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ORIGINAL ARTICLE *Infertility*

Frozen–thawed embryo transfer in a natural or mildly hormonally stimulated cycle in women with regular ovulatory cycles: a RCT

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STUDY QUESTION: Can ovarian stimulation with low dose hMG improve the implantation rate (IR) per frozen–thawed embryo transferred (FET) when compared with natural cycle in an FET programme in women with a regular ovulatory cycle?

SUMMARY ANSWER: Both IR and live birth rate (LBR) per FET were similar in the group with mild ovarian stimulation and the natural cycle group.

WHAT IS KNOWN ALREADY: Different cycle regimens for endometrial preparation are used prior to FET: spontaneous ovulatory cycles, cycles with artificial endometrial preparation using estrogen and progesterone hormones, and cycles stimulated with gonadotrophins or clomiphene citrate. At present, it is not clear which regimen results in the highest IR or LBR. More specifically, there are no RCTs in ovulatory women comparing reproductive outcome after FET during a natural cycle and during a hormonally stimulated cycle.

STUDY DESIGN, SIZE, DURATION: A total of 410 women scheduled for FET during 579 cycles (December 2003–September 2013) were enrolled in an open-label RCT to natural cycle (NC FET group, $n = 291$) or to a cycle hormonally stimulated with s.c. gonadotrophins (hMG FET group, 37.5–75 IU per day, $n = 288$). A total of 672 embryos were transferred during 434 cycles (332 embryos and 213 cycles in the NC FET group; 340 embryos and 221 cycles in the hMG FET group). Assuming a = 0.05 and 80% power, it was calculated that 219 frozen–thawed embryos were required for transfer in each group to demonstrate a difference of 10% in IR.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Women were eligible according to the following inclusion criteria: regular ovulatory cycle, female age ≥21 years and ≤45 years, informed consent. FET cycles with preimplantation genetic screening were excluded. The primary outcome was IR per embryo transferred. Secondary outcomes included IR with fetal heart beat (FHB), LBR per embryo transferred and endometrial thickness on the day of hCG administration. Statistical analysis was by intention to treat and controlled for the presence of multiple measures, as eligible women could be randomized in more than one cycle. Chi-square and independent *t*-test were used to compare categorical and continuous variables. The relative risk (RR) was estimated using a Poisson model with log link. Hierarchical models with random intercepts for patient and cycle were considered to account for clustering of cycles within patients and of embryos within cycles.

MAIN RESULTS AND THE ROLE OF CHANCE: The primary outcome, IR per embryo transferred, was not statistically different between the NC FET group (41/332 (12.35%)) and in the hMG FET group (55/340 (16.18%)) (RR 1.3 (95% confidence interval (CI) 0.9–2.0), $P = 0.19$). Similarly, the secondary outcome, IR with FHB per embryo transferred, was 34/332 (10.24%) in the NC FET group and 48/340 (14.12%) in the hMG FET group (RR 1.4 (95% CI 0.9–2.1), $P = 0.15$). The LBR per embryo transferred was 32/332 (9.64%) in the NC FET group and 45/340 (13.24%) in the hMG FET group (RR 1.4 (95% CI 0.9–2.2), $P = 0.17$). Endometrial thickness was also similar in both groups [8.9 (95% CI 8.7–9.1) in the NC FET group and 8.9 (95% CI 8.7–9.1) in the hMG FET group]. The duration of the follicular phase was significantly shorter ($P < 0.001$) in the hMG FET group [13.7 days (95% CI 13.2–14.2)] than in the NC FET group [15.4 days (95% CI 14.8–15.9)].

LIMITATIONS, REASONS FOR CAUTION: Randomization of cycles instead of patients; open-label design; relatively long period of recruitment.

WIDER IMPLICATIONS OF THE FINDINGS: Our observation that the IR per embryo transferred is not significantly increased after FET during natural or gonadotrophin stimulated cycle, suggests that the effect of mild hormonal stimulation with gonadotrophins is smaller than what was considered clinically relevant with respect to reproductive outcome after FET. These data suggest that endometrial receptivity is not relevantly improved, but also not impaired after hormonal stimulation with gonadotrophins. Since FET during a natural cycle is cheaper and more patient-friendly, we recommend this regimen as the treatment of choice for women with regular cycles undergoing FET.

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Key words: frozen–thawed embryo transfer / natural cycle / HMG / RCT / ART

Introduction

Cryopreservation of human embryos was first described in 1983 (Trounson and Mohr, 1983). Ever since, frozen–thawed embryo transfer (FET) has been widely used to increase the cumulative pregnancy rate per IVF-cycle allowing additional chances of pregnancy without a subsequent oocyte retrieval procedure. Embryo cryopreservation allows us to perform single embryo transfer after an oocyte aspiration cycle, avoiding multiple gestations (De Neubourg *et al.*, 2014), and is a strategy to prevent ovarian hyperstimulation syndrome or to delay embryo transfer if endometrial preparation is not optimal (D'Angelo and Amso, 2002; El-Toukhy *et al.*, 2008; Gera *et al.*, 2010).

In Belgium, laboratory costs are reimbursed during six fresh assisted reproductive technology (ART) cycles for female patients younger than 43 years with a Belgian insurance number since 2003 (Belgisch Staatsblad, 2003), on the condition that only a limited number of embryos can be transferred depending on female age and cycle rank, as described before (Debrock *et al.*, 2005; De Neubourg *et al.*, 2013). According to Belgian law, supernumerary frozen embryos need to be thawed and used before a new oocyte aspiration cycle with IVF is allowed to create new embryos (Belgisch Staatblad, 2007). This has led to a drastic increase in FET cycles in our centre (436 in 2006, 745 in 2009 and 832 in 2012) and in all Belgian centres (6020 in 2006, 8878 in 2009 and 9939 in 2012) (http://www.belrap.be; De Neubourg *et al.*, 2013).

In most ART registries, pregnancy rates after FET are reported to be lower than following fresh embryo transfer (De Mouzon *et al.*, 2009; De Neubourg *et al.*, 2013; Kupka *et al.*, 2014; registration data from the Society for Assisted Reproductive Technology: http://www.cdc.gov/mmwr/). This can be explained because firstly the best embryos are usually selected for embryo transfer in the fresh ART cycle, and secondly embryo freezing and thawing is associated with ice crystal formation which can reduce embryo quality (Mandelbaum *et al.*, 1988; Check *et al.*, 2001; Wang *et al.*, 2001; Ashrafi *et al.*, 2011; Veleva *et al.*, 2013; Eftekhar *et al.*, 2014). However, the recently established method of embryo vitrification, associated with 90–100% embryo survival rates after warming and an increased live birth rate (LBR) per frozen/thawed embryo when compared with slow freezing (Balaban *et al.*, 2008; Wilding *et al.*, 2010; Fasano *et al.*, 2014) has allowed us to maximize the probability of live birth within six reimbursed egg aspiration cycles.

Embryo implantation is the most critical step of ART and relies on three important parameters: embryo quality, endometrial receptivity and optimal synchronization between endometrial growth and embryonic development (Achache and Revel, 2006). Different cycle regimens are used worldwide in order to create the optimal conditions for implantation. However, until recently there was still no evidence that one cycle regimen is superior to others (Ghobara and Vandekerckhove, 2008; Glujovsky *et al.*, 2010; Groenewoud *et al.*, 2013). The options range from natural cycle FET, over ovarian stimulation, to artificial preparation of the endometrium (hormonal substitution) with estrogens and progesterone. Indeed, in the latest Cochrane review (Ghobara and Vandekerckhove, 2008), based on seven RCTs comparing different cycle regimens for FET, it was concluded that no regimen was superior to another regimen.

It is controversial whether ovarian stimulation during the follicular phase may benefit or reduce embryo implantation during the subsequent luteal phase, when compared with a natural cycle environment. Obviously, such comparisons are only possible in women with a regular ovulatory cycle. It has been hypothesized that ovarian stimulation may improve certain defects in the follicular and subsequent luteal phase, resulting in a better endometrial preparation for embryo implantation (Van der Auwera *et al.*, 1994; Levi *et al.*, 2001). On the other hand, other data suggest that ovarian stimulation can reduce endometrial quality and lead to reduced embryo implantation (Bourgain and Devroey, 2003; Kolibianakis *et al.*, 2003; Andersen and Ezcurra, 2014; Ezoe *et al.*, 2014). In fact, observational data in our centre (February 2002–August 2003) suggested a 10% higher implantation rate (IR) per embryo after FET during a cycle with ovarian stimulation (18%) than after FET during a natural cycle (9%). However, in several prospective cohort studies comparing natural cycles and stimulated cycles in FET cycles, no significant differences in reproductive outcome were found between both

groups (Testart et al, 1987; Mandelbaum et al., 1988; Dor et al., 1991; Imthurn et al, 1996; Tanos et al., 1996; Konc et al., 2010).

Since, to the best of our knowledge, no RCTs have been published comparing reproductive outcome after FET cycles during natural cycles and cycles mildly stimulated with gonadotrophins, we tested the hypothesis that, in ovulatory women, the IR per frozen–thawed embryo transferred is 10% higher during a cycle stimulated with gonadotrophins than during a natural cycle.

Materials and Methods

Patients

Between December 2003 and September 2013 we performed an open-label prospective RCT at the Leuven University Fertility Centre (LUFC), Department of Obstetrics and Gynecology at the University Hospital Gasthuisberg (Leuven, Belgium). The study protocol was approved by the Institutional Review Board of the Ethical Committee of the University Hospitals Leuven (ML2436). Since trial registration was not mandatory at the start of the study in 2003, registration was done later in 2007 at ClinicalTrials.gov (NCT00492934).

Women undergoing FET were eligible for the study when they had a regular cycle (between 21 and 35 days) and were 21–45 years old. Furthermore, a written informed consent was required before randomization. The exclusion criterion was a FET after PGD.

All of the couples received a complete infertility evaluation before starting ART treatment. This included a medical history, physical examination, serum hormone assays (FSH, LH, 17-beta-estradiol) on Day 2–5 of the menstrual cycle, luteal phase determination of serum progesterone concentration, pelvic ultrasound, hysteroscopy, endometrial biopsy, genetic analysis and semen analysis. Tubal patency was documented by either hysterosalpingography or methylene blue tubal testing during laparoscopy.

Ovarian stimulation, oocyte aspiration, embryo culture, cryopreservation and thawing/warming procedures

In the fresh ART cycles, ovarian stimulation, oocyte aspiration, IVF, embryo culture and embryo transfer was performed as described before (Debrock et al., 2010, 2011). Briefly, ovarian stimulation was carried out with gonadotrophins (Menopur, Ferring, Copenhagen, Denmark; Gonal-F or Metrodin HP, Merck-Serono, Geneva, Switzerland; Puregon, Organon, Oss, The Netherlands) and GnRH agonists (GnRHa) (Busereline acetate, Suprefact®, Hoechst, Frankfurt, Germany or Triptorelin, Decapeptyl®, Ispen, Signes, France) during a long or short protocol. The follicular response was monitored by regular gynaecological ultrasound measurements and peripheral blood measurements for estradiol. A subcutaneous injection of HCG (10.000 IU; Pregnyl, Organon, Oss, The Netherlands) was given when at least three follicles had reached a follicular diameter of 17 mm. Ultrasound guided oocyte retrieval was carried out 35 h after hCG injection. Luteal supplementation was given by intravaginal application of progesterone (600 mg/day, Utrogestan®, Besins, Drogenbos, Belgium) started at the evening of the HCG injection. Supernumerary embryos were cryopreserved by slow freezing procedure or vitrification as described before (Debrock et al., 2011) if their quality was sufficient (2 pronuclei zygotes on Day 1; embryos containing ≥2 cells on Day 2 with ≤25% fragmentation, no multinucleation and with symmetric or slightly asymmetric blastomeres; embryos containing ≥6 cells on Day 3 with ≤25% fragmentation, no multinucleation and with symmetric or slightly asymmetric blastomeres; on Day 5: embryos reaching the blastocyst stage (minimum expansion: early blastocyst, inner cell mass and trophectoderm layer: score A or B (Gardner and Schoolcraft, 1999)).

In FET cycles, the thawing/warming procedures were performed as described before (Debrock et al., 2011). Embryo survival was defined as the presence of ≥50% of cells intact immediately after thawing (Alpha Scientist in Reproductive Medicine, 2012). Blastocyst survival was based on the integrity of inner cell mass and trophectoderm cells. Degeneration or arrest development was characterized by darkening of cytoplasm or no re-expansion of the blastocoele 24 h after thawing. After thawing/warming, embryo quality was defined on the day of transfer as follows: on transfer day Day 2 and Day 3, embryo development was evaluated according to the number of blastomeres, the percentage of fragmentation and the symmetry of the blastomeres. On Day 4–6, embryo quality was evaluated based on compaction formation (including both embryos that initiated but not yet completed compaction, and embryos that formed a dense morula) and on blastocyst formation and expansion (Debrock et al., 2010). Good embryo quality was defined on the day of transfer as follows: on transfer day 2: >4 cells with ≤25% fragmentation, no multinucleation and with symmetric or slightly asymmetric blastomeres; on transfer day 3: ≥7 cells with ≤10% fragmentation, no multinucleation and with symmetric to slightly asymmetric blastomeres; on transfer day 4: morula or more; on transfer day 5: blastocyst ranging between expanding blastocysts and hatching blastocysts with blastocoele formation in >50% of the embryo and a good inner cell mass (score A) and a trophectoderm layer (score A) (Gardner and Schoolcraft, 1999).

Study design

We randomized eligible couples at the level of the treatment cycle. Study participants were randomized for FET in either natural cycle (NC FET group) or in a cycle with mild ovarian stimulation using gonadotrophins (hMG) (hMG FET group) by an independent investigator after written informed consent was obtained. Treatment allocation for each participant was performed at the start of the FET cycle by opening opaque sealed envelopes only. We used blocked randomization per 10 envelopes, containing five in the NC FET group and five in the hMG FET group. A patient was allowed to participate more than once; in that case she was randomized again to one of both groups.

Patients of the hMG FET group started s.c. injections of gonadotrophins (Menopur, Ferring, Copenhagen, Denmark) on Day 2 of the menstrual cycle. The starting dose of gonadotrophins (37 or 75 IU) was determined by the treating clinician, based on patient's age, BMI, basal (Day 2–5) serum FSH and (if applicable) the response to previous ovarian stimulation. On Day 6 or 7 of the menstrual cycle a first ultrasound and serum hormonal analysis (17 Beta-estradiol, progesterone, LH, FSH) were performed. Based on these results the dose of gonadotrophins could be adjusted if needed. Patients of the NC FET group underwent a first pelvic ultrasound and blood analysis around Day 10–12 of the menstrual cycle. In both NC FET and hMG FET groups, the follicular response was monitored by regular vaginal ultrasound and serum hormonal analysis. HCG was administered when the leading follicle had a mean diameter of ≥17 mm and endometrial thickness ≥7 mm with serum estradiol levels preferably >150 ng/l. The day of ovulation was calculated 2 days after hCG administration in the absence of an LH surge and 1 day after hCG administration if an LH surge was observed on the day of HCG administration. Embryo transfer was cancelled when endometrial thickness was less than 5 mm.

The timing of the embryo transfer was based on the day of embryo freezing, and on the day of hCG injection. When a serum LH surge was detected, the scheduled embryo transfer was advanced by 1 day. Day 0 was defined as the estimated day of ovulation. From here, the date of embryo thawing and transfer was calculated in order to achieve synchronization between embryo and endometrium.

Embryo transfer was performed the same way in both groups. A Cook embryo replacement catheter (Cook, Sydney IVF Embryo Transfer Catheter, Brisbane, Australia) was used and embryo transfer was performed with

abdominal ultrasound guidance. One or more embryos were transferred into the middle of the uterine cavity according to the Belgian law (De Neubourg *et al.*, 2013). Afterwards, the embryo transfer catheter was checked in the lab to confirm that the embryo did not accidentally remain in the catheter. Luteal supplementation was given by intravaginal application of progesterone (600 mg/day, Utrogestan®, Besins, Drogenbos, Belgium) starting on the day after hCG injection. Although the use of luteal support is still controversial it is standard practice in our fertility centre, supported by the results of a retrospective study (Veleva *et al.*, 2013) and a RCT (Bjuresten *et al.*, 2011) both showing a higher LBR in natural FET cycles with luteal supplementation than in natural FET cycles without luteal supplementation, even though these results were not confirmed by two other studies (Kyrou *et al.*, 2010; Eftekhar *et al.*, 2013).

A blood test was performed 14–16 days after ovulation to check the hCG levels. Pregnancy was determined as positive serum hCG levels (≥25 IU/l). In case of pregnancy, progesterone was continued until 12 weeks of pregnancy. In case of a negative pregnancy test, progesterone administration was stopped. Live birth was defined as the live birth of a child beyond 24 weeks of gestation (Zegers-Hochschild *et al.*, 2009). Follow-up of pregnancies and deliveries was performed in our hospital and in other hospitals. There was no specific protocol for follow-up of pregnancies in our study design. All obstetrical data were reported according to the compulsory registration of IVF cycles to the Belgian Register for Assisted Procreation (BELRAP) and therefore available in our databank (http://www.belrap.be; De Neubourg *et al.*, 2013).

Outcome parameters

The primary outcome was IR per frozen–thawed embryo transferred (the presence of a gestational sac (intrauterine (IU) or extrauterine (EU)) on ultrasound at 6–8 weeks of gestation). Secondary outcomes included IR with fetal heart beat (FHB), LBR per frozen–thawed embryo transferred and endometrial thickness on the day of hCG administration. A sub-analysis was carried out including only FET cycles with embryos that had been cryopreserved on Day 3 and a second sub-analysis reported reproductive outcome according to different age groups (<36 years, 36–39 years, >40 years).

Additional outcome variables included: the clinical pregnancy rate (PR) (defined as the presence of a gestational sac, IU or EU, on ultrasound at 6–8 weeks of gestation), the ongoing clinical PR (defined as viable pregnancy with FHB at 12 weeks of gestational age) and LBR per embryo transfer cycle; the LBR per started FET cycle; the duration of the follicular phase and reasons for treatment discontinuation.

Statistical methods

Sample size calculation

The aim of the study was to test the hypothesis that ovarian stimulation with low dose gonadotrophins would increase the IR per FET when compared with natural cycle in an FET programme in women with a regular ovulatory cycle. We assumed that the IR per FET would be 10% higher in the hMG-FET group than in the NC-FET group. We based this assumption on observational data in our centre reflecting our clinical practice before the initiation of this study, showing a 9% higher IR per embryo transferred after FET during a cycle with ovarian stimulation (18%) than after FET during a natural cycle (9%). Assuming a = 0.05 and 80% power, it was calculated that 219 frozen–thawed embryos were required for transfer in each group to demonstrate a difference of 10% in IR per embryo transferred.

Statistical considerations

Both groups were compared with respect to baseline characteristics (Table I), as well as endometrial thickness, cycle duration, number of previous fresh ART cycles and type of freezing (slow freezing versus vitrification) (Tables I and Table II). Reasons for treatment discontinuation were also compared between both groups (Table III). Statistical analysis was done based on intention to treat, including also cycles that were cancelled due to treatment discontinuation, or cycles that were marked by violation of the study protocol (Fig. 1). Chi-square and independent t-test were used to compare categorical and continuous patient or clinical variables, respectively, between the hMG-FET and NC-FET groups. Since couples could participate more than once, the data showed clustering of cycles within couples, and since analysis was performed at the embryo level, the data additionally showed clustering of embryos within cycles. Therefore we applied statistical methods that took into account both levels of clustering, using a similar approach as published recently by our group (Peeraer *et al.*, 2015). The relative risk (RR) was estimated using a Poisson model with log link. Hierarchical models with random intercepts for patient and cycle were considered to account for clustering of cycles within patients and of embryos within cycles. All analyses have been performed using SAS software (version 9.3 of the SAS System for Windows; SAS Institute Inc., Cary, NC, USA). A value of $P < 0.05$ was considered significant.

Table I Baseline characteristics at cycle level (all randomized cycles).

Variable	Natural cycle (*N* = 291)	hMG (*N* = 288)
Patients randomized	235	237
Mean age of females (± SD)	33.0 ± 4.39	33.2 ± 4.23
Mean BMI (± SD)	24.0 ± 4.13	23.6 ± 3.74
Mean cycle duration (± SD)	28.9 ± 2.46	28.9 ± 3.04
Previous fresh cycles, Mean (Range)	1.6 (0–9)	1.4 (0–7)
Mean duration of infertility, Months (± SD)	37.0 ± 26.29	38.1 ± 29.38
Type of infertility, No. (%)		
Primary	138/291 (47%)	143/288 (50%)
Secondary	153/291 (53%)	145/288 (50%)
Indication for treatment, No. (%)		
Anovulation*	4/291 (2%)	3/288 (1%)
Endometriosis	30/291 (10%)	42/288 (15%)
Genetic	2/291 (1%)	4/288 (1%)
Implantation	2/291 (1%)	3/288 (1%)
Tubal factor	26/291 (9%)	24/288 (9%)
Male factor	147/291 (50%)	139/288 (48%)
Mixed	42/291 (14%)	43/288 (15%)
Unexplained	38/291 (13%)	30/288 (10%)
No transfer, No. (%)	78/291 (27%)	67/288 (23%)

All patients had regular menses at the start of the frozen–thawed cycle.
*Anovulation means that the patient had anovulatory cycles before IVF/ICSI treatment.

Results

Between December 2003 and September 2013, 410 couples were randomized who started 579 FET cycles; 291 cycles were allocated to the NC-FET group and 288 cycles were allocated to the hMG-FET group (Fig. 1). Most cycles were recruited between 2007 and 2010 (402/579 cycles); in the period before 2007 and after 2010 several other ART

Table II Characteristics of cycles with transfer—cycle level.

Variable	Natural cycle (*N* = 213)	hMG (*N* = 221)	*P*-value
Number of cycles according to method of freezing over number of randomized cycles with embryo transfer (%)			0.546
Slow freezing	170/213 (80%)	175/221 (79%)	
Vitrification	41/213 (19%)	41/221 (19%)	
Mixed	2/213 (1%)	5/221 (2%)	
Endometrial thickness at the time of HCG injection (mm): mean (95% CI)	8.9 (8.7–9.1)	8.9 (8.7–9.1)	0.974
Duration of follicular phase (days): mean (95% CI)	15.4 (14.8–15.9)	13.7 (13.2–14.2)	<0.001
Total number of embryos transferred,	332	340	
17-beta-estradiol at time of hCG injection (ng/l): mean (95% CI)	247 (230–264)	325 (308–341)	<0.001
Progesterone at time of hCG injection (μg/l): mean (95% CI)	0.7 (0.6–0.7)	0.6 (0.5–0.7)	0.251
LH at time of hCG injection (IU/l): mean (95% CI)	19 (16.5–20.5)	11 (9.1–13.0)	<0.001

The chi-square test was used for categorical variables, and the independent *t*-test was used for continuous variables.
CI, confidence interval.

Table III Reasons for treatment discontinuation after randomization.

Variable	Natural cycle (*N* = 291)	hMG (*N* = 288)	*P*-value
Number of cycles marked by treatment discontinuation over total number of randomized cycles (%)	78/291 (26.8%)	67/288 (23.3%)	0.326
Reasons of treatment discontinuation (%)			0.276
No embryo survival	69/78 (88.5%)	65/67 (97.0%)	
Insufficient ovarian follicular development	3/78 (3.9%)	0/67 (0.0%)	
Patient based non-medical reason	2/78 (2.6%)	0/67 (0.0%)	
Serum LH a/o progesterone rise	1/78 (1.3%)	0/67 (0.0%)	
Medical reason (ovarian cyst, illness, diagnosis of breast cancer)	2/78 (2.6%)	2/67 (3.0%)	
Spontaneous pregnancy	1/78 (1.3%)	0/67 (0.0%)	

The chi-square test was used to compare groups.

studies were conducted in our centre, which had an influence on the recruitment for this study. Both groups were comparable with respect to female age, BMI, cycle duration, type and duration of infertility, indication for IVF of ICSI, number of previous fresh ART cycles, and number of cycles without FET (Table I). The majority (289 couples) participated in one cycle, whereas 87 couples participated in 2 cycles, 26 couples participated in 3 cycles, 2 couples participated in 4 cycles and 6 couples participated in 5 cycles. No differences in cancellation rate and cancellation reasons per started FET cycle were observed between the NC-FET (78/291 or 26.8%) and the hMG-FET group (67/288 or 23.3%) ($P = 0.326$). More details regarding treatment discontinuation are described in Table III.

A total of 672 embryos were transferred in 317 patients (332 in the NC-FET group and 340 in the hMG-FET group) (Fig. 1 and Table II). Although serum estradiol levels were significantly higher ($P < 0.001$, Table II) in the hMG FET group (325 ng/l (range 308–341)) than in the NC FET group (247 ng/l (range 230–264)), endometrial thickness was comparable between both groups, and histograms with Gauss curves of the estradiol levels for both treatment groups demonstrated largely overlapping distributions (Supplementary Fig. S1). The type of cryopreservation used, slow freezing or vitrification, was comparable in both groups ($P = 0.546$) (Table II). Furthermore, the distribution of all embryos according to the day of transfer ($P > 0.999$) and the distribution of good quality embryos according to the day of embryo transfer ($P = 0.640$) were also comparable in both groups (Supplementary Table SI).

Our primary outcome, IR per embryo transferred, was not statistically different between groups [NC 41/332 (12.35%) versus hMG 55/340 (16.18%), RR 1.3 (95% confidence interval (CI) 0.9–2.0), $P = 0.19$)]. A similar result was found for the IR with FHB per embryo transferred [34/332 (10.24%) in the NC FET group and 48/340 (14.12%) in the hMG FET group (RR 1.4 (95% CI 0.9–2.1), $P = 0.15$)] and for the LBR per embryo transferred [32/332 (9.64%) in the NC FET group and 45/340 (13.24%) in the hMG FET group (RR 1.4 (95% CI 0.9–2.2), $P = 0.17$)] (Table IV). The same observations where made when we limited our analysis to cycles with only Day 3 frozen–thawed embryos transferred (Table IV) and when we compared the reproductive outcome in different age groups in accordance with the Belgian legislation (Belgisch Staatsblad, 2003; <36 years, 36–39 years, >40 years) (Supplementary Table SII). In order to

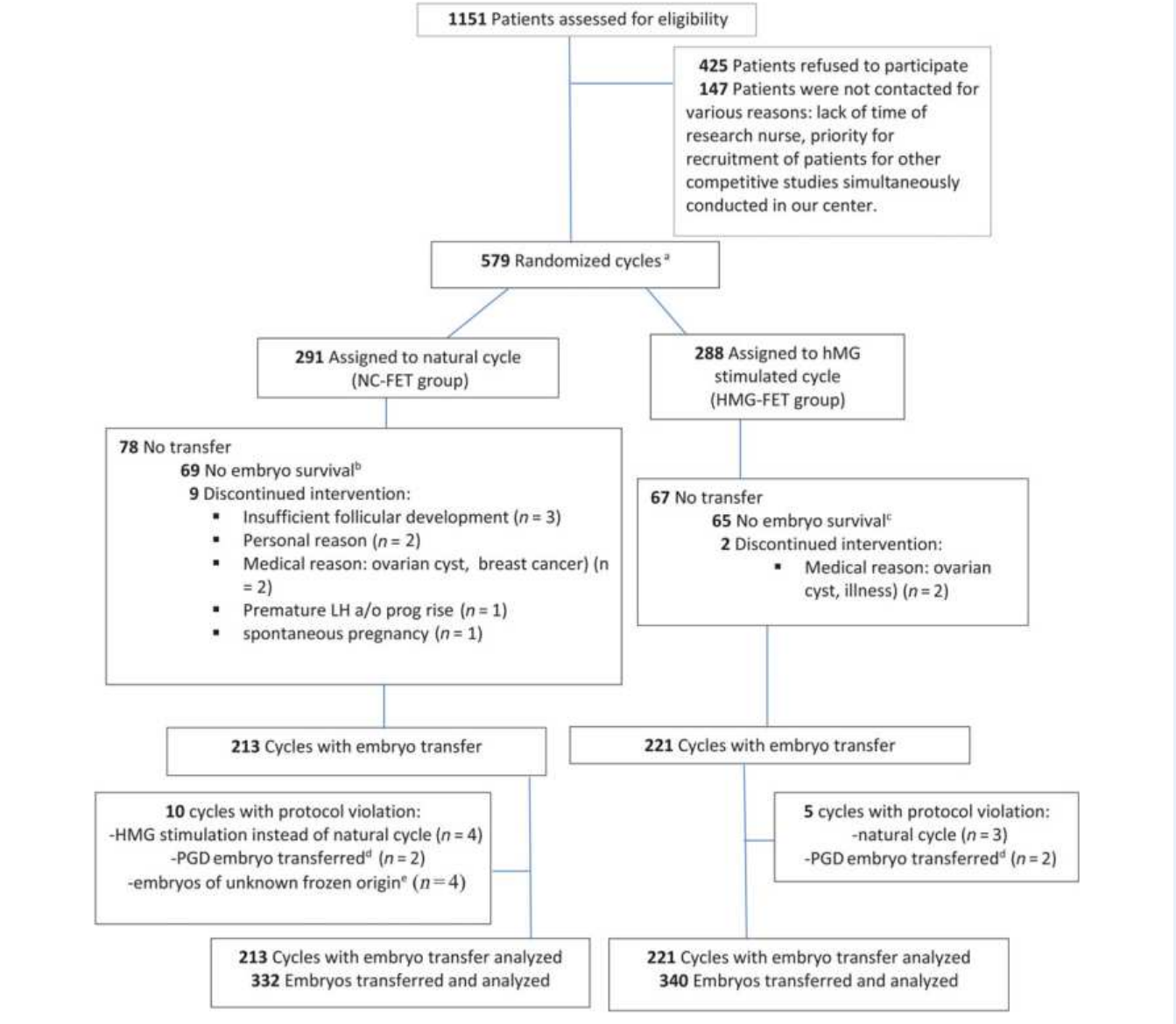

Figure 1 CONSORT diagram of screening, randomization, and follow-up of study participants. [a]Each patient was allowed to participate more than once in either one or both of the NC and hMG groups. [b]Included five cycles with protocol violation (stimulation taken ($n=1$), PGD embryo ($n=4$)). [c]Included two cycles with protocol violation (PGD embryo ($n=2$)). [d]Embryo with preimplantation genetic diagnosis. [e]Embryos of unknown frozen origin due to transport from another IVF centre to our centre.

incorporate cancellation rates due to 'no embryo survival', we also calculated the LBR per started frozen–thawed embryo transfer cycle and observed no difference (1.5 (95% CI 0.9–2.4), $P = 0.097$) between the NC (10.3%) and HMG (15.3%) group.

In view of the effort put into the extensive data collection, a multivariate analysis was carried out to find out whether the main results (broadly 4% absolute, 1.4 relative differences) moved away from or moved towards neutrality after adjusting for baseline covariates at individual level. After multivariate analysis of the treatment effect correcting for the most important clinical variables (age, type of infertility, duration of infertility, BMI, estradiol level, endometrial thickness, duration of follicular phase), results were very similar to the results of the univariate analysis with 1.4 relative differences (Supplementary Table SIII).

Endometrial thickness was also similar in both groups [8.9 (95% CI 8.7–9.2) in NC FET group and 8.9 (95% CI 8.7–9.1) in hMG FET group]. However, the duration of the follicular phase was significantly shorter ($P < 0.001$) in the hMG FET group [13.7 (95% CI 13.2–14.2) than in the NC FET group (15.4 days (95% CI 14.8–15.9)] (Table II).

Overall, no adverse events and hospitalizations were reported in any participant during the trial. In the obstetrical data outcome we observed four dichorial diamniotic twins (4/77(5%)): two in the NC group (2/32 (6%)) and two in the hMG group (2/45(4%)), and one monochorial diamniotic twin was found in the NC group (1/32 (3%)). Perinatal mortality was observed in one baby allocated to the hMG-FET group due to a neonatal sepsis. In newborns, congenital malformations were absent.

Table IV Reproductive outcome per embryo transferred and per embryo transfer.

	Natural cycle	hMG	Relative risk	*P*-value[a]
Reproductive outcome per embryo transferred				
Total *N* embryos transferred	*n* = 332	*n* = 340		
Implantation rate (IU + EU)[b]: % (95% CI)	12.4 (9.1–16.8)	16.2 (12.4–21.1)	1.3 (95% CI 0.9–2.0)	0.191
Implantation rate with FHB[c]: % (95% CI)	10.2 (7.3–14.3)	14.1 (10.6–18.7)	1.4 (95% CI 0.9–2.1)	0.153
Live birth rate: % (95% CI)	9.6 (6.8–13.6)	13.2 (10–17.7)	1.4 (95% CI 0.9–2.2)	0.171
Reproductive outcome per embryo transfer cycle	*n* = 213 cycles	*n* = 221		
Clinical pregnancy rate (IU + EU): % (95% CI)	17.4 (12.6–24.0)	23.5 (17.9–30.9)	1.4 (95% CI 0.9–2.1)	0.159
Clinical pregnancy rate with FHB[b]: % (95% CI)	14.6 (10.2–20.7)	20.8 (15.6–27.8)	1.4 (95% CI 0.9–2.3)	0.124
Live birth rate: % (95% CI)	14.1 (9.8–20.2)	19.9 (14.8–26.8)	1.4 (95% CI 0.9–2.3)	0.145
Reproductive outcome per embryo transferred on Day 3				
Total *N* embryos transferred after cryopreservation on Day 3	*n* = 287	*n* = 293		
Implantation rate (IU + EU)[b]: % (95% CI)	12.5 (9.0–17.4)	16.7 (12.6–22.1)	1.3 (95% CI 0.9–2.1)	0.191
Implantation rate with FHB[c] : % (95% CI)	10.1 (7.0–14.6)	15.0 (11.1–20.2)	1.5 (95% CI 0.9–2.4)	0.098
Live birth rate: % (95% CI)	9.8 (6.7–14.1)	14.0 (10.3–19.0)	1.4 (95% CI 0.9–2.3)	0.142

[a]The statistical analysis is performed using a hierarchical Poisson model with log link for estimating the relative risk, and accounting for repeated measurements per individual.
[b]Presence of a gestational sac intrauterine (IU) or extrauterine (EU).
[c]FHB: fetal heart beat.

Admission to the neonatal unit was observed in 12 babies (12/77 (16%), 6 babies from the hMG-FET group and 6 from the NC-FET group). These admissions were mostly related to preterm birth and difficult neonatal adaptation.

Discussion

In this RCT, we did not confirm our hypothesis that ovarian stimulation with low dose hMGs improves the IR per FET when compared with natural cycle in a FET programme in women with a regular ovulatory cycle. However, there was a trend towards a slightly higher IR (4% higher), clinical PR (4% higher) and LBR (4% higher) in the hMG-FET group.

A strength of our study is that it is applicable in daily clinical practice because recruitment at cycle level reflects real life clinical practice where often the type of endometrial preparation in FET cycles is selected individually on the cycle level, not on the patient level and is based on dynamic and shared decision making between doctors and patients. A potential bias is caused by the fact that each patient could participate in more than one cycle in the study in both treatment groups. In our design, repeated randomization after each cycle allowed couples to have both treatments, thus increasing the willingness to participate, as reported before (Peeraer *et al.*, 2015). The repeated randomization makes our study principally different from a crossover study, in which randomization determines the allocation in the first cycle, but then 'crosses over' to the other intervention, thus generating bias (Khan *et al.*, 1996). Repeated randomization prevented this type of bias. Furthermore, our statistical analysis accounts for patient participation in multiple cycles, no matter whether cycles of the same patient appeared in the same or different treatment groups. The clustered study design (multiple observations per couple) does not lead to a bias in the sense of an over-or underestimated treatment effect. A possible problem with clustered data might be related to the precision of the estimated effect. The precision could be over- or underestimated, depending on whether couples were randomized more likely within the same or different treatment groups. This could, respectively, lead to too narrow or too wide CIs for the treatment effect, and hence too liberal or too conservative *P*-values. However, statistical techniques to correct for such clustering effect are nowadays commonly used and were applied in this study (Aerts *et al.*, 2002). Therefore, we would like to argue that the results of our study are both unbiased and with correct precision estimates (Peeraer *et al.*, 2015). Indeed, our results regarding treatment effect based on univariate analysis were confirmed by analysis using a multivariable model correcting for the most important clinical variables, with relative differences remaining around 1.4 (Supplementary Table SIII).

A limitation of or study is its long duration. Since our study lasted 10 years, and protocols, techniques and reproductive outcome of treatment with medically assisted reproduction may vary over time, a *post hoc* analysis was performed to compare reproductive outcome during early (2003–2008) versus late (2009–2013) recruitment periods. Results were similar for clinical IR (12.6% early versus 16.6% late, RR 1.3 (95% CI 0.9–2.0), $P = 0.186$), clinical IR with positive FHB (10.8% early versus 14.1% late, RR 1.3 (95% CI 0.8–2.0), $P = 0.234$), and LBR per embryo transferred (10.1% early versus 13.4% late, 1.3 (95% CI 0.9–2.1), $P = 0.210$). An alternative to a single-centre RCT of long duration would have been to consider a multi-centre trial over a shorter period of time. However, a multi-centre trial would have introduced more heterogeneity and variability between centres regarding clinical and laboratory practices.

In a Cochrane review, it was concluded, based on seven RCTs comparing different cycle regimens for FET, that all regimens had similar reproductive outcome (Ghobara and Vandekerckhove, 2008). Conventional hormonal substitution with estrogen and progesterone (O+P) was compared with four different regimes: natural cycle, ovarian stimulation with FSH, ovarian stimulation with clomiphene citrate and hormonal substitution (O+P) with added GnRHa. Clomiphene citrate was also compared with hormonal substitution (O+P)

Table V Overview of studies comparing natural cycle versus hormonal stimulation with gonadotrophins in frozen–thawed embryo transfer cycles.

First author	Study design	No. of patients	No. of FET cycles with embryo transfer	Patient selection	Regimens used for endometrial preparation	Implantation rate per embryos transferred	Pregnancy rate per embryo transfer	Live birth rate	Mean number of embryos transferred	Conclusion
Testart *et al.* (1987)	Cohort study	NS	100	Not described	Natural cycle (NC) ($n = 62$)	NA	18/62 (29%)[a]	18%	1.2	No significant difference.
					hMG 75 IU day 6–8–10 ($n = 11$)	NA	5/11 (45.5%)[a]		1.5	
					Natural cycle + hCG ($n = 3$)	NA	0/3 (0%)[a]		1.3	
					hMG + hCG ($n = 24$)	NA	3/24 (12.5%)[a]		1.3	
Mandelbaum *et al.* (1988)	Cohort study	NS	249	Dysovulation in case of stimulated cycles	Natural cycle ($n = 172$)	NA	26/172 (15%)[c]	NA	NA	No significant difference.
					Hormonal stimulated cycles[b] ($n = 36$)	NA	10/36 (28%)[c]	NA		
					Artificial cycles ($n = 41$)	NA	7/41 (17%)[c]	NA		
Dor *et al.* (1991)	Cohort study	109	124	Ovulatory cycle only	Natural cycle ($n = 56$)	9/95 (9.5%)	9/56 (16.1%)[c]	7/56 (12.5%)	1.7	No significant difference.
					hMG 150 IU daily from Day 3 + hCG ($n = 44$)	5/76 (6.6%)	5/44 (11.4%)[c]	4/44 (9.1%)	1.7	
					Artificial cycle ($n = 42$)	4/74 (5.4%)	4/42 (9.5%)[c]	2/42 (4.8%)	1.8	
Imthurn *et al.* (1996)	Cohort study	123	24	Regular cycle (NC) versus anovulatory cycle (hMG)	NC + hCG ($n = 16$)	2/42 (4.8%)	2/16 (13%)[d]	NA	2.6	No significant difference.
					GnRHa + hMG daily + hCG ($n = 8$)	3/18 (16.7%)	3/8 (38%)[d]	NA	2.3	
Tanos *et al.* (1996)	Cohort study	236	381	Regular cycles (NC), irregular cycles (artificial cycle or stimulation)	Natural cycle ($n = 219$)	43/772 (5.6%)	37/219 (16.9%)[c]	NA	3.5	No significant difference.
					GnRHa + artificial cycle ($n = 85$)	17/306 (5.6%)	14/85 (16.5%)[c]	NA	3.6	
					GnRHa + hMG daily + hCG ($n = 77$)	12/260 (4.6%)	12/77 (15.6%)[c]	NA	3.4	
Konc *et al.* (2010)	Cohort study	NS	831	Not described	Natural cycle + hCG ($n = 315$)	NA	110/315 (34.9%)[e]	NA	NA	No significant difference.
					hMG or recFSH daily + hCG ($n = 282$)	NA	78/282 (27.6%)[e]	NA	NA	
					GnRHa + artificial cycle ($n = 234$)	NA	58/234 (24.7%)[e]	NA	NA	
Our study, 2015	RCT	410	434	Regular cycle	Natural cycle + hCG ($n = 213$)	41/333 (12.3%)	31/213 (14.6%)[f]	30/213 (14.1%)	1.56	No significant difference.
					hMG daily = hCG ($n = 221$)	55/340 (16.2%)	46/221 (20.8%)[f]	44/221 (19.9%)	1.54	

Note: +hCG: ovulation was triggered using hCG; NA: not available; GnRHa: GnRH agonist; FET: frozen embryo transfer.
[a]Pregnancy rate: hCG value >20 mIU/ml 9 days post transfer.
[b]Not specified which ovarian stimulation was used.
[c]Pregnancy rate not further defined.
[d]Clinical pregnancy rate defined as an ultrasonographic detection of a gestational sac.
[e]Pregnancy rate defined as a spontaneous rise in the hCG 10 days post transfer.
[f]Fetal heart beat positive pregnancy rate.

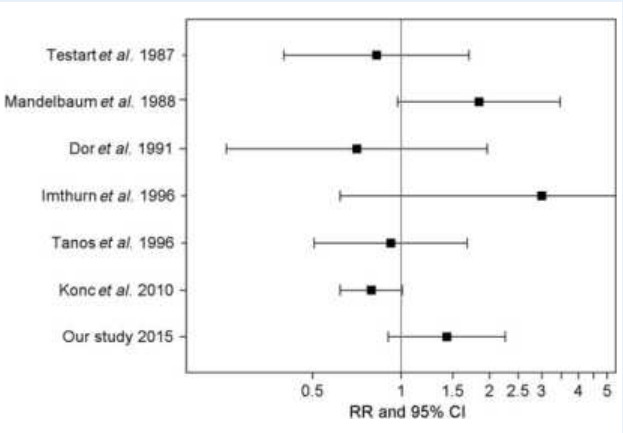

Figure 2 Forest plot for all studies comparing natural cycle versus hormonal stimulation with gonadotrophins in frozen-thawed embryo transfer cycles on the pregnancy rate per embryo transfer.

with added GnRHa. The Cochrane included also one RCT (van der Auwera *et al.*, 1994) previously performed in our centre, in order to compare reproductive outcome in FET cycles (188 FET cycles between November 1991 and April 1993) stimulated with a combination of clomiphene citrate 100 mg daily from Day 2 to 6 and gonadotrophins (hMG 150 IU) daily from Day 6 with FET cycles stimulated with hMG alone (150 IU daily from Day 2). In that study (van der Auwera *et al.*, 1994), the IR per embryo was higher after FET cycles stimulated with hMG alone (15%) when compared with FET cycles stimulated with the combination of clomiphene citrate and hMG (10%). Interestingly, the IR per embryo transferred in the FET-hMG group (15%) (Van der Auwera *et al.*, 1994) is similar to the result in the FET-hMG group in our study. In a more recent Cochrane review (Glujovsky *et al.*, 2010), comparing endometrial preparation for women undergoing embryo transfer with frozen embryos or embryos derived from donor oocytes, it was also concluded that there is insufficient evidence to recommend any particular protocol for endometrial preparation over another. Finally, in the most recently published systematic review and meta-analysis on the most effective method of endometrial preparation prior to FET, it was also concluded that it is not possible to identify one method of endometrium preparation in FET as being more effective than another (Groenewoud *et al.*, 2013), also after the inclusion of retrospective studies not included in a previous Cochrane review (Ghobara and Vandekerckhove, 2008). Since none of the Cochrane reviews or systematic reviews published on this topic (Ghobara and Vandekerckhove, 2008; Glujovsky *et al.*, 2010; Groenewoud *et al.*, 2013) identified any RCTs comparing reproductive outcome after natural cycle FET with FET cycles prepared by mild hormonal stimulation using gonadotrophins, our study is the first randomized trial addressing this clinical question. Interestingly, our data confirm the results of six non-randomized prospective cohort studies addressing the same question (reviewed in Table V and Fig. 2).

In our series we observed that the duration of the follicular phase was 1 day longer in the NC FET group (15 days) than in the HMG FET group (14 days). This observation is intriguing but hard to explain, and probably without clinical relevance in view of the similar reproductive outcome in both groups. We hypothesize that ovarian stimulation with gonadotrophins may stimulate a faster development of the dominant follicle(s) when compared with a natural cycle, and as such reduce the duration of the follicular phase when compared with a natural cycle.

In view of the lack of differences in reproductive outcome observed between one or another protocol for endometrial preparation prior to FET, the decision should be made based upon other factors such as number of monitoring visits needed, side-effects and cost of medication, and most importantly patient preference. Daily s.c. injections could also be less acceptable than natural cycle from the patient point of view.

Indeed, there is a growing awareness of patient-centeredness in fertility treatment and decision making (Dancet *et al.*, 2010, 2011, 2014). In daily practice, patients are insufficiently involved in choosing between treatment options, as treatment decisions are predominantly made by professionals. Patients' treatment preferences, should be taken into account when choosing the right treatment option for an individual patient especially if no differences in PRs are observed between treatment options, as observed in this paper. Similarly, clinical evaluation research primarily focuses on the effectiveness of treatments (secondarily on treatment safety and costs and less on burden) and conceptualizes outcomes from the viewpoint of professionals rather than patients (Dancet *et al.*, 2014). Therefore, not only an economic analysis may be useful to determine which protocol of endometrial preparation prior to FET is more cost-effective when compared with another but also safety, burden and patient preferences need to be addressed in next trials.

In conclusion, our study shows that, for women with a regular cycle, FET after ovarian stimulation with gonadotrophins does not result in significantly improved IR and LBR when compared with FET in a natural cycle. The advantage of natural cycle is that it is easy for the patient since there are no daily s.c. injections and it is inexpensive. Therefore, based on our data that confirm earlier prospective controlled cohort studies, we recommend a natural cycle regimen for FET as the treatment of choice in patients with a regular ovulatory cycle.

Supplementary data

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

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Authors' roles

T.D. and K.P. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: T.D. Acquisition of data: All authors. Analysis and interpretation of data: K.P., S.D., I.C., D.D.N., T.D. Drafting of the manuscript: K.P. Critical revision of the manuscript for important intellectual content: T.D. Statistical analysis: A.L. Administrative, technical or material support: M.W.

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Conflict of interest

None declared.

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