.*, 2013; Marconi *et al.*, 2022).

Since the trend towards extended embryo culture began, several studies have been published to compare the clinical outcomes of blastocyst-stage transfer vs cleavage-stage transfer. A meta-analysis (Papanikolaou *et al.*, 2008) based on eight randomized controlled trials (RCTs) suggested that, when an equal number of embryos are transferred in a fresh IVF cycle, the probability of both live birth and clinical pregnancy is significantly higher when performing transfer at the blastocyst stage compared with the cleavage stage. A review of Wang and Sun (2014) demonstrated that blastocyst transfer in a fresh IVF/ICSI cycle significantly increased clinical pregnancy, implantation, ongoing pregnancy, and live birth rates and lowered miscarriages rate in comparison with cleavage stage embryo transfer in seven RCTs. A Cochrane systematic review (Glujovsky *et al.*, 2022) showed that the live birth rate after fresh blastocyst transfer was higher compared with cleavage stage transfer based on 15 RCTs, with low-quality evidence. However, they suggested that cumulative pregnancy rates provide a more realistic assessment of success rates by taking into account the transfer of all fresh and frozen embryos. Only five RCTs out of 27 in this Cochrane systematic review have reported cumulative pregnancy rates after fresh and frozen transfers and have shown no significant difference after one oocyte retrieval (OR). Only one study by Fernandez-Shaw *et al.* (2015) used vitrification for the cryopreservation of supernumerary embryos while four other studies have used a slow freezing protocol. A retrospective analysis by De Vos *et al.* (2016) also concluded similar cumulative live birth rates for cleavage-stage and blastocyst-stage transfers but significantly fewer transfers were necessary until live birth for blastocyst-stage embryos. A recent study of Clua *et al.* (2022), using vitrification for cryopreserving supernumerary embryos/blastocysts, showed higher cumulative live birth rates and shorter times to achieve a live birth after blastocyst-stage transfer. However, the study was underpowered and prematurely stopped due to poor results in the Day 3 group.

Since time to pregnancy might be shorter, transferring blastocysts may seem more effective from a patient's point of view. Freezing cleavage-stage embryos provides couples with more opportunities to achieve pregnancy but additional transfers may also increase the burden for the patient (Glujovsky *et al.*, 2022). On the other hand, the increased embryo transfer cancellation rate, as described in 17 RCTs in the Cochrane systematic review, might also cause emotional harm to the patient.

In this RCT, we compared the cumulative pregnancy rates of two strategies: fresh embryo transfer on Day 3 followed by vitrification of supernumerary embryos on Day 3 compared with fresh embryo transfer on Day 3 followed by vitrification of supernumerary embryos on Day 5/6. We performed a superiority trial thereby assuming cumulative pregnancy rates are higher if supernumerary embryos are vitrified on Day 5/6. We aimed to determine whether cumulative pregnancy rates are different if supernumerary embryos are vitrified on Day 5/6 vs Day 3. Additionally, we examined the cost implications of both strategies. Prior research performed in Spain by Clua *et al.* (2022) has shown that the average cost per live birth with cleavage-stage (Day 3) transfers is 24% higher than with blastocyst-stage (Day 5/6) transfers (Clua *et al.*, 2022). We investigated whether the cost implications are greater for the vitrification Day 3 group than for the vitrification Day 5/6 group in the Belgian context.

Materials and methods

Patient selection

The study protocol was approved by the Institutional Review Board (Clinical-Trials.gov ID: NCT04196036). Patients were recruited at UZ Leuven from January 2018 until the number of patients required according to power analysis was reached by August 2020. Patients stayed in study until a clinical pregnancy occurred or if no clinical pregnancy occurred, until all fresh and frozen embryos of two sequential IVF cycles were transferred or

until patient discontinuation. Follow-up of clinical outcome was performed until December 2021.

Patients fulfilling the following inclusion criteria were eligible for participation: female age <38 years, using own oocytes, planning a first or second OR for an IVF/ICSI treatment, and with normal FSH and Anti-Mullerian Hormone levels. Patients planning for preimplantation genetic testing, patients with BMI >30, and patients with endometriosis stage III or IV stages (according to the revised system of the American Society of Reproductive Medicine, 1996) were excluded.

After providing informed consent, patients were randomized at the start of the first fresh cycle using a computerized system through a randomization website. Allocation to the vitrification Day 3 vs Day 5/6 group was based on a 1:1 blocked randomized computer algorithm. Once randomized and allocated to a group, the supernumerary embryos per couple of two sequential IVF cycles were frozen on the respective day. A fresh embryo transfer in both groups was performed on Day 3.

Ovarian stimulation during fresh ART cycles, OR, IVF/ICSI procedure, embryo culture, and embryo transfer

In the fresh cycles, ovarian stimulation was performed as previously described (Debrock *et al.*, 2010). All oocytes and embryos were cultured in a single-step medium (GM501, Gynemed, Lensahn, Germany; Global Total LP, Cooper Surgical©, Arizona, USA) covered with mineral oil (Gynemed, Lensahn, Germany). Oocytes were fertilized using conventional IVF or ICSI. ICSI was performed either with fresh/frozen ejaculated or frozen testicular sperm. Sperm preparation, OR, and standard IVF/ICSI procedures were performed as previously described (Paternot *et al.*, 2010). On Day 1 (at 16–20 h post-insemination), oocytes were examined for the presence of two pronuclei. Further individual development of fertilized oocytes was evaluated on Day 2 (at 41–44 h post-insemination), Day 3 (at 66–71 h), and Day 5/6 (at 115–121 h/139–145 h). On Days 2 and 3, embryos were evaluated according to the number and the size of blastomeres (equal or unequal, i.e. >25% or >50% difference in size) and the degree of fragmentation (0%, <10%, 10–25%, 26–50%, >50%). Blastocysts were evaluated on Day 5/6 according to the inner cell mass (ICM), the trophectoderm layer (TE), and the expansion of the blastocoel (Gardner and Schoolcraft, 1999). A fresh embryo transfer was performed on Day 3, thereby selecting the best embryo based on developmental and morphological characteristics (Paternot *et al.*, 2010). One or two embryos were transferred according to the Belgian law (Belgisch Staatsblad, 2003). Supernumerary embryos of sufficient quality were cryopreserved on Day 3 (study group: vitrification Day 3) or after extended culture on Day 5/6 (study group: vitrification Day 5/6) depending on randomization. Sufficient quality of cleavage-stage embryos was defined as embryos with ≥6 cells, with ≤25% fragmentation, and with ≤25% difference in blastomere size on Day 3. Day 5/6 embryos of sufficient quality are described as blastocyst-stage embryos with a clear ICM (Score A or B) and TE (Score A or B) (Gardner and Schoolcraft, 1999). Cryopreservation was performed by vitrification.

Vitrification/warming procedure of cleavage-stage (Day 3) and blastocyst-stage (Day 5/6) embryos

The vitrification procedure was performed as previously described (Debrock *et al.*, 2015) using dimethylsulphoxide, ethylene glycol, and sucrose as the cryoprotectants (Irvine Scientific® Vitrification Freeze kit, Newtownmountkennedy, County Wicklow, Ireland). Embryos were vitrified one by one and loaded onto CBS-VIT-High Security (HS) straws (CBS, Cryo Bio System, L'Aigle, France).

For the warming procedure, the straws were warmed one at the time using commercially available thawing media (Irvine Scientific®, Vitrification Thaw Solution, Newtownmountkennedy, County Wicklow, Ireland) as previously described (Debrock *et al.*, 2015). Embryos were warmed in order of embryo quality.

After warming, cleavage-stage embryos were examined for the number/regularity of blastomeres and the degree of fragmentation. Cleavage-stage embryos were defined to have survived if ≥50% of the cells survived the warming procedure upon inspection immediately after this procedure; embryos were scored as fully intact if 100% of the cells survived. After warming, surviving Day 3 embryos were kept in culture for 24 h. All of the surviving embryos with or without further cleavage were transferred. In case of degeneration after overnight culture, if there were no vitrified embryos left, the embryo transfer was cancelled (Debrock *et al.*, 2015).

After warming, the morphological survival of the blastocyst was evaluated immediately. Only blastocysts with >50% of blastomeres intact were eligible for transfer. If the blastocyst was severely (>50% of the cells damaged) or completely damaged, an extra one was warmed immediately. Blastocysts were transferred only if they showed no further impairment between the time of warming and the moment of transfer. If there was impairment and no vitrified blastocysts were left, the embryo transfer was cancelled.

Hormonal monitoring and stimulation during frozen embryo transfer cycle

A frozen embryo transfer (FET) cycle is defined as a cycle with the intention to transfer a frozen/warmed embryo. Frozen/warmed embryos were transferred in natural cycles, stimulated cycles (gonadotrophin or letrozole), or hormonal replacement cycles (Debrock *et al.*, 2015).

In natural FET cycles, cleavage-stage embryos were transferred on Day 6 after the ovulation trigger and blastocysts were transferred on Day 7 after ovulation trigger. Embryo transfer was 1 day earlier when a spontaneous LH surge was detected on the intended day of the ovulation trigger. In hormonal replacement FET cycles, cleavage-stage embryos were transferred on Day 5 of progesterone administration and blastocysts were transferred on Day 6 of progesterone administration. A maximum of two embryos were replaced as determined by Belgian law (Belgisch Staatsblad, 2007).

Power calculation and outcome variables

The primary objective was to investigate if the cumulative ongoing pregnancy rate (cOPR) could be improved by vitrifying supernumerary embryos on Day 5/6 compared to Day 3. The primary outcome was a time-to-event variable with ongoing pregnancy as the event of interest and the number of transfers as the time component. Ongoing pregnancy was defined as 12 weeks of pregnancy at ultrasound. Patients were followed during all transfers (fresh and/or FET cycles) of two subsequent IVF cycles until pregnancy or until all embryo transfers were performed. Patients who did not reach pregnancy were censored at their last transfer. A better cOPR would imply a lower median number of transfers needed. The power calculation, performed for a two-sided log-rank test assuming six planned transfers per patient, a median time to drop-out (censoring) of four transfers in both arms, and a 5% significance level, revealed that 75 patients were needed in each group, assuming a median of four transfers in the

vitrification Day 3 group vs a median of two transfers in the vitrification Day 5/6 group to achieve ongoing pregnancy. The statistical comparison was performed by a Cox proportional hazards model. The proportional hazards assumption was tested using the Supremum test.

The secondary objective was a comparison of both treatment arms on the cumulative clinical pregnancy rate (cCPR) where the clinical pregnancy was defined as a pregnancy with foetal heart rate on ultrasound at 6–8 weeks of pregnancy (Zegers-Hochschild *et al.*, 2009). The analyses were performed were similar to those for the primary objective.

Cost analysis

To ensure accuracy of our cost estimates, we only included patients with a complete cost record for their entire Day 3 or Day 5/6 treatment trajectory. Patients whose ovarian stimulation and/or hormonal monitoring was performed outside UZ Leuven were excluded from analysis since this obscured our ability to retrieve complete data on care consumption.

The RCT collected information on all relevant medical procedures, i.e. stimulation (fresh and FET cycles), ORs (fresh cycles), and transfers (fresh and FET cycles). Resources utilized per patient were collected in terms of medical/technical procedures (ultrasounds, consultations, anaesthesia, OR, transfers, supplements, and other), clinical biology laboratory assessments (blood samples for hormonal assessments and other), and fertility laboratory fees (health insurance covered and not health insurance covered). Total costs were calculated from four perspectives: the hospital, the healthcare payer, the patient, and a societal perspective. The hospital perspective considered the total cost of all resources used during fertility treatment. The healthcare payer perspective only considered the share covered by health insurance (i.e. RIZIV in Belgium) whereas the patient perspective only included the patient's out-of-pocket shares. Finally, to explore the societal perspective, productivity losses of undergoing treatment were added to the total hospital cost, based on a previous study by Fiddelers *et al.* (2006), who, in an RCT comparing single embryo transfers with double embryo transfers (DET), also considered productivity costs using the friction cost method. For this study, an average productivity loss of €768 per embryo transfer was inferred.

Average and median cost were calculated for each perspective. The distribution of costs for all patients by perspective is shown in boxplot figures. An average total cost per pregnancy was also calculated based on the cumulative pregnancy rates found in the RCT. Mann–Whitney *U* tests for independent samples were used to compare if there is a significant difference. Since the distribution of costs from each perspective differed in the two groups, the result of the Mann–Whitney *U* test was based on the rank sums.

Results

Patient and treatment characteristics

Between January 2018 and August 2020, 161 patients provided informed consent and were randomized and allocated to the vitrification Day 3 (n = 80) or the vitrification Day 5/6 (n = 81) group (Fig. 1). In total, seven patients were excluded from analysis after randomization due to reasons of being included by mistake (not meeting inclusion criteria after all) (n = 3), no oocytes/fertilization in the first cycle and no second cycle started (n = 3) and stopping treatment before first embryo transfer (n = 1) (Fig. 1). There were 154 patients included analysis: 77 in the vitrification Day 3 group and 77 in the vitrification Day 5/6 group.

There were 125 patients who stayed in study until all fresh and frozen embryos of two sequential IVF cycles were transferred or until clinical pregnancy occurred. There were 29 patients who dropped out after not reaching pregnancy in the first cycle after all fresh and frozen transfers, meaning that only one cycle was available for analysis. This included 19 patients who did not start a second cycle due to: stopping treatment (n = 5), a second opinion in another centre (n = 10), or a spontaneous pregnancy between the first and second cycle (n = 4), as well as 7 patients, for whom the day of transfer/cryopreservation changed in the second cycle (at request of treating physician or patient) and 3 patients who declined further participation after the first cycle.

The majority of the patients were in their first IVF cycle at the beginning of the study: 55/77 (70.5%) in the vitrification Day 3 group vs 59/77 (76.6%) in the vitrification Day 5/6 group. The mean female age at the first OR was 31 years in both groups (31.5 ± 3.6 vs 31.6 ± 3.5) (Table 1).

The present analysis included 110 vs 108 fresh cycles in the vitrification Day 3 and the vitrification Day 5/6 group respectively. Table 2 summarizes all of relevant data on the fresh cycles. The type of fertilization was similar in both groups. The number of oocytes retrieved (11.5 ± 5.7 vs 12.3 ± 6.7), mature oocytes (9.7 ± 5 vs 10.6 ± 6.2), and fertilized oocytes (6.9 ± 3.9 vs. 7.4 ± 4.7) did not differ between the vitrification Day 3 and the vitrification Day 5/6 group respectively. The fresh transfer rate was comparable (96/110, 87.3%) between the vitrification Day 3 group and in the vitrification Day 5/6 group (88/108, 81.5%) and no difference was found in the number of freeze-all cycles (13/110 (11.9%) vs (17/108) 15.7%) and the number of cycles without cryopreservation (30/110 (27.3%) vs 31/108 (28.7%)). The utilization rate, defined as the number of embryos utilized (transferred or cryopreserved) per number of fertilized oocytes in the same cycle (Vienna Consensus, 2017) was significantly higher in the vitrification Day 3 group (58%) compared with the vitrification Day 5/6 group (47.9%; $P < 0.05$).

A total of 255 FET cycles were included in the analysis: 140 in the vitrification Day 3 group and 115 in the vitrification Day 5/6 group. Table 2 summarizes all relevant data on FET cycles. The frozen transfer rate was comparable between both groups ((137/140 (97.9%) in the vitrification Day 3 group vs 113/115 (98.3%) in the vitrification Day 5/6 group) but more DET were performed in the vitrification Day 3 group (27/137 (19.7%)) compared with in the vitrification Day 5/6 group (9/113 (8%); $P < 0.05$). In total, 175 Day 3 embryos and 138 Day 5/6 blastocysts were warmed. A higher embryo survival rate was observed in the vitrification Day 3 group (170/175 (97.1%)) compared with the vitrification Day 5/6 group (122/138 (88.4%); $P < 0.05$).

Clinical outcome of fresh embryo transfer and FET

By December 2021, 233 transfers (96 fresh + 137 frozen) in 77 patients had been performed in the vitrification Day 3 group and 201 transfers (88 fresh + 113 frozen) had been performed in the vitrification Day 5/6 group. Fresh transfer rates were comparable between the two groups: 96/110 (87.3%) in the vitrification Day 3 group vs 88/108 (81.5%) in the vitrification Day 5/6 group).

Fresh embryo transfer was performed on Day 3 in both study arms. Although not significant, the ongoing pregnancy rate (OPR) from the fresh transfers in either the first or second OR cycles was higher in the vitrification Day 3 group (24.0%) compared with the vitrification Day 5/6 group (18.2%). Performing a FET at the blastocyst stage (vitrification Day 5/6 group) resulted in higher OPRs (28/113 (24.8%)) compared to FET at the cleavage stage (vitrification Day 3 group) (19/137 (13.9%); $P < 0.05$).

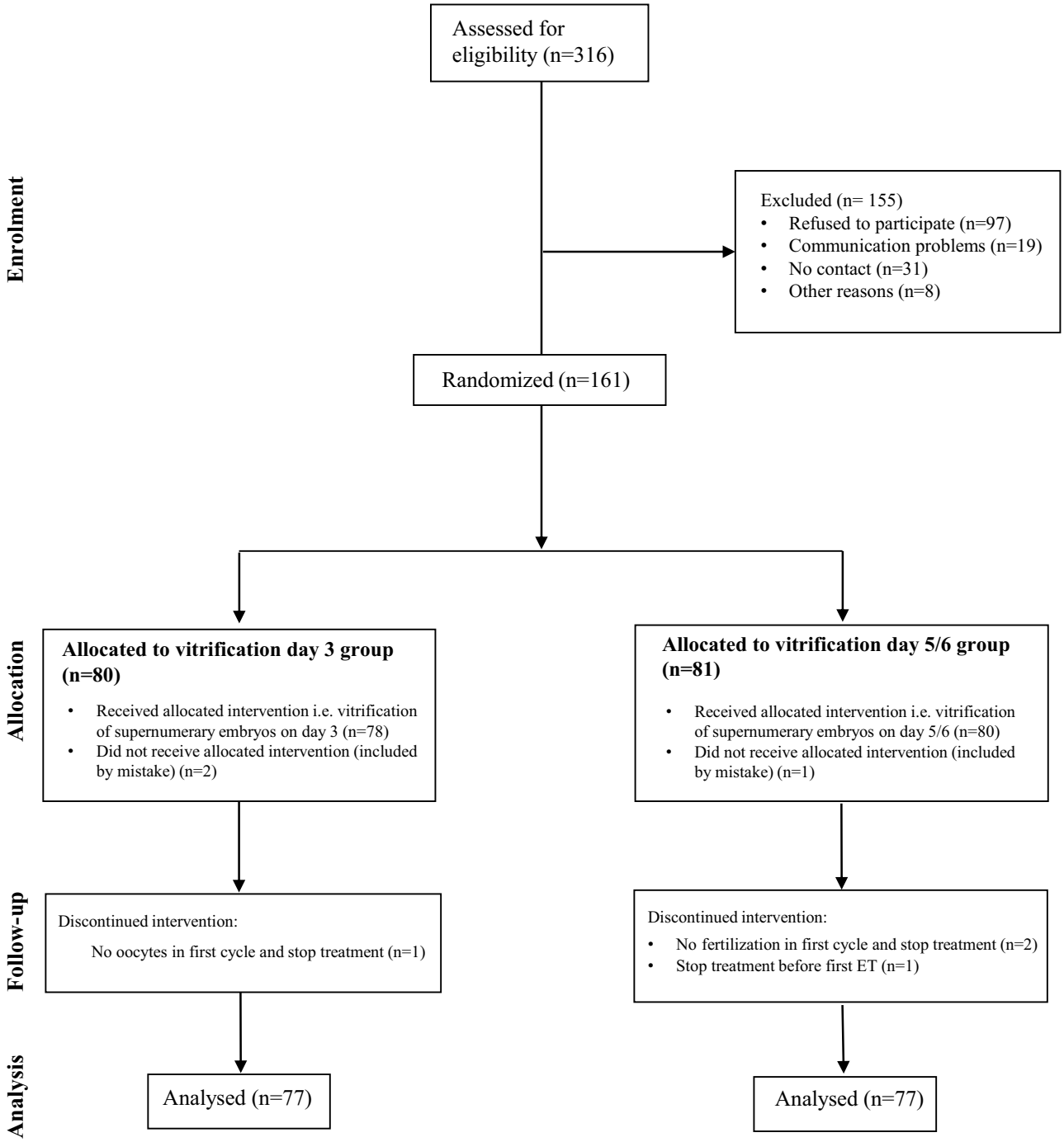

Figure 1. **Flow diagram of patient flow.**

Table 1. Patient characteristics.

	Vitrification d3 group (n = 77)	Vitrification d5 group (n = 77)
N patients	77	77
In Cycles 1–2, n (%)	55 (70.5)	59 (76.6)
In Cycles 2–3, n (%)	22 (29.5)	18 (23.4)
Female age (years) at first OR, mean ± SD	31.5 ± 3.6	31.6 ± 3.5
Primary infertility, n (%)	53 (68.83)	45 (58.4)
Infertility cause		
Male, n (%)	35 (46.45)	28 (36.4)
Female, n (%)	18 (23.38)	22 (28.6)
Mixed, n (%)	14 (18.18)	18 (23.4)
Unexplained, n (%)	10 (12.99)	9 (11.7)

OR, oocyte retrieval.

Table 2. Cycle characteristics.

	Vitrification d3 group (n = 110)	Vitrification d5 group (n = 108)	Statistical significance
Stimulation protocol			
Agonist long, n (%)	57 (51.8)	38 (35.2)	0.09
Agonist short, n (%)	5 (4.55)	8 (7.4)	
Antagonist, n (%)	48 (43.6)	62 (57.4)	
Culture medium			
GM 501, n	77	66	0.2
Global total LP, n	33	42	
Oocytes retrieved, n (mean ± SD)	1269 (11.5 ± 5.7)	1324 (12.3 ± 6.7)	0.43
Mature oocytes, n (mean ± SD)	1069 (9.7 ± 5)	1145 (10.6 ± 6.2)	0.29
Fertilized oocytes, n (mean ± SD)	759 (6.9 ± 3.9)	798 (7.4 ± 4.7)	0.46
Type of fertilization			
IVF, n (%)	30 (27.3)	32 (29.6)	0.73
ICSI, n (%)	71 (64.6)	70 (64.8)	
IVF/ICSI, n (%)	9 (8.2)	6 (5.6)	
Freeze all cycles, n (%)	13 (11.9)	17 (15.7)	0.42
Cycles with no cryopreservation, n (%)	30 (27.3)	31 (28.7)	0.83
Embryo transfers, n (%)	96 (87.3)	88 (81.5)	0.25
Utilization rate %	58.02	47.88	**0.0065**
FET cycles	**d3 group (n = 140)**	**d5 group (n = 115)**	
Endometrial preparation			
Natural cycles, n (%)	107 (75.43)	80 (69.57)	0.75
Ovarian stimulation cycle, n (%)	1 (0.71)	1 (0.01)	
Hormonal replacement, n (%)	32 (22.86)	34 (29.57)	
Embryos/blastocysts warmed, n	175	138	
Embryos/blastocysts survived, n (%)	170 (97.14)	122 (88.41)	**0.0035**
Embryo transfers, n (%)	137 (97.86)	113 (98.26)	0.81
SET, n (%)	110 (80.29)	104 (92.03)	**0.0264**
DET, n (%)	27 (19.71%)	9 (7.96)	

DET, double embryo transfer; FET, frozen embryo transfer; SET, single embryo transfer.

Table 3. Cumulative ongoing pregnancy rates by group + HR.

	% pregnancy (95% CI)			
Embryo transfer	**Vitrification d3 group**	**Vitrification d5 group**	**Hazard ratio (95% CI)**	***P*-value**
1	22.08 (13.61, 31.85)	22.08 (13.61, 31.85)	1.252 (0.816, 1.922)	0.3042
2	33.97 (23.64, 44.57)	33.77 (23.50, 44.31)		.
3	38.25 (27.37, 49.02)	45.45 (34.12, 56.10)		.
4	45.89 (34.18, 56.83)	53.68 (41.89, 64.10)		.
5	50.78 (38.64, 61.70)	55.05 (43.22, 65.40)		.
6	55.68 (43.27, 66.41)	56.42 (44.56, 66.68)		.
7	55.68 (43.27, 66.41)	56.42 (44.56, 66.68)		.
8	57.31 (44.85, 67.94)	57.79 (44.56, 66.68)		.

HR, hazard ratio. HR and *P*-value from Cox proportional hazard model. HR > 1 indicates higher pregnancy rate for the vitrification d5 group. Median nr of embryo transfers: vitrification d3 group=5 and virification d5 group = 4.

Cumulative pregnancy rates and number of transfers needed

The time-to-event analysis did not show a difference in cOPR as the primary endpoint between the vitrification Day 3 and Day 5/6 groups. The cOPR after 8 embryo transfers was 57.3% in the vitrification Day 3 group vs 57.8% in the vitrification Day 5/6 group (hazard ratio [HR] 1.25, 95% CI 0.82; 1.92, $P = 0.3$) (Table 3). The median number of transfers until ongoing pregnancy was 5 vs 4 respectively (Fig. 2). Regarding the secondary outcome, the time-to-event analysis showed similar results: the cCPR after 8 embryo transfers was 61.7% in the vitrification Day 3 group vs 59.1% in the vitrification Day 5/6 group (HR 1.19, 95% CI 0.78; 1.81, $P = 0.41$) (Table 4). The median number of embryo transfers until clinical pregnancy was 4 in both groups (Fig. 3). There was no evidence for a violation of the proportional hazards assumption in the primary or secondary outcome ($P = 0.4$ and $P = 0.5$, respectively).

Cost analysis

Our cost analysis included a total of 25 patients in the Day 3 and 26 patients in the vitrification Day 5/6 group and explored the medical resource costs associated with these two groups. This sample size is limited and smaller than the one used in the study by Clua *et al.* (2022) (69 patients in the vitrification Day 3 group and 65 patients in the vitrification Day 5/6 group) but it allows for an exploration of cost differences.

Table 5 provides an overview of the medical resource costs associated with the vitrification Day 3 and Day 5/6 groups. The analysis revealed that both groups had comparable numbers of medical/technical procedures and clinical biology laboratory assessments related to fresh cycle stimulations, OR, and fresh embryo transfer, whereas the vitrification Day 3 group had a higher average number of ultrasounds, consultations and clinical biology laboratory assessments related to FET cycle stimulations per patient. Similarly, both groups had comparable costs per

patient for resource categories related to fresh cycles (ultrasounds, consultations, clinical biology laboratory assessments), ORs (anaesthesia, fertility laboratory fees), and medical/technical performances related to fresh embryo transfers. The vitrification Day 3 group had a higher mean cost per patient for most resource categories related to stimulation and embryo transfer in FET cycles (i.e. ultrasounds, consultations and clinical biology laboratory assessments).

Figure 4 shows boxplots for each perspective to visualize cost distributions. Table 6 presents the median and average total costs per patient for all four perspectives. Our results show that the vitrification Day 3 group incurred higher costs per patient compared to the vitrification Day 5/6 group. From a hospital perspective, including all direct medical costs, the costs related to the vitrification Day 3 group were higher by 3.6% per patient. From a healthcare payer perspective, the costs were higher by 1.3% per patient. From a patient perspective, the costs were higher by 24.0% per patient. From a societal perspective, the vitrification Day 3 group incurred costs that were higher by 7.9% per patient compared to the vitrification Day 5/6 group. Furthermore, Table 6 also displays the average and median total cost per pregnancy for both the vitrification Day 3 and Day 5/6 groups. Out of the 25 and 26 patients in the vitrification Day 3 and Day 5/6 group respectively, 17 and 16 achieved pregnancy, respectively. The average and median total cost per pregnancy was found to be higher for the vitrification Day 3 group compared to the vitrification Day 5/6 group for all four perspectives. However, potentially because of the low sample size, the results of the Mann–Whitney *U* tests indicated no statistically significant difference between the costs of the two groups for any of the perspectives, neither for the average total cost per patient nor for the average total cost per pregnancy.

Discussion

Extended embryo culture and blastocyst-stage transfer are well adapted in fertility clinics worldwide but it remains unclear whether this approach improves the cumulative pregnancy rate. Cumulative pregnancy rates provide a more realistic assessment of success rates by taking into account transfers of all fresh and frozen embryos. In our study cumulative pregnancy rates were similar in the vitrification Day 3 group (57.3%) and the vitrification Day 5/6 group (57.8%) after eight transfers if supernumerary embryos were vitrified on Day 5/6 instead of Day 3 after a fresh cleavage-stage transfer was performed on Day 3. The strength of this study is that it is the first powered RCT reporting cumulative pregnancy rates as a primary outcome and using vitrification as the method for cryopreservation. Previous studies by Rienzi *et al.* (2002) and Emiliani *et al.* (2003) reported cumulative pregnancy rates but used the slow freezing method for cryopreservation, although these studies were published 20 years ago and improvements in culture conditions (incubators, culture media) have been made in the meantime. A study of Fernandez-Shaw *et al.* (2015) reporting cumulative pregnancy rate was the only study to use vitrification for cryopreservation. However, none of these RCTs were powered to prove a difference in cumulative pregnancy rate between cleavage- and blastocyst-stage transfers.

Our finding is different from Fernandez-Shaw *et al.* (2015) who found a higher cumulative pregnancy rate in the Day 5 group, yet the difference with the Day 3 group was not significant. Although

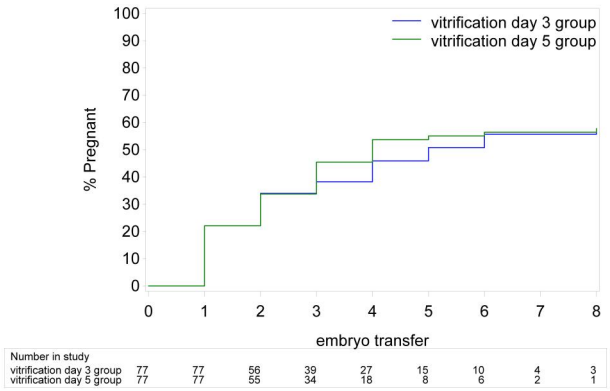

Figure 2. Cumulative ongoing pregnancy curve by group. Time-to-event analysis with ongoing pregnancy as the event of interest (Y-axis) and the number of cycles with embryo transfer as the time component (X-axis).

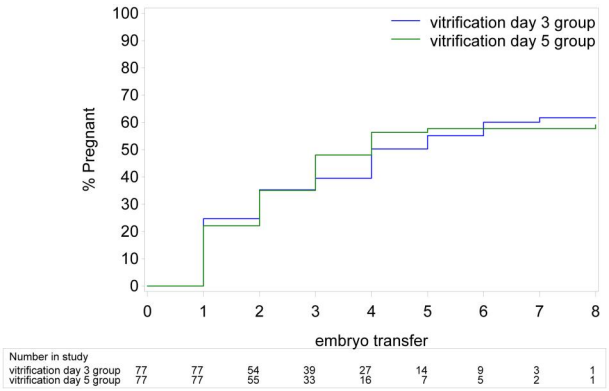

Figure 3. Cumulative clinical pregnancy curve by group. Time-to-event analysis with clinical pregnancy with foetal heart rate as the event of interest (Y-axis) and the number of cycles with embryo transfer as the time component (X-axis).

Table 4. Cumulative clinical pregnancy rates by group + HR.

	% pregnancy (95% CI)			
Embryo transfer	**Vitrification d3 group**	**Vitrification d5 group**	**Hazard ratio (95% CI)**	***P*-value**
1	24.68 (15.73, 34.69)	22.08 (13.61, 31.85)	1.190 (0.783, 1.809)	0.4145
2	35.26 (24.78, 45.89)	35.06 (24.64, 45.65)		.
3	39.53 (28.54, 50.31)	48.05 (36.56, 58.63)		.
4	50.24 (38.23, 61.10)	56.33 (44.47, 66.60)		.
5	55.16 (42.81, 65.89)	57.71 (45.83, 67.89)		.
6	60.08 (47.57, 70.50)	57.71 (45.83, 67.89)		.
7	61.72 (49.20, 72.01)	57.71 (45.83, 67.89)		.
8	61.72 (49.20, 72.01)	59.09 (45.83, 67.89)		.

HR, hazard ratio. HR and *P*-value from Cox proportional hazard model. HR>1 indicates higher pregnancy rate for the vitrification d5 group. Median nr of embryo transfers: vitrification d3 group 3 = 4 and virification d5 group = 4.

Table 5. Overview of resource use and cost implications for the vitrification Day 3 and Day 5 groups.

			Vitrification d3 group		Vitrification d5 group	
			Mean (median) resource use per patient	Mean (median) cost per patient (€)	Mean (median) resource use per patient	Mean (median) cost per patient (€)
Stimulation Fresh						
	Medical technical performance					
		Ultrasound	6 (5)	149 (132)	6 (5)	145 (119)
		Supplement	3 (2)	40 (32)	3 (2)	45 (32)
		Consultation	6 (5)	135 (127)	5 (4)	141 (105)
		Other	1 (1)	67 (67)	1 (1)	35 (35)
	Clinical biology laboratory					
		Hormonal assessment	21 (9)	66 (64)	22 (17)	67 (52)
		Other	1 (1)	2 (2)	2 (1)	3 (2)
FET						
	Medical technical performance					
		Ultrasound	8 (7)	210 (185)	4 (4)	113 (106)
		Supplement	3 (2)	43 (32)	3 (2)	51 (32)
		Consultation	8 (8)	198 (178)	5 (4)	119 (107)
		Other	2 (2)	122 (122)	2 (2)	24 (24)
	Clinical biology laboratory					
		Hormonal assessment	31 (25)	95 (76)	19 (18)	58 (55)
		Other	3 (3)	6 (6)	/	/
OR						
	Medical technical performance					
		Anaesthesia	4 (3)	134 (104)	4 (3)	141 (105)
		OR	1 (1)	309 (236)	1 (1)	318 (237)
		Supplement	1 (1)	26 (15)	1 (1)	19 (15)
		Other	1 (1)	0 (0)	1 (1)	0 (0)
	Fertility laboratory fee					
		Covered by RIZIV	1 (1)	2.085 (1.589)	1 (1)	2.136 (1.602)
		Not covered	2 (2)	133 (165)	1 (1)	105 (85)
Follow-up/PID						
	Medical technical performance					
		Ultrasound	2 (2)	53 (53)	/	/
		Supplement	5 (5)	75 (75)	/	/
		Consultation	2 (2)	53 (53)	/	/
		Other	2 (2)	0 (0)	/	/
	Clinical biology laboratory					
		Hormonal assessment	12 (12)	40 (40)	/	/
		Other	96 (96)	58 (58)	/	/
Follow-up / OHSS						
	Medical technical performance					
		Ultrasound	1 (1)	31 (26)	1 (1)	33 (26)
		Supplement	2 (2)	20 (20)	/	/
		Consultation	2 (2)	36 (27)	1 (1)	33 (27)
	Clinical biology laboratory					
		Hormonal assessment	3 (2)	11 (7)	3 (2)	11 (8)
		Other	24 (16)	14 (9)	16 (16)	9 (10)
Embryo transfer Fresh						
	Medical technical performance					
		Ultrasound	1 (1)	34 (26)	1 (1)	36 (26)
		Embryo transfer	1 (1)	215 (169)	1 (1)	228 (169)
		Supplement	2 (2)	108 (100)	2 (2)	109 (100)
		Consultation	1 (1)	32 (27)	1 (1)	34 (26)
FET						
	Medical technical performance					
		Ultrasound	3 (2)	81 (53)	2 (2)	51 (40)
		Embryo transfer	3 (2)	519 (343)	2 (2)	330 (254)
		Supplement	3 (2)	161 (100)	3 (2)	129 (100)
		Consultation	3 (2)	82 (77)	2 (2)	51 (40)
	Clinical biology laboratory					
		Hormonal assessment	3 (3)	10 (10)	/	/

OHSS, ovarian hyperstimulation syndrome; OR, oocyte retrieval; PID, pelvic inflammatory disease; RIZIV, rijksinstituut voor Ziekte- en Invaliditeitsverzekering. In the clinical biology laboratory, the 'Other' category encompasses tasks such as setting up aerobic cultures and conducting operations following blood collection. Under medical technical performance, the 'Other' category includes procedures like placing an intravenous line for medication administration, performing punctures/biopsies, and providing paramedical care

this is the only study using vitrification, it could be argued that it is not a valid comparison to our study for several reasons: (i) fresh embryo transfer in our study was performed on Day 3 in both study groups whereas Fernandez-Shaw performed fresh transfer on Day 3 or Day 5 according to the study group, (ii) their interim analysis reached a statistical power of 65%, and (iii)

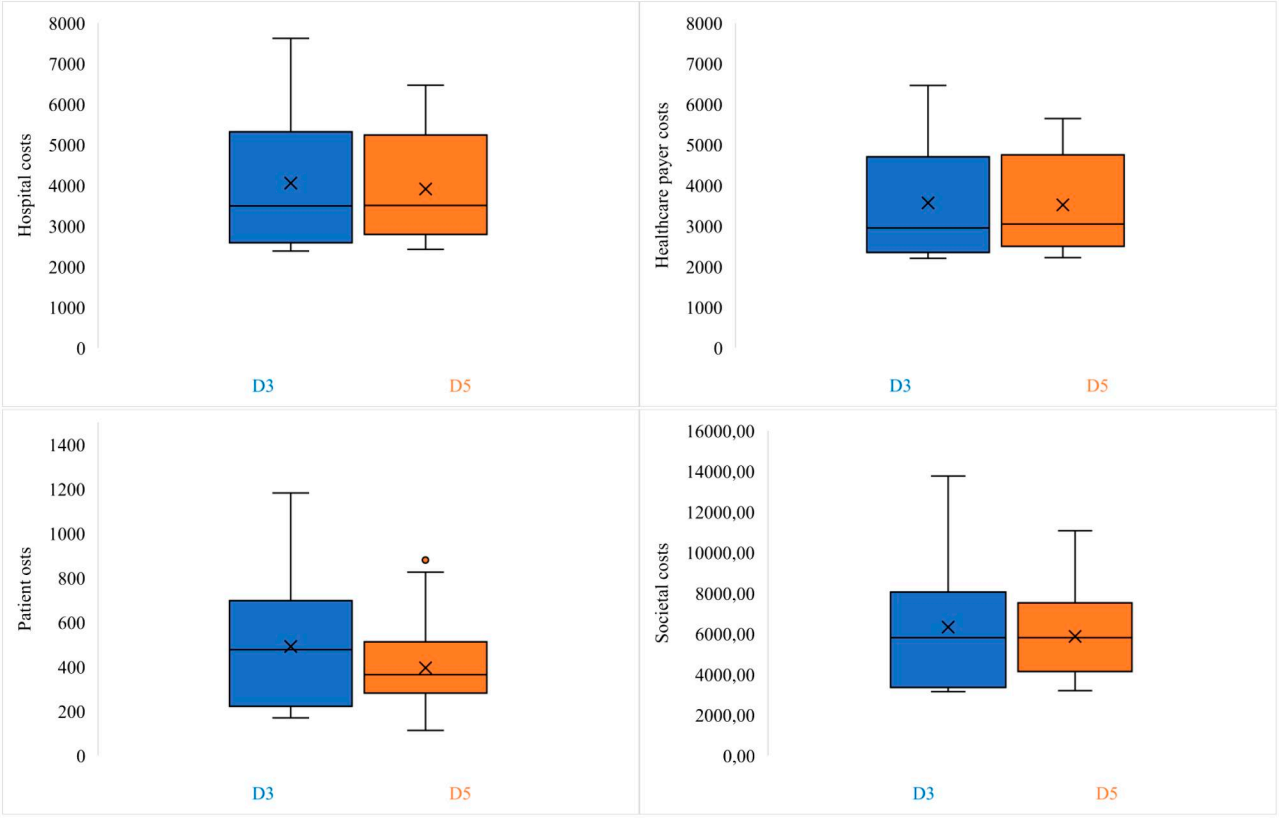

Figure 4. Boxplot of cost implications for the Day 3 vs Day 5 vitrification groups. The median and mean cost per patient are shown by the line and the cross inside the boxplot respectively. The upper and lower quartiles are indicated by the edges of the boxplot and the range of the data is indicated by the whiskers.

patients were randomized on Day 1 thereby only selecting patients with more than 4 zygotes. The same applies to studies using the slow freezing method: patients expected to do well with blastocyst cultures were selected based on the number of oocytes, fertilized oocytes, and/or top quality embryos. Studies using the slow freezing method report a benefit for cleavage-stage transfer (Rienzi *et al.*, 2002; Emiliani *et al.*, 2003). One of the strengths of our RCT is the study design that patients were randomized prior to the start of the cycle, irrespective of the number of oocytes, fertilized oocytes, or good-quality embryos on Day 3.

The time-to-event analysis showed that the median number of transfers until ongoing pregnancy was 5 vs 4 in the vitrification Day 3 and Day 5/6 group respectively. Although not significantly different between both groups, the Day 5/6 strategy seems to be more effective. The utilization rate on the other hand, presenting information about the comparative number of pregnancy opportunities that each treatment provides, was significantly higher in the vitrification Day 3 group than in the vitrification Day 5/6 group. This can be explained by the process of self-selection: only the most viable embryos will develop into blastocysts. Although the number of transfers needed to obtain ongoing pregnancy is not significantly different if supernumerary embryos are vitrified on Day 5/6 instead of Day 3, more pregnancy opportunities are available if supernumerary embryos are vitrified on Day 3. In Belgium where the laboratory costs for six fresh ART cycles and consecutive FET cycles are reimbursed (Belgisch Staatsblad, 2003), the Day 3 approach might be a better strategy from the patient perspective whereas the Day 5/6 approach might be more interesting from healthcare payer perspective. This also applies to most European countries since 39 out of 43 countries performing ART provide public funding (Calhaz-Jorge *et al.*, 2020). On the other hand, we should take into account that physical and emotional health of the patient might be more compromised the longer the journey takes, which can be an argument in favour of extended embryo cultures.

From the perspective of private clinics, it is important to reduce the financial burden for the patient by focusing on getting the best results from one single OR. The Day 5/6 approach might be the preferred strategy in this case. Cost-effectiveness analyses are needed to draw complete conclusions on this part.

The OPR after the first fresh embryo transfer was not significantly different between the vitrification Day 3 group and the vitrification Day 5/6 group which makes sense since the fresh transfer was performed on Day 3 in both study groups. A logistic regression model was used accounting for data clustering on the patient level. The OPR after FET was significantly higher in the vitrification Day 5/6 group (24.8%) compared to the vitrification Day 3 group (13.9%) even though significantly more DET was performed in the vitrification Day 3 group (19.7%) than the vitrification Day 5/6 group (8%). Fernandez-Shaw (2015) showed similar OPRs after FET on Day 3 and Day 5 but their results were calculated per patient making comparison with our study irrelevant. Our results were calculated accounting for data clustering on patient and cycle level.

According to the Cochrane systematic review, the failure rate to transfer any embryo leading to cycle cancellation was significantly higher in the blastocyst-stage group. Our study could overcome this harm by performing the fresh transfer on Day 3 in the two study groups. As expected no difference was found in the fresh embryo transfer rate (87.3% in the vitrification Day 3 group vs 81.5% in the vitrification Day 5/6 group). Also, frozen transfer rates were similar in both groups (97.9% in the vitrification Day 3

Table 6. Overview of cost implications for different perspectives for the vitrification Day 3 and Day 5 groups.

	Vitrification d3 group (€)	Vitrification d5 group (€)	Statistical significance*
Hospital perspective			
Average total cost per patient (median)	4.056 (3.498)	3.915 (3.509)	0.96
Average total cost per pregnancy	3.874 (3.142)	3.614 (2.910)	0.99
Healthcare payer perspective			
Average total cost per patient (median)	3.566 (2.951)	3.519 (3.049)	0.99
Average total cost per pregnancy	3.438 (2.773)	3.258 (2.589)	1.00
Patient perspective			
Average total cost per patient (median)	491 (477)	395 (364)	0.34
Average total cost per pregnancy	436 (315)	356 (295)	0.66
Societal perspective			
Average total cost per patient (median)	6.329 (5.802)	5.864 (5.813)	0.73
Average total cost per pregnancy	5.907 (4.678)	5.294 (4.446)	0.87

* Based on Mann–Whitney *U* test comparing the rank sums between two independent samples.

group vs 98.3% in the vitrification Day 5/6 group) although the embryo survival rate was significantly higher in the vitrification Day 3 group (97.1%) than in the vitrification Day 5/6 group (88.4%). A reduced blastocyst survival rate was also reported by studies using the slow-freezing method. Vitrification has been reported to have similar survival rates for Day 3 and Day 5/6 embryos (Cobo *et al.*, 2012). This finding could not be confirmed by our study.

Another consequence of extended embryo culture is that it increases the complexity of an IVF cycle translated into additional time requirements for proper and safe completion of laboratory tasks (Alikani *et al.*, 2014). The increased number of procedures impacts the workload at the fertility lab with additional scoring on Days 5 and 6, artificial collapse of expanded blastocysts before vitrification (Van Landuyt *et al.*, 2015), and renewal dishes and medium on Day 3. Therefore additional staff and equipment might be required when changing the embryo transfer/vitrification policy to Day 5/6 (Alikani *et al.*, 2014). However, fewer blastocysts need to be vitrified and fewer blastocyst FET cycles need to be scheduled. This should also be taken into account when determining the optimal strategy.

The cost analysis aimed to comprehensively evaluate the medical interventions and resources used per patient in the vitrification Day 3 or Day 5/6 group, but due to the limited sample size, our findings remain explorative. While we observed a higher cost for the vitrification Day 3 group, consistent with the study by Clua *et al.* (2022), we did not find statistically significant differences between the two groups. The inclusion criteria were limited to patients who received their entire treatment at UZ Leuven and had complete data, which substantially reduced the sample size compared to the RCT. We also excluded foreign patients, as their proportion of patient shares was substantial as they did not receive reimbursement from public health insurance (RIZIV). Consequently, the sample size and number of patients in the economic analysis were considerably smaller than in the RCT, which may have reduced the power of statistical tests and therefore complicated our ability to detect statistically significant differences. The presence of outliers in our cost analysis suggests that factors such as the need for multiple embryo transfers or additional follow-up procedures for OHSS may have contributed to variations in costs among patients. Moreover, our cost analysis only considered clinic costs and did not account for broader hospital overhead costs. Future research with a larger sample size is needed to confirm the findings of our study regarding the differences in costs between the vitrification Day 3 and Day 5/6 groups.

Last but not least, although literature shows conflicting results on preterm birth (PTB) and large-for-gestational-age (LGA) babies after blastocyst transfer, perinatal outcomes should be accounted for when determining the optimal strategy. Studies by Maheshwari *et al.* (2013), Wang *et al.* (2017), and Alviggi *et al.* (2018) suggest blastocyst transfers are associated with higher risks of PTB and LGA babies. These results could not be confirmed by Litzky *et al.* (2018), Marconi *et al.* (2019), and Shi *et al.* (2019). A systematic review of Marconi *et al.* (2022) concluded that blastocyst-stage embryo transfer is associated with a higher risk of LGA and an increased risk of PTB, although the quality of evidence ranged from low to very low.

The first limitation of our study is that overall pregnancy rates are low considering the young patient population. Also, the lower survival rate in the vitrification Day 5/6 group vs Day 3 embryos may negatively affect the results of our study. According to the Vienna consensus meeting on the development of ART laboratory performance indicators (2017), the blastocyst cryosurvival rate should be ≥90% (competency value) whereas the blastocyst cryosurvival rate in our study was 88%.

Secondly, although in line with expectations, approximately 30% of eligible patients refused to participate. This can be explained by the fact that our study requires an extensive explanation to the patient and a well-informed consideration since patients stay in the study for two subsequent fresh cycles. This could impact the generalisability of the study.

Thirdly, performing a fresh transfer on Day 3 in both groups was a thoughtful decision to reduce the embryo transfer cancellation rate, but performing a fresh embryo transfer on Day 3 or Day 5/6 according to the study group would complete the picture.

Last, the fertility lab was relocated mid-trial to a cleanroom in another building during the study. Theoretically culture conditions (low oxygen, incubator with individual chambers) and air quality in the lab remained unchanged. The effect of relocation was assessed by an interaction effect between group and randomization before or after relocation. No evidence was found for a differential treatment effect according to relocation ($P = 0.12803$).

In conclusion, cumulative clinical and OPRs were similar whether supernumerary embryos were vitrified on Day 5/6 or on Day 3. Our cost analysis indicated non-significantly higher costs for the vitrification Day 3 group compared to the vitrification Day 5/6 group; however, the small sample size limits the certainty of the results.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

Study design: A.M., C.S., A.L., S.D., J.L. Acquisition of data: A.M., S.D. Statistical Analysis: A.L. Clinical Analysis: A.M., S.D. Health economic analysis: A.V.M., J.L. Writing of the manuscript: A.M., A.V.M. Interpretation of data, critical review of manuscript: A.M., C.S., A.L., S.D., A.V.M., J.L., K.P.

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Conflict of interest

The authors declare no conflict of interest related to this study.

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