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ORIGINAL ARTICLE *Infertility*

Biopsying, fragmentation and autotransplantation of fresh ovarian cortical tissue in infertile women with diminished ovarian reserve

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STUDY QUESTION: Can ovarian biopsying *per se* and/or autotransplantation of fragmented ovarian cortical tissue activate dormant follicles and increase the number of recruitable follicles for IVF/ICSI in women with diminished ovarian reserve (DOR)?

SUMMARY ANSWER: Ovarian biopsying followed by immediate autotransplantation of fragmented cortical tissue failed to increase the number of recruitable follicles for IVF/ICSI 10 weeks after the procedure either at the graft site or in the biopsied ovary, but 12 of the 20 women subsequently had a clinical pregnancy during the 1-year follow-up.

WHAT IS KNOWN ALREADY: Infertile women with DOR constitute a group of patients with poor reproductive outcome mainly due to the low number of mature oocytes available for IVF/ICSI. Recent studies have shown that *in vitro activation* of residual dormant follicles by both chemical treatment and tissue fragmentation has resulted in return of menstrual cycles and pregnancies in a fraction of amenorrhoeic women with premature ovarian insufficiency.

STUDY DESIGN, SIZE, DURATION: This is a prospective clinical cohort study including 20 women with DOR treated at the fertility clinic, Rigshospitalet, Denmark, during April 2016–December 2017. Non-pregnant patients were on average followed for 280 days (range 118–408), while women who conceived were followed until delivery. Study follow-up of non-pregnant patients ended in September 2018.

PARTICIPANTS, MATERIALS, SETTING, METHODS: The study included infertile women aged 30–39 years with preserved menstrual cycles, indication for IVF/ICSI and repeated serum measurements of anti-Müllerian hormone (AMH) $\leq$ 5 pmol/L. Patients were randomized to have four biopsies taken from either the left or the right ovary by laparoscopy followed by fragmentation of the cortical tissue to an approximate size of 1 mm^3 and autotransplanted to a peritoneal pocket. The other ovary served as a control. Patients were followed weekly for 10 weeks with recording of hormone profile, antral follicle count (AFC), ovarian volume and assessment for ectopic follicle growth. After 10 weeks, an IVF/ICSI-cycle with maximal ovarian stimulation was initiated.

MAIN RESULTS AND THE ROLE OF CHANCE: No difference in the number of mature follicles after ovarian stimulation 10 weeks after the procedure in the biopsied versus the control ovaries was observed (1.0 vs. 0.7 follicles, $P = 0.35$). In only three patients, growth of four follicles was detected at the graft site 24–268 days after the procedure. From one of these follicles, a metaphase II (MII) oocyte was retrieved and fertilized, but embryonic development failed. Overall AMH levels did not change significantly after the procedure ($P = 0.2$). The AFC increased by 0.14 (95% CI: 0.06;0.21) per week ($P < 0.005$), and the biopsied ovary had on average 0.6 (95% CI: 0.3;−0.88) follicles fewer than the control ovary ($P = 0.01$). Serum levels of androstenedione and testosterone increased significantly by 0.63 nmol/L (95% CI:

0.21;1.04) and 0.11 nmol/L (95% CI: 0.01;0.21) 1 week after the procedure, respectively, and testosterone increased consecutively over the 10 weeks by 0.0095 nmol/L (95% CI: 0.0002;0.0188) per week ($P = 0.045$). In 7 of the 20 patients, there was a serum AMH elevation 5 to 8 weeks after the procedure. In this group, mean AMH increased from 2.08 pmol/L (range 1.74–2.34) to 3.94 pmol/L (range 3.66–4.29) from Weeks 1–4 to Weeks 5–8. A clinical pregnancy was obtained in 12 of the 20 (60%) patients with and without medically assisted reproduction (MAR) treatments. We report a cumulated live birth rate per started IVF/ICSI cycle of 18.4%.

LIMITATIONS, REASON FOR CAUTION: Limitations of the study were the number of patients included and the lack of a non-operated control group. Moreover, 9 of the 20 women had no male partner at inclusion and were treated with donor sperm, but each of these women had an average of 6.8 (range 4–9) unsuccessful MAR treatments with donor sperm prior to inclusion.

WIDER IMPLICATIONS OF THE FINDINGS: Although 12 out of 20 patients became pregnant during the follow-up period, the current study does not indicate that biopsying, fragmenting and autotransplanting of ovarian cortical tissue increase the number of recruitable follicles for IVF/ICSI after 10 weeks. However, a proportion of the patients may have a follicular response in Weeks 5–8 after the procedure. It could therefore be relevant to perform a future study on the possible effects of biopsying *per se* that includes stimulation for IVF/ICSI earlier than week 10.

STUDY FUNDING/COMPETING INTEREST(S): This study is part of the ReproUnion collaborative study, co-financed by the European Union, Interreg V ÖKS. The funders had no role in the study design, data collection and interpretation, or decision to submit the work for publication. None of the authors have a conflict of interest.

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Introduction

Infertile women with diminished ovarian reserve (DOR) are among the most challenging patients to successfully treat with assisted reproductive technology (ART). As recently reviewed by Pastore *et al.* (2018), such patients are clinically categorized somewhere between patients with premature ovarian insufficiency (POI) and patients with poor ovarian response (POR) and are best defined by low levels of anti-Müllerian hormone (AMH), low antral follicle counts (AFCs) and preserved menstrual cycles, in contrast to women with manifest POI. Typically, patients with DOR fulfil the Bologna consensus criteria to characterize patients with POR due to their reduced ovarian reserve (Ferraretti *et al.*, 2011). Thus, the key problem is that women with DOR have few recruitable follicles despite aggressive gonadotropin stimulation. Several protocols and treatment modalities have been tested to improve the outcome for patients with POR, but currently only marginal or no beneficial effects have been documented compared with standard ART treatment as recently reviewed (Jeve and Bhandari, 2016; Papathanasiou *et al.*, 2016).

In 2013, Kawamura and colleagues were the first to introduce the concept of *in vitro activation* (IVA) of ovarian follicles in a clinical setting. Kawamura and colleagues reported that ovaries of women with manifest POI in some instances harboured residual 'dormant follicles' which could be recruited for growth (Kawamura *et al.*, 2013). After a two-step procedure, involving both mechanical (fragmentation) and chemical (IVA drug) treatment of biopsied cortical tissue, 9 out of 37 women developed pre-ovulatory follicles, and 3 patients obtained a positive serum human chorionic gonadotropin (hCG) following IVF treatment (Suzuki *et al.*, 2015).

One of the mechanisms behind the mechanical activation of dormant follicles could be inhibition of the Hippo pathway. The Hippo pathway is active in many organs regulating organ size by cell proliferation, growth, differentiation and cell death in metazoan animals (reviewed in Pfleger, 2017). In a murine model, Kawamura and colleagues showed that the Hippo pathway also operates in granulosa cells in the ovaries. Fragmentation of ovarian tissue from juvenile mice followed by allotransplantation led to increased graft size, graft weight and more follicles being recruited for growth and development. Mature oocytes were retrieved, and healthy pups were born after IVF (Kawamura *et al.*, 2013).

Many of the components involved in the Hippo pathway are unknown, but it has been suggested that physical changes, e.g. changes in the extracellular matrix and the actin cytoskeleton, play an important role in regulating the pathway (Dupont *et al.*, 2011; Reddy *et al.*, 2013).

We therefore aimed to investigate whether ovarian biopsying *per se* and/or fragmentation and autotransplantation of ovarian cortical tissue could lead to activation of follicular growth in patients with DOR with a subsequent increase in the number of recruitable follicles after ovarian stimulation and possible pregnancy after medically assisted reproduction (MAR) treatments.

Infertile women with DOR were randomized to have four small biopsies taken by laparoscopy from either the left or the right ovary followed by immediate autotransplantation of the fresh fragmented tissue.

Materials and Methods

Study design

A clinical cohort study was conducted at The Fertility Clinic, The Laboratory of Reproductive Biology and Department of Gynaecology at Rigshospitalet, Copenhagen, Denmark. Data were collected prospectively from April 2016 to September 2018. Final data on live births were obtained May 2019.

Study population and inclusion criteria

The study included infertile patients with a very low ovarian reserve treated at the Fertility Clinic at Rigshospitalet between April 2016 and December 2017. The inclusion criteria were infertility with an indication for IVF/ICSI, repeated serum AMH measurements

Table I Reproductive history and Cycle Days 2–5 baseline sonography and endocrine findings.

Patients, $n = 20$	**Mean ± SD (range)**[a]
Age, years	37.4 ± 2.47 (30–39)[b]
Smoking (%)	15%
Hypothyroidism (treated with levothyroxine) (%)	20%
TPO antibodies (%)	15%
Infertility diagnoses, number	
DOR only	3
Male factor	8
Single/female partner	9
Tubal factor	2
Infertility duration, months (range)[c]	
Single/female partner	16.8 (6–39)
Male partner	31 (12–60)
Earlier treatments, number (range)[d]	
Started cycles per patient (all types)	4.75 (0–9)
Inseminations per patient (cycles = 15)	0.75 (0–6)
Donor inseminations per patient (cycles = 39)	1.95 (0–6)
IVF/ICSI per patient (cycles = 13)	0.65 (0–5)
IVF/ICSI with donor sperm per patient (cycles = 22)	1.10 (0–4)
Endocrinology	
AMH, pmol/L	2.10 ± 1.33 (0.22–4.43)[e]
FSH, IU/L	11.6 ± 6 (4.8–25.9)
LH, IU/L	7.4 ± 3.3 (3.0–16.7)
Oestradiol, nmol/L	0.27 ± 0.24 (0.09–1.01)
Testosterone, nmol/L	0.76 ± 0.21 (0.41–1.30)
Prolactin, IU/L	288 ± 161.69 (170–558)
Ovarian sonography	
AFC, number of follicles	5.1 ± 1.7 (2–9)
AFC, biopsied ovary	2.6 ± 1.6
AFC, control ovary	2.5 ± 1.2
Ovarian volume, mL	3.7 ± 1.6 (1.4–8.1)

TPO antibodies, thyroid peroxidase antibodies; DOR, diminished ovarian reserve; AMH, anti-Müllerian hormone; AFC, antral follicle count.
[a]If nothing else stated.
[b]Age at the time of ovarian biopsying and autotransplantation of ovarian cortical tissue.
[c]For women who were single or had a female partner, duration of infertility was the time from first visit in a fertility clinic/first treatment, whatever documented in the patient records. For women with a male partner, it was based on patient recall as part of the inclusion questionnaire.
[d]Earlier treatments are based on a mixture of patient recall and patient records.
[e]AMH 2.1 pmol/L ≈ 0.3 ng/mL.

≤5 pmol/L (0.7 ng/mL) (at least two independent measurements within 1 year prior to screening) and age 25–39 years. The exclusion criteria were any ovarian pathology (endometriosis, cysts, malignancy), known chromosomal abnormalities at the time of screening, known autoimmune disease (except thyroid peroxidase-antibodies) and contraindications for laparoscopy. Initially, only patients with bilateral AFC ≤ 5 were included, but this criterion was later abandoned, and patients were included when they had consistent AMH levels below 5 pmol/L.

The search for eligible patients was completed in collaboration with hospitals in the greater Copenhagen area. In total, 24 patients were screened, and 20 patients fulfilled the inclusion criteria. All screenings were done at Rigshospitalet and included ovarian sonography with AFC and blood sampling for analysis of serum concentrations of AMH, follicle-stimulating hormone (FSH), luteinizing hormone (LH), oestradiol, progesterone, testosterone, androstenedione, 17-hydroxy-progesterone, dehydroepiandrosterone sulfate (DHEAS) and sex hormone-binding globulin (SHBG) on Cycle Days 2–5. Blood sampling for analysis of plasma ovary antibodies, plasma thyroid peroxidase antibodies, plasma adrenal antibodies, karyotype and fragile X mental retardation 1 (DNA (spec)-FMR1) was performed regardless of cycle day.

The reproductive history is described in Table I. In addition to DOR, the causes of infertility were tubal factor ($n = 2$), male factor ($n = 8$)

and single status or women with a female partner ($n = 9$). Seventeen of the 20 patients had more than one infertility diagnosis.

As a result of previous repeated donor insemination (DI) treatments, eight of the patients had at least one IVF/ICSI with donor sperm (single or with female partner) prior the procedure (range 1–4 IVF/ICSI). One woman had only four DI treatments prior to inclusion. However, she did not become pregnant. All patients had received on average 4.75 failed MAR treatments (0–9 treatments per patient).

Patients who were single or with a female partner had a mean of 16.8 months (range 6–39 months) of fertility treatments prior the procedure. Patients with a male partner had on average been infertile for 31 months (range 12–60 months).

Laparoscopy and biopsies

After inclusion, the 20 patients were randomized by a computerized generated randomization list to have laparoscopy with four biopsies of ovarian cortex taken from either the left or right ovary. The contralateral ovary therefore served as an intra-individual control for the biopsied ovary. The biopsies were taken at random locations from the surface of the randomized ovary.

Each biopsy had a volume of $\sim 5 \times 5 \times 3$ mm. After removal of the four biopsies, three of the biopsies were cut into small fragments ($\sim 1 \times 1 \times 1$ mm) at the Laboratory for Reproductive Biology and the fragments were then placed under the peritoneal serosa underneath the right ovary, irrespective of the randomization. The surgical procedure as such, including the procedure of fragmentation and autotransplantation of the fragmented cortical tissue, has recently been published as a video article (Lunding *et al.*, 2018).

The fourth biopsy was stored and used for evaluation of follicular distribution and density as described by Schmidt *et al*. (2003).

Blood samples

The serum concentration of AMH was analysed by the Elecsys AMH assay (Cobas® 8000, e801 module, Roche Diagnostics GmbH, Mannheim, Germany). The limit of detection and the limit of quantification were 0.071 and 0.21 pmol/L, respectively (1 pmol/L = 0.14 ng/mL). The intra- and inter-assay coefficients of variation were 0.5–1.4 and 0.7–1.9%, respectively (Li *et al.*, 2016).

Serum levels of oestradiol, FSH, LH, progesterone and SHBG were analysed using the routine methods based on fully automated measurements using assays from Roche Diagnostics A/S (Cobas e801). Detection and quantification limits were 18.4 pmol/L, 0.3 mIU/mL, 0.3 mIU/mL, 0.159 nmol/L, 0.8 nmol/L and 91.8 pmol/L, 1 mIU/mL, 1 mIU/mL, 0.636 nmol/L, 2 nmol/L, respectively.

Serum levels of testosterone, androstenedione, DHEAS and 17-hydroxy-progesterone were analysed by liquid chromatography-mass spectrometry. The measuring range of the analyses was 0.2–40 nmol/L, 0.5–60 nmol/L, 0.1–25 µmol/L and 0.5–75 nmol/L, respectively.

Ovarian sonography

Transvaginal sonography was performed by the same four physicians (S.A.L., A.N.A., S.K.L. and K.T.M.). Antral follicles were counted and grouped according to size into four categories: 2–4, 5–7, 8–10 and 10–12 mm. Echo-free structures over 12 mm were recorded separately and were not included in the AFC. The volume of an ovary was estimated as the volume of a prolate ellipse (Cohen *et al.*, 1990). If an ovary contained a follicular structure ≥12 mm, the volume was not included in the analysis.

Physicians doing the sonography were blinded throughout the study in relation to the side of the biopsied ovary. The operator of the procedure (A.T.P.) was excluded from the follow-up AFC evaluation.

Follow-up, fertility treatments and pregnancies

Postoperatively, all patients were followed weekly for 10 weeks with recording of AFC, ovarian volume, AMH, FSH, LH, oestradiol, progesterone, testosterone, androstenedione, 17-hydroxy-progesterone, DHEAS and SHBG.

Search for ectopic follicle growth was performed by transvaginal sonography under the right ovary at the transplant site.

After 10 weeks, an IVF/ICSI cycle was initiated. Patients had maximal stimulation using Corifollitropin alfa 100–150 mg, according to bodyweight. If needed, an additional recombinant follicle-stimulating hormone (rFSH) (Puregon, MSD, Denmark) of 200–300 IU was administered until hCG administration. The oocytes were retrieved 34–36 h after hCG, and patients had conventional IVF or ICSI as appropriate. Embryo transfer was done on day 2, and luteal support was given in form of vaginal progesterone 100 mg × 3 daily (Lutinus, Ferring, Denmark).

In non-pregnant patients, follow-up beyond the 10th week was done on Cycle Days 2–5 up to 1 year. During this period, patients were offered intrauterine insemination (IUI), DI, IVF or ICSI as appropriate. Blood sampling and AFC after the 10-week period were only included in the data analysis if the patient had no preceding ovarian stimulation.

Ovulations were determined by progesterone levels above 12 ng/mL and/or a corpus luteum visualized by ultrasound during the mid-luteal phase.

A clinical pregnancy was defined as an intrauterine gestational sac containing an embryo with foetal heartbeat at Week 7. Time to pregnancy was defined as number of days from the procedure to a positive s-hCG.

Endpoints

The primary predefined endpoint was the number of follicles ≥12 mm in response to ovarian stimulation with Corifollitropin alfa for IVF/ICSI from the biopsied ovary versus the control ovary 10 weeks after autotransplantation of the fragmented ovarian tissue. Secondary endpoints were frequency of ovulations occurring from the biopsied ovary versus the control ovary (in the 10 stimulation-free weeks); AFC in the biopsied ovary versus the control ovary changes in serum AMH, gonadotrophins, steroid hormones and ectopic follicular growth within 6 months post laparoscopy; clinical pregnancy (natural or by MAR); and live births.

Statistical analyses

Summary data are presented as mean ± SD (range) or median for continuous variables and as frequencies and/or percentages for categorical variables. Range is defined as lowest and higest value.

To assess whether serum levels of AMH, DHEAS, androstenedione and testosterone changed following the procedure, the hormones in relation to time after the procedure were fitted with a linear-mixed model. The (log)-linear-mixed model enables evaluation

of multiple measurements over time for each patient, allowing for each patient to have her own hormone level (i.e. random intercept). Furthermore, a (log)-linear-mixed model allows to evaluate if the baseline value was different from the postoperative development. A different baseline value could be interpreted as an immediate effect of the procedure.

To investigate if AMH serum levels correlated with androgens (DHEAS, androstenedione and testosterone), we performed a linear-mixed model with AMH and androgens adjusted for time.

To assess whether AFC changed following the procedure and if AFC differed between the biopsied and the control ovary, the linear-mixed model was used. All fitted models were tested for non-linearity with non-linear natural splines. To find the best description of possible changes after the procedure, a stepwise elimination approach was used. *P* values of less than 0.05 were considered statistically significant.

AMH levels were logarithmically transformed before analysis, to account for a right-skewed distribution. AMH serum levels below the quantification limit were given the value of the quantification limit (= 0.21 pmol/L).

To evaluate whether more mature follicles developed in the biopsied ovary compared with the control ovary in response to ovarian stimulation, a log-linear mixed Poisson model was used. For comparing frequency of ovulations in the biopsied ovary and the control ovary, a logit mixed binominal model was used.

Comparison of androgen levels between pregnant and non-pregnant patients was done by adjusting for pregnancy in our linear mixed model. Additionally, comparison of androgen levels between the pregnant and non-pregnant after 5–8 weeks and 10 weeks was done by standard *t*-test. One patient displayed a DHEAS serum level on average 5 µmol/L higher than the rest of the patients (Patient Number 9, $P = 0.004$) and was therefore defined as an outlier and excluded from the DHEAS analyses. We were able to compare the oestradiol serum levels in pregnant and non-pregnant patients prior to the protocolled Corifollitropin alfa treatment, since this was the only time where blood sampling was done on Cycle Days 2–3. For comparison, we used a Wilcoxon exact test for non-parametric data. A logistic regression model adjusted for age was made to examine if baseline FSH correlated with pregnancy. Furthermore, by a paired *t*-test, baseline FSH and FSH prior to the protocolled Corifollitropin treatment at Cycle Days 2–3 were compared.

For post hoc analyses of the two subgroups of the patients according to the increase in AMH and AFC, a simple regression model was used for comparison of baseline AMH, AFC, LH, FSH and androgens. For comparing the number of pregnancies, logistic regression was used. To avoid too selective presentations of the results, additional statistical analyses on the two subgroups were not preformed.

Statistical tests were done using R version 3.4.4 (https://www.r-project.org/).

Ethical approval

The study was approved by the Scientific Ethical Committee of the Capital Region of Denmark (Journal No.: H-15011975) and The Danish Data Protection Agency (Journal No.: 2012-58-0004).

All patients signed written consent according to the Declaration of Helsinki for Medical Research involving Human Subject before entering the study.

Results

Baseline characteristics

Table I includes baseline data at Cycle Days 2–5 and characteristics of the 20 included patients. The mean age was 37.4 years (range 30–39 years). Mean AMH at the screening visit was 2.1 pmol/L ≈ 0.3 ng/mL (range 0.2–4.4 pmol/L), mean total AFC was 5.1 follicles (range 2–9), mean ovarian volume was 3.7 mL (range 1.4–8.1 mL) and mean serum FSH was 11.6 IU/L (range 4.8–25.9 IU/L). Patients had between one and four earlier AMH measurements ≤ 5 pmol/L within the last 6 months before the procedure. One patient had a single AMH measurement of 5.2 pmol/L before the procedure. Five patients had an AFC > 5 (Patients 11, 13, 15, 18 and 20 in Supplementary Figure SI); however, compared with patients with AFC ≤ 5, mean AMH levels did not differ (2.03 pmol/L (range 0.46–3.4 pmol/L) vs. 2.10 pmol/L (range 0.22–4.4 pmol/L), $P = 0.92$). Fifteen of the patients fulfilled the Bologna consensus criteria for POR. Of these, 12 (80%) had ≥2 episodes of POR after maximal stimulation (range, two to five episodes). Five patients did not fulfil the criteria as they had only had IUI treatments ($n = 4$) or no treatment ($n = 1$) prior to study inclusion. These five patients had a mean age of 37.1 years (range 33–38 years) and a mean baseline AMH of 1.64 pmol/L (range 0.22–3.6 pmol/L) equivalent to 0.23 ng/mL with the Elecsys assay, which is below the Bologna AMH cut-off value (0.5–1.1 ng/mL) (Ferraretti *et al.*, 2011). Furthermore, 11 out of 20 patients had a shift towards larger antral follicles (>8 mm) at Cycle Days 2–5 corresponding to a physiologically aged ovary with low ovarian reserve and early follicular recruitment (Bentzen *et al.*, 2013).

In one patient, the blood sample for karyotyping taken at the screening visit revealed at low-grade Turner mosaicism with 7 affected cells out of 31 cells (1 cell had 47,XXX and 6 cells had 45,X). This patient remained included but withdrew from the follow-up after the first protocolled Corifollitropin alfa stimulation (Patient Number 20 in Supplementary Figure SI).

Biopsy procedure and histology

No complications were observed in the 20 patients undergoing laparoscopy. Four biopsies were taken from each patient. The distribution of randomization to the left versus right ovary was 10 and 10. The mean volume of the four biopsies was 45 mm^3, and a mean of 134 fragments (range 71–271) were autotransplanted per patient.

In 90% of the patients, residual follicles were present in the fourth biopsy at histological assessment. The median follicular density was 1.9 follicles/mm^3 (range 0–93.7 follicles/mm^3). One patient had an extremely high follicular density (93.7 follicles/mm^3); however, her AMH serum levels were low (see Patient Number 3 in Supplementary Figure SI) and she did not become pregnant.

Weekly follow-up

Weekly development of antral follicles and reproductive endocrinology

Figure 1 shows the mean AFC ± SEM in the biopsied and the non-biopsied (control) ovary during the weekly follow-up for 10 weeks. No difference in AFC in the biopsied ovary versus control ovary was found at baseline ($P = 0.84$). In both sides, AFC increased significantly

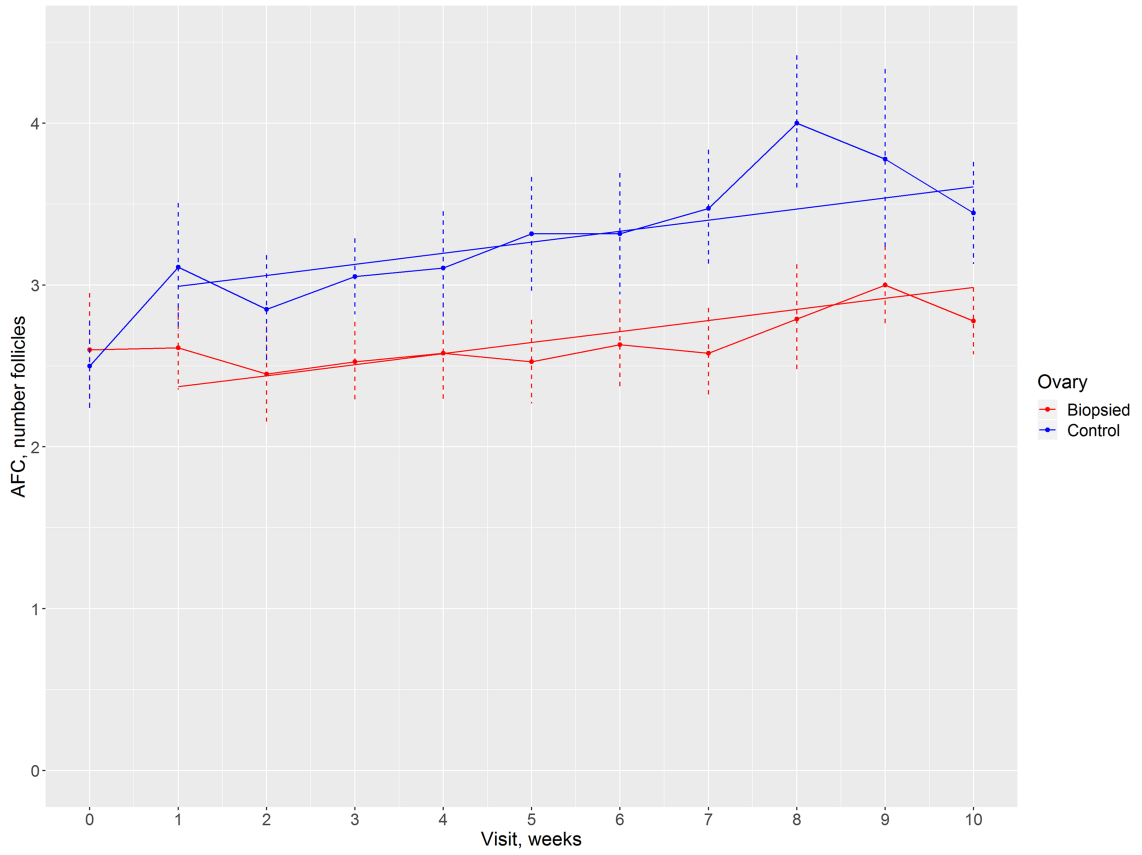

Figure 1 Mean antral follicle count (AFC) in the biopsied ovary (red) and the control ovary (blue) during the 10 weeks follow-up after the biopsying and autotransplantation. The joint points represent the mean AFC for each visit. The dotted vertical lines represent the standard error of the mean. The straight lines represent the best fitted line.

by 0.07 (95% CI: 0.03;0.11) follicles per week ($P = 0.001$), but the biopsied ovary had on average 0.6 (95% CI: 0.36; 0.88) follicles less than the control ovary ($P = 0.01$). The total number of AFC increased by 0.14 (95% CI: 0.06;0.21) follicles per week ($P < 0.005$) (Table II).

Table II gives data on crude mean serum AMH, androstenedione, testosterone levels and AFC during the 10-week follow-up. Overall serum levels of AMH did not change significantly during the follow-up ($P = 0.2$). Based on a linear regression analysis, serum levels of androstenedione increased with 0.63 nmol/L (95% CI: 0.21;1.04) from baseline to 1 week after the procedure ($P < 0.005$). At baseline, serum levels of testosterone were 0.87 nmol/L (95% CI: 0.74;1.00). One week after the procedure, the fitted mean testosterone level had increased by 0.11 nmol/L (95% CI: 0.01;0.21) and increased consecutively by 0.0095 nmol/L (95% CI: 0.0002;0.0188) per visit ($P = 0.04$). DHEAS did not change significantly during the 10 weeks ($P = 0.3$). There was no correlation between AMH serum levels and DHEAS and testosterone; however, we did see a significant correlation between AMH and androstenedione serum levels. For every one-unit increase in AMH androstenedione increased by 0.15 nmol/L ($P = 0.043$).

During the 10-week follow-up, the mean serum levels of oestradiol, FSH and LH were 0.26 nmol/L (range 0.09–1.07), 14.17 IU/L (range 4.8–43.8) and 10.49 IU/L (range 2.3–46.6) in the early follicular phase (Cycle Days 1–7), respectively.

Individual AMH serum level development

Supplementary Figure S1 shows the development of AMH serum levels during the 10 weeks for each patient. It is noted that both inter- and intra-individual variabilities of AMH serum levels were huge. It seems that for seven patients (Patients 1, 2, 3, 10, 18, 19 and 20), a rise in AMH serum levels occurred 5–8 weeks after the procedure (Fig. 2A). For this subgroup of seven patients (35%), mean AMH increased from 2.08 pmol/L (range 1.74–2.34) to 3.94 pmol/L (range 3.66–4.29) from Weeks 1–4 to Weeks 5–8. This positive development was accompanied by an increase in AFC as seen in Figure 2B.

Post hoc analyses of the seven patients showed that baseline mean AMH in this subgroup was 2.43 pmol/L (range 1.06–4.40) versus 1.89 pmol/L (range 0.22–4.43) in the remaining 13 patients, though not significant ($P = 0.41$). The two groups did not differ in terms of number of pregnancies or baseline age, AFC, FSH, LH, oestradiol and androgens (data not shown).

Ovulations

Nineteen out of 20 patients had at least one natural ovulation during the 10-week follow-up. The side of ovulation could be determined in 44 cycles and in 17 cycles (39%) (95% CI: 32%;45%); this occurred in the biopsied ovary versus 27 cycles (61%) (95% CI: 55%;68%) in the control ovary. The difference was not significant ($P = 0.16$).

Table II Antral follicle count and reproductive hormones during 10 weeks after the ovarian biopsying and autotransplantation of ovarian cortical tissue. Data are represented in crude means ± SD.

	Antral follicle count			Reproductive hormones		
Week	Biopsied ovary[a]	Control ovary[a]	Total, both ovaries[c]	AMH, pmol/L[d]	Androstenedione, nmol/L[e]	Testosterone, nmol/L[f]
0	2.6 ± 1.6[b]	2.5 ± 1.2[b]	5.1 ± 1.7	2.1 ± 1.3	3.0 ± 0.9	0.76 ± 0.2
1	2.6 ± 1.1	3.1 ± 1.7	5.7 ± 2.0	1.6 ± 1.1	3.4 ± 1.1	0.89 ± 0.3
2	2.5 ± 1.3	2.9 ± 1.5	5.3 ± 2.3	1.7 ± 1.1	3.8 ± 1.7	0.86 ± 0.3
3	2.5 ± 1.1	3.0 ± 1.0	5.6 ± 1.6	1.9 ± 1.3	3.3 ± 1.0	0.86 ± 0.3
4	2.6 ± 1.4	3.1 ± 1.5	5.7 ± 1.2	1.7 ± 1.2	3.5 ± 1.2	0.90 ± 0.3
5	2.5 ± 1.1	3.3 ± 1.5	5.8 ± 1.7	2.5 ± 2.3	3.7 ± 1.5	0.95 ± 0.3
6	2.6 ± 1.2	3.3 ± 1.6	5.9 ± 2.3	2.4 ± 2.1	3.6 ± 1.6	0.93 ± 0.3
7	2.6 ± 1.2	3.5 ± 1.6	6.1 ± 2.2	2.0 ± 1.8	3.4 ± 1.1	0.95 ± 0.3
8	2.8 ± 1.5	4.0 ± 1.8	6.8 ± 2.5	2.4 ± 2.0	3.6 ± 1.1	1.00 ± 0.4
9	3.0 ± 1.0	3.8 ± 2.4	6.8 ± 2.4	1.9 ± 1.4	3.8 ± 1.5	0.99 ± 0.4
10	2.8 ± 0.9	3.4 ± 1.3	6.2 ± 1.8	1.7 ± 1.2	3.2 ± 1.5	0.87 ± 0.3

AFC, antral follicle count; AMH, anti-Müllerian hormone; DHEAS, dehydroepiandrosterone.
[a]AFC increased on average by 0.07 follicles per week in both biopsied and control ovaries ($P = 0.001$). The biopsied ovary had on average 0.6 follicles less than the control ovary ($P = 0.01$).
[b]AFC in the biopsied vs. the control ovary was not statistical different at baseline ($P = 0.84$).
[c]The total AFC increased by 0.14 follicles pr. week ($P < 0.005$).
[d]AMH serum levels did not change significantly during the 10-week follow-up ($P = 0.2$).
[e]Based on a linear regression, model serum levels of androstenedione increased with 0.63 nmol/L from baseline to Week 1.
[f]The fitted serum levels of testosterone had increased by 0.11 nmol/L at Week 1 and increased consecutively by 0.0095 nmol/L per week ($P = 0.04$).

Follicular growth in autotransplanted tissue

In one patient, spontaneous ectopic growth compatible with a follicle measuring 9 and 9.5 mm was detected during the 10-week follow-up (24 and 43 days) after the procedure. Additionally, three ectopic structures measuring 8.5, 11 and 12 mm at the graft site were detected during ovarian stimulation 149–268 days after the procedure in two other patients. One of the three patients had successful retrieval of an metaphase II (MII) oocyte from an ectopic follicle at Day 268 after the procedure. This follicle was successfully fertilized by ICSI, but further embryonic development failed.

Primary endpoint: response to first ovarian stimulation

Ten weeks after the procedure, 17 of the 20 patients started stimulation according to the protocol with Corifollitropin alfa at Cycle Days 2–3. The mean number of days of stimulation was 8.8 days (range 6–14), and a mean of 543 IU rFSH (range 0–2100) was administered after Day 8. In three patients (17.6%), the cycle was cancelled due to lack of follicular response. Stimulation was not initiated in three patients due to pregnancy ($n = 2$) and consistent cyst development ($n = 1$).

The primary endpoint was the number of follicles >12 mm after Corifollitropin alfa stimulation. This was 1.0 (95% CI: −1.08;3.08) and 0.71 (95% CI: −0.64;2.05) in the biopsied versus the control ovary, respectively. The difference was not significant ($P = 0.35$).

Long-term follow-up

Non-pregnant patients were on average followed for 279.5 days (range 118–408). Two patients were followed less than 6 months. In one of these patients the karyotype revealed a Turner mosaicism, the other patient dropped out of the study.

Development of antral follicles and reproductive endocrinology

During the follow-up from 10 weeks to 1 year after the procedure, serum levels of AMH did not change. Supplying a natural spline (non-linear line) was not meaningful as the number of hormone measurements decreased notably (due to pregnancies and treatments). The number of antral follicles increased significantly over time after the procedure. The increase was 0.006 follicles (95% CI: 0.003;0.010) per day ($P < 0.005$) (data not shown). Serum testosterone levels had a non-linear course over time, but overall no increase or decrease occurred. Serum levels of DHEAS did not change during the long-term follow-up. Androstenedione serum levels declined by −0.0028 nmol/L (95% CI: −0.005; −0.001) per day ($P < 0.005$) during the long-term follow-up.

Treatments and pregnancies

In total, 55 MAR treatments were initiated as part of this study (Table III). Fourteen patients had subsequent MAR treatments after the first Corifollitropin alfa stimulation. In 38 initiated cycles for IVF/ICSI, 13% (5/38) were converted into an insemination; in 5% (2/38) no oocytes were retrieved, and 13% (5/38) were cancelled due to lack of follicular response ($n = 4$) and premature ovulation ($n = 1$). In 16 patients, oocyte retrieval was done 28 times. An average of 2.2 oocytes (range 0–5) were retrieved and 1.2 embryos (range 1–2) transferred in 10 patients, who had a total of 18 transfers. Three patients each had one frozen blastocyst stored. Only one of these had a frozen embryo transferred and got pregnant.

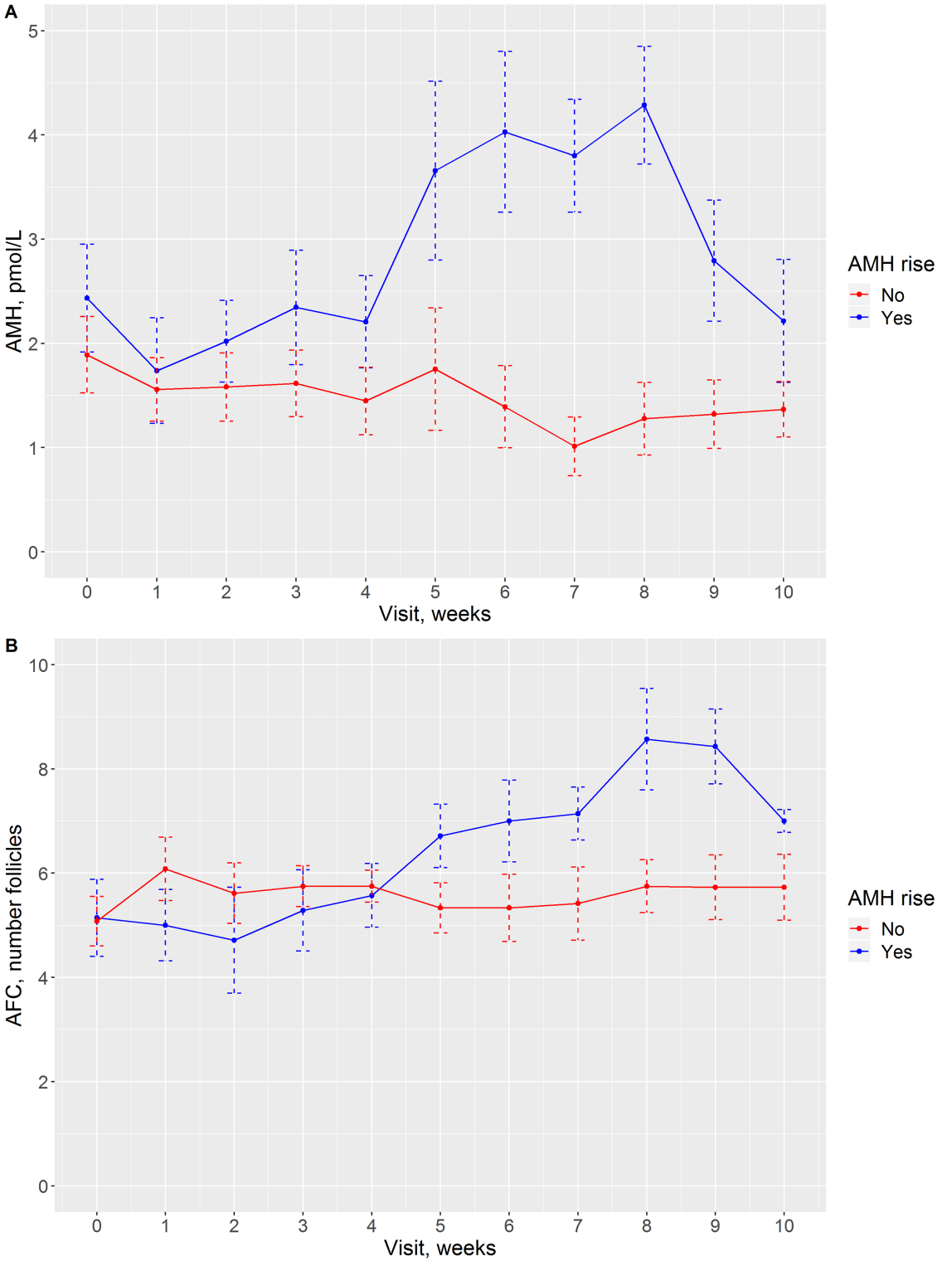

Figure 2 Variation in anti-Müllerian hormone (AMH) and antral follicle count (AFC) in two subgroups of the patients during the 10-week follow-up after the biopsy and autotransplantation. (**A**) Mean AMH. Seven of the 20 patients were classified as having a rise in AMH serum levels 5–8 weeks after the procedure (blue line), and 13 patients were classified as having no rise in AMH (red line). The dotted vertical lines represent the standard error of the mean. (**B**) Mean AFC. The blue line shows AFC for the seven patients classified as having a rise in AMH 5–8 weeks after the procedure; the red line shows AFC for the 13 patients classified as having no rise in AMH. The dotted vertical lines represent the standard error of the mean.

Table III Cumulative reproductive outcome after MAR treatments within 1 year after ovarian biopsying and autotransplantation in 20 patients.

Started MAR cycles, all types (*n* = 55 cycles*)	Number
IVF/ICSI cycles	38
Insemination cycles (partner)	6
Insemination cycles (donor)	10
Frozen embryo transfer cycles	1
Cycles per patient, mean (range)	3 (1–7)
IVF/ICSI outcome (*n* = 38 cycles)	
Oocyte retrievals	28
Oocytes per retrieval, mean (range)	2.2 (0–5)
Embryo transfers	18
Embryos transferred per transfer, mean (range)	1.2 (1–2)
Patients with one cryopreserved blastocyst	3
Pregnancies	
Clinical pregnancies (all)	12 (60%)
Live births	10[a] (50%)
Cumulated live birth rate per started IVF/ICSI cycle, *n* = 38	7 (18.4%)
Live birth rate per IUI cycle, *n* = 21[b]	1 (4.8%)
Live births after natural conception	2

*Including the Corifollitropin alfa stimulation 10 weeks after ovarian biopsying and autotransplantation.
[a]One patient had a spontaneous abortion in Week 9, another patient terminated the pregnancy in the second trimester due to a large foetal hernia of the diaphragm.
[b]Five of the 21 cycles started as IVF/ICSI cycles but were converted to IUI.

Overall, 12 clinical pregnancies were obtained 177.75 days (range 35–288 days) after the procedure, 3/20 patients conceived naturally (15%), 2/20 patients became pregnant as a result from the first Corifollitropin alfa ovarian stimulation followed by IVF/ICSI (10%) and 7/20 patients became pregnant during the additional MAR treatments (35%). Two out of seven had inseminations, and five of seven had IVF/ICSI/FET treatments resulting in pregnancy. One patient aborted spontaneously in Week 9, but she continued in the study. Another patient terminated a second trimester pregnancy due to a large foetal hernia of the diaphragm.

Pregnancy outcome in relation to baseline characteristics is shown in Supplementary Table SI. When comparing androgen levels in pregnant and non-pregnant patients during the 10-week follow-up, no significant difference was found (data not shown). In patients who became pregnant, baseline serum FSH was 10.9 IU/L (range 4.8–22.5) compared with 12.6 IU/L (range 5.6–25.9) in patients who did not become pregnant. This difference was not significant ($P = 0.45$). A small, but non-significant, increase was seen in FSH levels from 12.2 IU/L (range 4.8–25.9) at baseline (Cycle Days 2–5) to 13.4 IU/L (range 3.7–27.7) prior to the Corifollitropin alpha treatment (Cycle Days 2–3) 10 weeks after the grafting ($P = 0.6$).

When comparing oestradiol levels at Cycle Days 2–3 prior to the Corifollitropin alfa treatment in those who became pregnant and those who did not become pregnant in the study, we found that non-pregnant patients had a significantly higher median serum level (0.28 vs. 0.09 nmol/L, $P = 0.012$), which fits a hypothesis of early follicular recruitment.

Discussion

In this study, we investigated whether ovarian biopsying *per se* and/or fragmentation and autotransplantation of ovarian cortical tissue could lead to activation of follicular growth in patients with DOR with a subsequent increase in the number of recruitable follicles responding to ovarian stimulation for IVF/ICSI 10 weeks after the procedure.

The study showed that, in terms of our primary endpoint, the follicular response to ovarian stimulation 10 weeks after the procedure was similar in the biopsied and control ovaries. Additionally, only 15% (3 of 20 patients) showed sonographic signs of ectopic follicle growth at the graft site and only one woman had a single oocyte retrieved from an ectopic follicle. Furthermore, the number of ovulations in the biopsied ovary versus the control ovary remained similar. Overall, these data suggest that neither biopsying nor autotransplantation of fragmented ovarian tissue are generally effective to augment follicular recruitment for IVF/ICSI at 10 weeks after the procedure. However, before abandoning this approach in infertile women with DOR, two findings should be considered. First of all, a fairly high percentage of patients (60%; 12/20) subsequently achieved a clinical pregnancy with or without MAR treatments. Secondly, based on our serial measurements of AFC and androgens and to a certain extent AMH, a subgroup of patients may respond to the procedure by some activation of early follicular growth around 5–8 weeks after ovarian biopsying. Therefore, it could be of relevance to do a large controlled trial of ovarian biopsying *per se*.

Based on our linear regression model, we found that after 10 weeks, the total AFC increased significantly by 1.4 follicles over the 10-week follow-up corresponding to an increase of 26% compared with baseline. Looking at the individual ovaries, we observed that AFC on average was 0.6 higher in the control ovary throughout the 10 weeks (Fig. 1). Baseline AFC on the two sides was similar (Table II), so a possible stimulatory effect on the control ovary occurred between baseline and 1 week after the procedure, most likely as an indirect result of the procedure. One hypothesis explaining the difference between the two sides over time could be that the effect of the procedure was a certain damage of the biopsied ovary due to tissue and follicle removal and that this effect may be persistent. However, over time there was a rise in AFC in both ovaries. A possible explanation could be a 'tissue damage and removal'-induced FSH increase followed by a rise in follicular growth. Indeed, it seemed that the FSH serum levels rose, although non-significantly, from 12.2 to 13.4 IU/L when comparing baseline FSH with FSH measured at Cycle Days 2–3 prior to the first stimulation in those women who started Corifollitropin after 10 weeks ($P = 0.6$). This could perhaps elicit new waves of follicular development that resulted in higher AFC over time.

Recently, Herraiz *et al.* showed in a pilot study that a subgroup of poor-responder patients experienced an increase in ovarian reserve markers after infusion of autologous bone marrow-derived stem cells into the ovarian artery (Herraiz *et al.*, 2018). Based on individual data and the theory that a proportion of our patients could experience activation of follicles, 7 (35%) out of 20 patients seemed to have a

rise in serum AMH from 2.08 pmol/L (range 1.74–2.34) at Weeks 1–4 to 3.94 pmol/L (range 3.66–4.29) around Weeks 5–8 after the procedure, and a corresponding rise in AFC slightly later, as illustrated in Fig. 2A and B. AFC may be difficult to accurately count, but an effect on the follicular recruitment in a proportion of patients is underlined by rises in both AMH and AFC (Fig. 2A and B) as larger antral follicles (up to 8–10 mm) are the main contributors to circulating serum AMH (Jeppesen *et al.*, 2013; Kristensen *et al.*, 2018). Silber and colleagues (Silber *et al.*, 2018) recently reported a similar discovery of rising serum AMH levels around 200 days after autotransplantation of thawed cryopreserved ovarian tissue. This supports our finding of an increase in serum AMH in a subgroup of our patients, when considering that the tissue autotransplanted in Silber *et al*. was thawed and not fresh tissue which is the case in this study.

Our decision to start ovarian stimulation at the first menstrual cycle 10 weeks after the procedure was based on experience from Schmidt *et al*., who found that ovarian function returned 8–19 weeks after transplantation of thawed cryopreserved ovarian tissue in cancer patients (Schmidt *et al.*, 2005). *In vivo*, the transition from primordial to the early antral stage (2 mm) takes around 6 months (Gougeon, 2010). One could speculate, whether the same time span applies when the ovarian tissue has been manipulated in one way or another. Therefore, an ovarian stimulation started around Weeks 5–8 may have resulted in a more positive response, but this knowledge was only established in hindsight. Other previous studies on transplantation of thawed cryopreserved ovarian tissue fragments in cancer survivors have revealed a recovery time span of ovarian function from 71 days to 5 months for follicles to emerge and 80 days to 6.5 months for menstrual cycle to recommence (Reviewed in Donnez *et al.*, 2013). Since development of primordial follicles to primary and early secondary follicles is independent of FSH (Edson *et al.*, 2009; Tingen *et al.*, 2009), a FSH-independent activation must take place e.g. induced by the preparation of the fragments before autotransplantation in cancer survivors. In women with POI, evident follicle growth, both spontaneous and stimulated, was seen as early as 20 days after IVA and autotransplantation of cortical ovarian tissue (Kawamura *et al.*, 2013; Zhai *et al.*, 2016; Fabregues *et al.*, 2018). These previous studies support that a temporary rise in both AMH and AFC could occur 34–72 days after the procedure, suggesting growth of follicles beyond the primordial stage. Based on xenografting of human ovarian tissue in mice, Kawamura *et al*. hypothesized that these early visible follicles in the POI women originated from secondary follicles (Kawamura *et al.*, 2013). Patients recruited in the present study were menstruating and not POI women, so maybe the rise was derived from later stages than secondary follicles.

Despite long-term follow-up up to 408 days, follicle growth in the autotransplanted tissue was only observed in three patients; therefore, we must conclude that autotransplantation of fragmented ovarian cortex does not increase the number of recruitable follicles. In one of these patients, no follicles were found in the histological preparations of one of the four cortical pieces excised, indicating that the number of remaining follicles probably was low. And she did not conceive. Furthermore, as mentioned, only one patient had an ectopic follicle that responded to ovarian stimulation. Even in women with normal ovarian reserve, primordial follicles are unevenly distributed and are grouped in clusters in the cortex (Schmidt, 2003; Da Silva-Buttkus *et al.*, 2009); thus, some of the fragments autotransplanted in this study could potentially have contained very few follicles. Furthermore, Soleimani *et al*. stated that revascularization of xenotransplanted ovarian tissue takes 10 days, and therefore some follicles will inevitably be lost to ischemia (Soleimani *et al.*, 2011). These factors contribute to a poor follicle development in the grafted tissue. One patient had a surprisingly high follicular density on 97.3 follicles/mm^3 despite a clear DOR phenotype with low AFC and low serum AMH; thus, we speculate whether her genotype is an FSH receptor mutation induced DOR (Meduri *et al.*, 2003; Ghezelayagh *et al.*, 2016).

Other parameters that may influence follicular survival in the ovarian fragments are the transplantation site and size of fragments. Data from Meirow *et al.* (2005) indicate that fragments can be too small to support follicle survival during the period in which revascularization takes place. However, in an animal study apoptosis was less prevalent in the smaller ovarian fragments (Gorricho *et al.*, 2018). Our decision to prepare fragments of 1 mm^3 was based on the experience by Kawamura *et al.* (2013). We chose the right ovarian fossa as site of transplantation due to laparoscopic accessibility, high vascularity and ease to retrieve oocytes from mature follicles. No golden standard has yet been established for the site of transplantation (Donnez *et al.*, 2004; Demeestere *et al.*, 2009).

Regarding pregnancy rates, our results are favourable, with a cumulated live birth rate (LBR) per started stimulated IVF/ICSI cycle of 18.4% (7/38). Fifteen of our 20 patients fulfilled the Bologna criteria for POR, and in general, our patients had a baseline mean AMH (0.3 ng/mL) below the lower Bologna AMH cut-off (0.5 ng/mL). In recent large studies of patients with POR, LBR was in the range 10.6–14.6% (Humaidan *et al.*, 2017; Yakin *et al.*, 2018). However, several other studies indicate that an LBR around 10% per treatment cycle is to be expected in women fulfilling the Bologna criteria (Busnelli *et al.*, 2015; La Marca *et al.*, 2015; Bozdag *et al.*, 2017).

The apparent overall good pregnancy rates in our study could theoretically be related to a change in oocyte quality and rescue of oocytes destined for atresia caused by alterations of the ovarian milieu. The theory may be supported by the findings that, following the procedure, levels of androstenedione and testosterone increased significantly, though not persistently. An *in vitro* study has shown that androstenedione is essential for oocyte growth and promotes meiotic division of the oocytes (Taketsuru *et al.*, 2012). In animal studies, androgens reduce atresia by increased expression of anti-apoptotic substances, stimulate primary to secondary follicle transition and moreover increase the number of FSH receptors (Yang and Fortune, 2006; Nielsen *et al.*, 2011; Sen *et al.*, 2014). Even in humans, a positive correlation between androgens and the number of mature and fertilized oocytes and developed embryos was found (Ferrario *et al.*, 2015; Chern *et al.*, 2018). Similarly, in our study we found that AMH serum levels correlated with androstenedione. This suggests that in women with follicular activity represented by higher AMH, androstenedione was correspondingly high. However, not all studies are unambiguous (Abide Yayla *et al.*, 2018). Other studies support the suggested rescue of oocytes destined for atresia by showing that mechanical stress regulates both meiosis II and meiotic maturation in the oocyte through actin and myosin II (Larson *et al.*, 2010; Shah *et al.*, 2018).

Limitations of the study were the limited number of patients included and the absence of an untreated control group. However, we did incorporate a control design in the study as we had the contralateral ovary as an intra-individual control ovary. Nevertheless, we acknowledge

the need for a future randomized controlled study to make clinically applicable conclusions. As sonography is subjective, we cannot exclude that the bilateral increase in AFC over time was due to physicians being biased, especially concerning the smallest follicles (2 mm) which may be difficult to record accurately. However, the concomitant increase in serum AMH in a subgroup of the patients supports an actual AFC increase. An additional limitation of the study was the discovery of a Turner mosaicism in one of the 20 patients. Due to the low number of mosaic cells, she remained in the study but did not have long-term follow-up. Furthermore, we deviated from the inclusion criterion on AFC ≤ 5 due to recruitment challenges. Patients with AFC > 5 were carefully handpicked with a mean of 4.2 MAR treatments (range 1–7) prior to inclusion, compared with 4.9 MAR treatments (range 0–9) in those with AFC ≤ 5, and consecutive baseline AMH serum levels <5 pmol/L (< 0.7 ng/mL) which did not differ from patients with AFC ≤ 5. Since pregnancy occurred in two of the patients with AFC > 5 (both with male partner) and in 10 patients with AFC ≤ 5, we do not believe that the inclusion of these five patients has affected our results (see Supplementary Table SI).

Nine out of 20 women included had no male partner at inclusion. However, each woman had on average 6.8 (range 4–9) MAR treatments with donor sperm prior to inclusion. Only one of the nine had not tried IVF with donor sperm prior the study; however, she had four previously failed DI treatments and did not become pregnant by the IVF/ICSI treatment offered in the study.

With a small study population, the risk of a type 2 error is substantial. This could mean that we were not able to detect a genuine effect of the procedure. Finally, the study was not designed to distinguish whether the oocyte that resulted in a pregnancy originated from the biopsied ovary, which would help to distinguish between a direct or indirect effect.

In conclusion, we were not able to show any direct effect of the procedure in the number of mature follicles in the biopsied ovary or at the site of transplantation after maximal ovarian stimulation. Thus, we did not find any convincing sign of the Hippo pathway actions 10 weeks after fragmentation. At this point, in our opinion, autotransplantation of the fragmented ovarian cortex should not be offered as treatment to patients with DOR. We do, however, suggest that there may be an effect with growth of more early follicles around 5–8 weeks after the procedure in a proportion of patients. Together with the fairly high number of pregnancies in 12 out of 20 women with a cumulated LBR of 18.4% (7/38) per started IVF/ICSI cycle, this warrants further studies on the possible effects of biopsying *per se* of ovarian cortical tissue in patients with DOR.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

A.N.A., K.T.M., A.T.P. and C.Y.A. planned the study. S.A.L., A.N.A, K.T.M., A.T.P., S.E.P., S.G.K. and A.P. executed the study. S.A.L., A.N.A., K.T.M. and S.K.L. collected the data. S.A.L. and E.M.F. did the statistical analyses. S.A.L. wrote the first draft of the manuscript. All authors participated in the critical discussion of the findings and revision of the manuscript.

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Conflict of interest

No conflicts of interest to declare.

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