Female reproductive ageing

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Chapter 7

Ovarian reserve and the morphology of preimplantation embryos of women of advanced reproductive age

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ABSTRACT

Background: Female reproductive ageing is characterised by a decrease in the quantity and quality of a woman's oocytes. The nature of the relation between oocyte quantity and quality is still unclear. To address this issue, we assessed the predictive value of five ovarian reserve parameters, representing oocyte quantity, for embryo morphology, which represents oocyte quality.

Methods: Prospective cohort of women aged 35-41 years undergoing their first IVF treatment in four Dutch clinics. Antral follicle count (AFC), anti-Müllerian hormone (AMH), follicle stimulating hormone, inhibin B, and the number of retrieved oocytes were assessed as ovarian reserve parameters. The main outcome measure was a morphological top quality embryo on day 3 of development.

Results: We included 294 women in this analysis. None of the ovarian reserve parameters had statistically significant predictive value for the chance that an oocyte would develop into a top quality embryo. The number of retrieved oocytes was strongly related with the chance that a patient had at least one top embryo. Additionally, women with high AFC and AMH levels had at least one top embryo significantly more often than women with low AFC or AMH levels.

Conclusions: Our findings indicate that ovarian reserve is not correlated with embryo quality. Women with higher ovarian reserve do significantly more often have at least one top quality embryo, but this is simply due to the fact that they generate more oocytes in an IVF cycle.

INTRODUCTION

Female reproductive ageing is characterised by a decrease in both the quantity and quality of a woman's oocytes[7;8;231]. The decline in oocyte quantity ultimately leads to menopause, the reproductive event representing depletion of the ovarian follicle pool[230]. Among women of the same age, ovarian reserve may vary considerably, a phenomenon reflected in the wide age range for the onset of menopause[104]. To assess the ovarian reserve, several ovarian reserve tests (ORTs) have been developed. Most ORTs are endocrine parameters, such as anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH) and inhibin B, or sonographic tests, such as antral follicle count (AFC). The number of follicles that develop in response to ovarian hyperstimulation during in vitro fertilisation (IVF) treatment can be regarded as a dynamic ovarian reserve test[72].

Years before menopause, fertility is already severely impaired: the chance to conceive gradually declines, while the chance of a chromosomally abnormal conception and miscarriage increases[117]. This phenomenon is attributed to a decrease in oocyte quality[7;230;231]. Compared to oocyte quantity, oocyte quality is a less solid characteristic and as such more difficult to assess clinically. Since the oocyte is a key player in early embryo development[127], embryo morphology may be used as a marker for oocyte quality.

The nature of the relation between oocyte quantity and oocyte quality as the two components determining reproductive capacity is still unclear[53;232]. Women with a diminished ovarian reserve are suggested to have reduced oocyte quality compared to their peers with normal ovarian reserve[8]. To address this issue, we studied the relation between five ovarian reserve parameters, representing oocyte quantity, and embryo morphology, representing oocyte quality, in a cohort of women aged 35-41 years undergoing IVF treatment. We aimed to determine whether parameters of ovarian reserve have a predictive value for the embryo morphology.

MATERIAL AND METHODS

Study design

All women participating in this study were enrolled in a randomised controlled trial evaluating the efficacy of preimplantation genetic screening (PGS). Details on the study design and outcome can be found elsewhere[253].

Briefly, women aged 35 to 41 years, starting their first IVF treatment in one of four Dutch clinics (Academic Medical Center, Onze Lieve Vrouwe Gasthuis in Amsterdam, University Medical Center Groningen and Medical Center Leeuwarden, all in the Netherlands) were eligible for the study. After providing written informed consent, the women were randomly assigned to undergo three IVF cycles either with of without PGS. For the present analysis we used data on embryo morphology scored on day three after follicle aspiration, just before biopsy (if applicable); all participants, whether assigned to PGS or not, underwent the same treatment up to that moment and were included in our study.

The study protocol was approved by the institutional review boards of all participating clinics and by the Central Committee on Research Involving Human Subjects in the Netherlands.

Ovarian reserve tests

The AFC of the participants agreeing to undergo ORTs was assessed in the early follicular phase of a natural cycle preceding treatment (cycle days 2-4). AFC was defined as the sum of antral follicles present in both ovaries measuring 2-10 mm on transvaginal ultrasound. Women with ovarian cysts were excluded. On the same day serum samples were drawn and stored at -20°C. After closure of the trial, serum levels of AMH, inhibin B and FSH were determined. AMH concentrations were measured using an in-house double-antibody enzyme-linked immunosorbent assay (ELISA), with intra- and interassay coefficients of variance of <5% and <10% respectively[254]. Dimeric inhibin B concentrations were assessed using an immunoenzymometric assay obtained from Serotec (Oxford, UK). Intra- and interassay coefficients of variance were less than 7% and 14%, respectively. FSH concentrations were determined with an electrochemiluminescence immunoassay using the Elecsys 2010 automated multi-analyser (Roche Diagnostics Nederland BV, Almere, the Netherlands). Intra- and interassay coefficients of variance were <3% and <5% respectively.

Treatment was not postponed to perform these ovarian reserve tests. If the patient preferred to start treatment as soon as possible and logistics allowed this, no natural cycle was awaited to perform the ovarian reserve tests.

Treatment protocol

The stimulation protocol included ovarian down-regulation with a gonadotropin-releasing hormone agonist (triptorelin; Decapeptyl®, Ferring, Hoofddorp, the Netherlands) starting in the mid-luteal phase. Ovarian hyperstimulation was conducted with recombinant FSH (rFSH) (follitropin; Gonal-F®, Merck-Serono International S.A., Geneva, Switzerland) starting on cycle day 5, at an initial dose of 150 IU. During the stimulation phase this dose was adjusted if appropriate. Follicular aspiration was performed 36 hours after ovulation induction with 10,000 U of human chorionic gonadotropin (Pregnyl®, Organon, Oss, the Netherlands). The oocytes were inseminated with 10,000 progressively motile spermatozoa (IVF) or injected with a single spermatozoon (intracytoplasmic sperm injection, ICSI) approximately 4 hours after follicular aspiration. Treatment protocols were the same for all participants up to the third day after follicle aspiration.

Embryo morphology

For the present analysis we used data on embryo morphology scored on day 3 after follicle aspiration, but before biopsy (if applicable). Embryos were assessed for number and regularity of blastomeres, percentage of fragmentation and presence of multinucleated blastomeres. Embryos were given a score of 1 to 4 depending on the regularity of blastomeres, percentage of fragmentation and the

presence of multi-nucleated blastomeres; the highest score represented the best morphological features[128]. An embryo was considered a top embryo if the product of the number of blastomeres and the embryo score, i.e. the cumulative embryo score, exceeded 23; for instance an eight cell embryo with less than 20% fragmentation (score 3) had a cumulative embryo score of 24[255]. For each participant we only used data from the first IVF cycle that resulted in follicle aspiration.

Statistics

We analysed the question whether parameters of ovarian reserve have a predictive value for embryo morphology on an oocyte and a patient level. We assessed the predictive value of parameters of ovarian reserve for (1) the chance that an oocyte will develop into a top embryo and (2) the chance that a patient has at least one top embryo on day 3 after follicle aspiration. We chose the outcome measure of top embryo as a reflection of embryo morphology to enable dichotomous analysis of the data. We chose the outcome measure 'at least one top embryo' instead of the number of top embryos per woman, since the distribution of this variable was too skewed. We analysed five parameters of ovarian reserve, i.e. AMH, FSH, inhibin B, AFC and the number of oocytes retrieved at follicle aspiration. Only patients with at least one retrieved oocyte at their first follicle aspiration and with results of at least one ovarian reserve test were used for the analyses.

We performed generalised estimating equations (GEE) to calculate the OR for an oocyte to develop into a top embryo; GEE takes the correlation between oocytes from the same patient into account. Next, we performed logistic regression analysis to calculate the OR for a patient having at least one top embryo on day 3 after follicle aspiration. In both analyses we calculated crude ORs and ORs adjusted for female age and total dose of rFSH (in IU), to correct for a possible influence of these values. Data were analysed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

RESULTS

In total, 408 patients were included of which 395 proceeded to follicle aspiration. One or more oocytes were retrieved at the first follicle aspiration in 388 patients. For 294 of them (75.8%), we had the results of at least one ovarian reserve test; these 294 women were included in the present analysis.

Women without ovarian reserve test results did not differ significantly from women with ovarian reserve test results for age, number of oocytes retrieved or number and proportion of top embryos (data not shown). Median total rFSH dose was lower in the women without ovarian reserve test results (2100 vs 2400 IU, $P<0.01$). However, the range of total rFSH dose used by the women without ovarian reserve test results fell within the range of total rFSH dose used in the study population.

In the study population the parameters of ovarian reserve related to each other as expected. The number of retrieved oocytes was statistically significantly correlated to AMH ($r = 0.59$), AFC ($r = 0.36$), FSH ($r = -0.33$) and inhibin B ($r = 0.24$) (P-values all <0.001).

Table I shows the basic characteristics of the study population. In total, 2386 oocytes were retrieved, of which 1568 fertilised (66.5%) and 295 developed into top embryos (12.4%). Table II shows the ORs of the five ovarian reserve parameters for the chance that an oocyte will develop into a top embryo. We found no significant relation between the ovarian reserve parameters and the chance of an oocyte developing into a top embryo.

Table III shows the predictive value of the parameters of ovarian reserve for the chance that a patient will have at least one top embryo on the third day after follicle aspiration. Patients in the highest

Table I. Patient and treatment characteristics

	N	Median or number*	10^{th}-90^{th} percentiles or percentage*
Patient characteristics			
Age at oocyte retrieval (years)	294	38.0	35.8-40.2
Smoking	284	59	20.8%
Body mass index (kg/m^2)	255	23.5	20.2-29.7
Main cause of infertility	294		
Tubal		59	20.1%
Male		105	35.7%
Unknown		101	34.4%
Other		29	9.9%
Ovarian reserve tests			
Anti-Müllerian hormone (µg/l)	284	1.21	0.31-3.47
Follicle stimulating hormone (IU/l)	285	8.27	5.44-14.32
Inhibine B (ng/l)	284	95	36-163
Antral follicle count	273	12	5-21
Number of retrieved oocytes	294	7	2-15
Treatment characteristics			
Total dose recombinant FSH (IU)	294	2400	1500-4594
Duration of stimulation (days)	294	13	10-17
Mode of fertilisation	294		
In vitro fertilisation		208	70.7%
Intracytoplasmic sperm injection		86	29.3%
At least one top embryo available	294	146	49.7%
Median number of top embryos	294	0	0-3

*For continuous variables median and 10^{th} to 90^{th} percentiles are presented; for categorical variables number and percentage are presented.

quartile of AMH (>2.22 µg/l) had a significantly higher chance of having at least one top embryo compared to patients in the lowest AMH quartile (<0.54 µg/l). After adjustment for rFSH dosage and age, this difference remained significant (OR 2.51, 95% CI 1.22-5.17). For AFC we found comparable results: patients in the highest quartile of AFC (>15) had a significantly higher chance of having at least one top embryo compared to patients in the lowest AFC quartile (<9), even after adjustment for rFSH dosage and age (OR 2.79, 95% CI 1.33-5.88). The number of retrieved oocytes was strongly

Table II. Odds ratios for an oocyte to develop into a top embryo

		Unadjusted OR			OR adjusted for rFSH* dose and age		
	*N***	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Anti-Müllerian hormone (µg/l)	2323						
< 0.54		1.00	Ref	-	1.00	Ref	-
0.54 - 1.21		0.58	0.34 -1.00	0.05	0.57	0.33-0.98	0.04
1.22 - 2.22		1.02	0.61-1.69	0.94	0.98	0.59-1.63	0.95
> 2.22		0.82	0.51-1.32	0.41	0.77	0.47-1.27	0.31
Follicle stimulating hormone (IU/l)	2330						
< 6.86		1.00	Ref	-	1.00	Ref	-
6.86 - 8.27		0.80	0.51-1.27	0.35	0.81	0.51-1.28	0.36
8.28 - 10.36		0.89	0.57-1.40	0.62	0.90	0.57-1.41	0.63
>10.36		0.82	0.47-1.41	0.46	0.85	0.47-1.53	0.59
Inhibin B (ng/l)	2323						
< 67		1.00	Ref	-	1.00	Ref	-
67 - 94		1.23	0.75-2.02	0.42	1.22	0.74-2.02	0.43
95 – 130		1.28	0.79-2.06	0.32	1.26	0.78-2.03	0.35
>130		1.33	0.82-2.14	0.25	1.30	0.80-2.12	0.30
Antral follicle count	2219						
< 9		1.00	Ref	-	1.00	Ref	-
9-12		0.68	0.39-1.17	0.16	0.67	0.39-1.16	0.15
13-15		0.94	0.57-1.54	0.80	0.91	0.55-1.53	0.73
>15		1.27	0.77-2.10	0.35	1.24	0.74-2.07	0.42
Number of retrieved oocytes	2386						
< 5		1.00	Ref	-	1.00	Ref	-
5-7		0.88	0.50-1.54	0.64	0.86	0.49-1.51	0.60
8-11		0.69	0.41-1.17	0.17	0.65	0.39-1.09	0.11
>11		0.77	0.47-1.29	0.32	0.73	0.44-1.21	0.22
Age at oocyte retrieval (years)	2386						
< 36.7		1.00	Ref	-			
36.8 - 37.9		0.89	0.54-1.47	0.65			
38.0 – 39.4		0.84	0.53-1.33	0.46			
> 39.4		1.01	0.65-1.58	0.96			
Total dose of recombinant FSH (IU)	2386						
< 1800		1.00	Ref	-			
1800-2374		1.43	0.90-2.25	0.13			
2375-3300		1.37	0.88-2.14	0.16			
> 3300		0.90	0.54-1.48	0.67			

*rFSH = recombinant follicle stimulating hormone; ** N = number of oocytes. In these analyses the correlation between oocytes from the same patient was taken into account. The number of patients was equal to the number of patients described in Tables I and III.

related to the chance of having at least one top embryo. Compared with the lowest quartile (<5 oocytes), women in the three higher quartiles had a significantly higher chance of having at least one top embryo. For the second quartile (5-7) the adjusted OR was 2.05 (95% CI 1.05-3.97), for the third quartile (8-11) it was 2.78 (1.40-5.52), and for the highest quartile (>11) it was 5.09 (2.43-10.63).

DISCUSSION

In our study population of women of advanced reproductive age, ovarian reserve parameters had no significant predictive value for the chance that an individual oocyte would develop into a morphologically top quality embryo. Higher values of anti-Müllerian hormone, antral follicle count and especially the number of retrieved oocytes did have predictive value for the chance that a patient would have at least one top embryo available for transfer, even after correction for female age and medication dose.

The ovarian reserve of a woman at any moment in her fertile life span mainly depends on the number of follicles she is born with and the rate of atresia of those follicles throughout life[22]. Based on studies that show a large agreement between the ages at menopause of mothers and daughters, it is assumed that genetic factors determine the size of the foetal follicle pool and its atresia rate[31;33;35]. Independent of the age-related decrease in follicle numbers, the increased rate of miscarriage and trisomic pregnancies with age may be caused by biological damage to the oocytes accumulating over the years[57;61;67]. The older a woman gets, the longer the exposure time and the larger the chance that individual oocytes become damaged. Other theories state that the processes of decreasing oocyte quantity and quality are in fact related. Physiological changes accompanying the depletion of the ovaries, such as an increase in FSH levels or impairment in ovarian blood flow, may hypothetically harm the residual oocytes[64;65;68]. Alternatively, the simple fact that fewer oocytes are present over the years may result in the selection of oocytes of lesser quality; if the absolute number of oocytes is limited, it is readily conceivable that in some cycles there may be no good quality oocytes available to choose from[63;232]. This latter hypothesis is in agreement with our findings. The chance of having at least one top quality embryo decreased linearly with declining oocyte numbers. The fact that higher levels of AMH and AFC were also related to the availability of at least one top embryo is explained by the fact that AMH and AFC are positively related to the ovarian response in IVF treatment.

Studies assessing the relation between oocyte quantity and quality are hampered by the lack of straightforward tests that can measure these entities directly. Determining the 'true' ovarian reserve by counting all follicles present in the ovaries is only possible by elaborate histological study following surgery. Ovarian reserve tests estimating oocyte quantity can be used in clinical practice, but have well-described drawbacks. For instance, FSH and inhibin B levels fluctuate within

and between menstrual cycles, and increased FSH levels have more causes than decreased ovarian reserve alone[75-77;194]. AFC supposedly overestimates the total number of follicles in women with reduced ovarian reserve as a relatively larger proportion of follicles is recruited monthly[19;107]. AMH is the most promising ovarian reserve test with steady values throughout the natural cycle and an age-related decrease in longitudinal studies[93;96;97;256]. Both AMH and AFC are related to the ovarian response in IVF treatment[111]. The number of retrieved oocytes in conventional IVF has convincingly been shown to be related to the size of the remaining follicle pool; women with a poor ovarian response in IVF treatment are at increased risk of early menopausal transition and menopause[109;110]. However, the exact number of oocytes retrieved from the same woman during consecutive IVF cycles varies considerably**.** Taking into account the shortcomings of the parameters of ovarian reserve, we cannot exclude the possibility that we may have missed a subtle relation between ovarian reserve and embryo morphology in our study.

An important advantage of our study was our population of women of 35-41 years of age, since the incidence of decreased ovarian reserve is higher in women of advanced reproductive age compared to the general IVF population. For instance, table III shows that one quarter of the participants had less than five retrieved oocytes at follicle aspiration, which is a high number of low responders compared to the younger IVF population[152]. If a relation between ovarian reserve and embryo quality does exist, this is the proper population to find it in.

Compared to oocyte quantity, oocyte quality is a less well-defined term and as such even harder to estimate correctly. The oocyte has a key role in early embryo development, since the genome of the embryo is not activated before day 3 (the day of our morphological assessment) and development up to that point largely depends on proteins and RNA stored in the oocyte[127]. Therefore it seems reasonable to use embryo morphology as a reflection of oocyte quality[128].

Another parameter suggested to reflect oocyte quality is embryonic aneuploidy, since miscarriage rates and the incidence of children born with chromosomal abnormalities increase with age[7;119]. However, recent studies have shown that the chromosomal analysis of a single cell from a cleavage stage embryo does not reflect the rest of the embryo. Because of this phenomenon, known as chromosomal mosaicism[131;132;257], in our view blastomere biopsy and analysis are not a suitable parameter for representing oocyte quality.

Ongoing pregnancy chances can be viewed as the main clinical parameter for oocyte quality, since diminished oocyte quality is held responsible for the lower pregnancy chances and increased miscarriage rates with age. However, systematic reviews show that ovarian reserve tests, such as FSH and AFC, have no clear predictive value for ongoing pregnancy after IVF[72;134], whereas the number of oocytes retrieved in conventional IVF treatment has been shown to be related to ongoing pregnancy chances. Women with a poor ovarian response to hyperstimulation have lower pregnancy chances than their peers with a normal response[140;258]. We found that AMH and AFC, but especially the number of retrieved oocytes were predictive for the chance of having at least one top

Table III. Odds ratios of a patient having at least one top embryo

		Unadjusted OR			OR adjusted for rFSH* dose and age		
	N	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Anti-Müllerian hormone (µg/l)	284						
< 0.54		1.00	Ref	-	1.00	Ref	-
0.54 - 1.21		0.93	0.48-1.83	0.84	0.90	0.45-1.79	0.76
1.22 - 2.22		1.91	0.98-3.72	0.06	1.75	0.88-3.47	0.11
> 2.22		2.73	1.39-5.37	<0.01	2.51	1.22-5.17	0.01
Follicle stimulating hormone (IU/l)	285						
< 6.86		1.00	Ref	-	1.00	Ref	-
6.86 - 8.27		1.03	0.53-1.98	0.94	1.05	0.54-2.03	0.89
8.28 - 10.36		1.26	0.65-2.44	0.50	1.31	0.67-2.57	0.43
>10.36		0.53	0.27-1.04	0.06	0.63	0.31-1.28	0.20
Inhibin B (ng/l)	284						
< 67		1.00	Ref	-	1.00	Ref	-
67 - 94		1.06	0.55-2.05	0.87	1.02	0.52-1.99	0.95
95 - 130		1.49	0.77-2.88	0.24	1.33	0.68-2.61	0.41
>130		1.40	0.73-2.72	0.31	1.22	0.62-2.40	0.57
Antral follicle count	273						
< 9		1.00	Ref	-	1.00	Ref	-
9-12		0.98	0.49-1.95	0.95	0.90	0.45-1.82	0.78
13-15		1.54	0.80-2.98	0.20	1.31	0.65-2.65	0.45
>15		3.15	1.54-6.44	<0.01	2.79	1.33-5.88	<0.01
Number of retrieved oocytes	294						
< 5		1.00	Ref	-	1.00	Ref	-
5-7		2.10	1.09-4.05	0.03	2.05	1.05-3.97	0.03
8-11		2.90	1.50-5.62	<0.01	2.78	1.40-5.52	<0.01
>11		5.53	2.69-11.34	<0.01	5.09	2.43-10.63	<0.01
Age at oocyte retrieval (years)	294						
< 36.7		1.00	Ref	-			
36.8 - 37.9		0.79	0.41-1.54	0.49			
38.0 - 39.4		1.08	0.57-2.06	0.82			
> 39.4		0.97	0.51-1.85	0.93			
Total dose of recombinant FSH (IU)	294						
< 1800		1.00	Ref	-			
1800-2374		1.19	0.61-2.34	0.61			
2375-3300		1.36	0.71-2.62	0.35			
> 3300		0.60	0.31-1.16	0.13			

*rFSH = recombinant follicle stimulating hormone

embryo available for transfer. Since our study shows that the chance that an oocyte will develop into a top quality embryo does not differ between women with a normal or decreased ovarian reserve, the absolute number of top embryos solely depends on the number of oocytes retrieved. A woman with a poor response due to decreased ovarian reserve will have similar chances of having a top embryo as a woman with a 'coincidental' poor response, for instance due to relative underdosing of rFSH. This is in line with studies showing that all poor responders have low pregnancy chances regardless of whether they were expected or unexpected poor responders. The difference between

'true' poor responders and 'coincidental' poor responders lies in their perspectives: the chance that a 'true' poor responder has another poor response with the associated low pregnancy chances in the next cycle is high compared to the 'coincidental' poor responder's chance[112;139;152].

The relation between ovarian reserve and embryo morphology has been studied before[148;166-169]. None of these previous studies, however, combined a prospective design, large study population, use of multiple distinctive parameters of ovarian reserve, or the analysis of the relation at both oocyte and patient level. Our findings are in accordance with previous studies on the subject by Lekamge *et al*., Ebner *et al*. and Lie Fong *et al.*[148;166;167]. They showed that, in conventional IVF treatment, AMH does not predict embryo morphology at oocyte level. De Sutter *et al.* found no difference in the proportion of embryos of good morphological quality between women with a low number of retrieved oocytes in conventional IVF (<5) compared to normal responders[152]. Silberstein *et al.* found that AMH, but not FSH, was associated with the morphological qualities of the embryos transferred; this is in line with our finding that the chance of having at least one morphologically top embryo is increased in women with higher AMH levels[169]. Smeenk *et al.* found no predictive value of various ovarian reserve tests for the morphology of transferred embryos[168]. However, a possible relation could well have been missed due to the size of their study population (N=80). Both Smeenk *et al.* and Silberstein *et al.* did not comment on the proportion of morphological top embryos in the total oocyte yield and the predictive value of the number of retrieved oocytes in embryo morphology.

In conclusion, our study shows that parameters of ovarian reserve had no predictive value for the chance that an oocyte would develop into a top quality embryo. The higher the values of AMH, AFC and especially the number of oocytes retrieved, the higher the chance that at least one top embryo would be available for transfer. Our study indicates that the proportion of top embryos in conventional IVF treatment is not related to a woman's ovarian reserve, but the absolute number is.