Female reproductive ageing

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Chapter 2

The number of small antral follicles (2–6 mm) determines the outcome of endocrine ovarian reserve tests in a subfertile population

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ABSTRACT

Background: Ovarian reserve is related to age and can be estimated by ovarian reserve tests (ORTs), such as antral follicle count (AFC) and various endocrine parameters. The endocrine function of a follicle is related to its size. The aim of this study is to evaluate which sizes of antral follicles are most closely correlated with age and the outcome of endocrine ORTs.

Methods: In total 474 subfertile, ovulatory patients, recruited from two fertility centers in the Netherlands, participated in this prospective cohort study. The following ORTs were performed: AFC (follicles from 2 to 10 mm), basal follicle stimulating hormone, basal inhibin B (bInhB), clomiphene citrate challenge test and inhibin B after stimulation with clomiphene citrate.

Results: The number of small follicles (2–6 mm) declined with age; the number of larger follicles (7–10 mm) remained constant. Independent of age, the number of small follicles was significantly related to all ORTs ($P<0.001$, except bInhB $P=0.005$). The number of larger follicles was only significantly related to bInhB ($P=0.009$).

Conclusions: The number of small antral follicles (2–6 mm) is significantly related to age and also, independent of age, to all endocrine ORTs tested, suggesting the number of small antral follicles represents the functional ovarian reserve.

INTRODUCTION

Reproductive ageing is considered to be the consequence of a decrease in quantity and quality of the ovarian follicle pool[8]. Autopsy studies in human ovaries show that the number of follicles decreases rapidly with female age, starting in foetal life and continuing until after menopause[15;19;174]. However, between women of the same chronological age the quantitative ovarian reserve may vary substantially[104]. To assess the individual quantitative ovarian reserve, during the last decades various ovarian reserve tests (ORTs) have been developed, which can roughly be divided into three groups. Most ORTs measure early follicular phase hormone levels, such as serum follicle stimulating hormone (FSH)[175;176], estradiol (E2)[177;178] and inhibin B[179;180]. Recently, anti-Müllerian hormone (AMH) has been added as a promising ORT[85;86;93]. Dynamic ORTs assess the endocrine response of the ovaries to exogenous stimuli. Examples of dynamic tests are the clomiphene citrate challenge test (CCCT)[81;82], the exogenous FSH ovarian reserve test (181) and the GnRH agonist stimulation test[182;183]. The third group of ORTs consists of sonographic parameters, such as the antral follicle count (AFC)[184;185] and measurement of the ovarian volume[186;187].

The way an AFC is performed differs between centres. Most often follicles of 2–5 mm or 2–10 mm are counted. Research in various fertile and IVF-treated populations demonstrates a close relation of AFC with age[105;106;188;189]. In the studies commenting on AFC and age, only total AFC is calculated and the different sizes of the individual follicles are not reflected on. The distinction of various size categories may be relevant since several studies show that the endocrine function of a follicle is related to its size. For instance, AMH is mainly produced by pre-antral and smaller antral follicles up to 4–6 mm[90]. In various studies, AMH is strongly associated with total AFC[85;86;93]. These data suggest that the number of smaller antral follicles up to 6 mm reflects ovarian reserve better than the total AFC, if follicles of 2–10 mm are counted. Magoffin and Jakimiuk[190] show that the production of inhibin B by the antral follicles increases with the size of the follicle up to 13 mm. Thus, the larger antral follicles may also be an important reflection of the remaining follicle pool. The relation between dynamic endocrine ORTs and antral follicle size has not been studied.

As part of a prospective study addressing the predictive value of ORTs for spontaneous and treatment-related pregnancy in a subfertile population, we examined which sizes of antral follicles are most closely associated with female age and which sizes of antral follicles determine the outcome of various endocrine ORTs.

MATERIALS AND METHODS

Study population

From December 1999 to July 2003, patients were recruited at the fertility centers of the University Medical Center Groningen, a tertiary fertility center, and the Martini Hospital, a teaching hospital, both in Groningen, the Netherlands. Patients were asked to participate after basal subfertility evaluation

including sonographic cycle analysis and measurement of midluteal progesterone, semen analysis, postcoital test and hysterosalpingography (HSG). Inclusion criteria were: (i) subfertility for at least 12 months, (ii) regular ovulatory cycle with a midluteal progesterone of >30 nmol/l and a cycle length between 21 and 42 days, (iii) at least one patent Fallopian tube at HSG and/or laparoscopy, (iv) semen analysis of the partner with a total motile count (volume x concentration x motility) >1 x 10^6, (v) no ovarian cysts, (vi) no history of gynaecological, hypothalamic or pituitary malignancy, (vii) no liver insufficiency, (viii) no history of thrombo-embolic processes and (ix) no sexual disorder leading to a coitus frequency of less than once a month. According to the inclusion criteria, 732 patients were eligible for this study (see flowchart, Fig. 1). One hundred thirty-six patients did not want to be enrolled in the study, and 27 patients were not asked to participate due to inadvertency. Fifty-four patients were excluded secondarily since eventually they did not meet the inclusion criteria; the main reason for this was two-sided tubal pathology on laparoscopy because of adhesions or severe endometriosis, despite one or two patent tubes on HSG. Furthermore, 32 patients became pregnant just after inclusion but before undergoing ORTs and 9 patients did not return after inclusion. In total, 474 patients actually participated, 353 in the University Medical Center Groningen and 121 in the Martini Hospital. Informed consent was obtained from all participants. The study protocol was reviewed and approved by the Medical Ethics Committee for Research Projects of both participating hospitals.

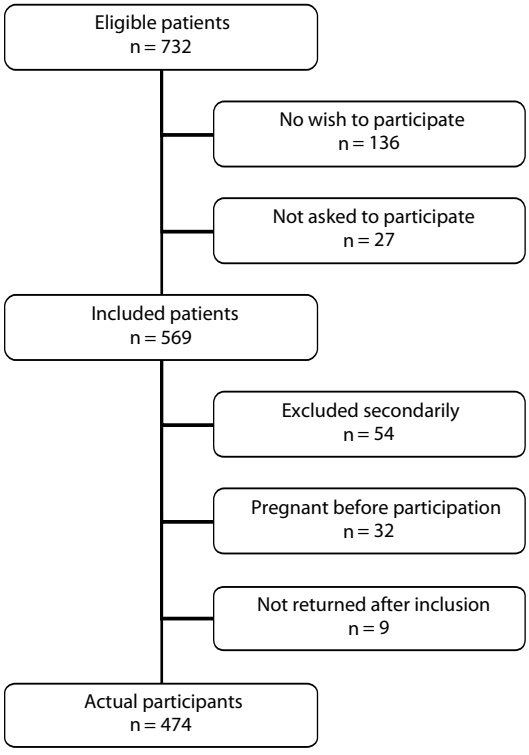

Figure 1. Flowchart of eligible patients

Study protocol

After inclusion, patients visited the outpatient clinic in the early follicular phase of the menstrual cycle (cycle day 2, 3 or 4). Transvaginal ultrasound was performed to measure size and number of the antral follicles as well as the volume of both ovaries. Peripheral blood was drawn to measure levels of basal FSH (bFSH) and basal inhibin B (bInhB). Subsequently, patients took 100 mg clomiphene citrate on cycle day 5–9. One week after the first visit, the transvaginal ultrasound was repeated and blood samples were drawn again to measure stimulated FSH and stimulated inhibin B levels (respectively, sFSH and sInhB in the CCCT). The result of the CCCT was defined as bFSH + sFSH.

Transvaginal ultrasound measurements

The volume of each ovary was determined by measuring the three perpendicular diameters and applying the formula for the volume of an ellipsoid: (L x W x D x π) / 6; the volumes of both ovaries were added for the total ovarian volume[186;189]. All follicles of 2–10 mm were counted and measured in two dimensions. The mean of these measurements was taken to be the follicle size. The numbers of follicles from both ovaries were added for the total AFC. All transvaginal ultrasound measurements were performed by four skilled gynaecologists using a 7.5 MHz vaginal probe on an Aloka SSD-1700 US machine. Several studies demonstrate that the intra-observer and inter-observer reproducibility of antral follicle measurements is adequate[191-193]. Intra-observer and inter-observer coefficients were not determined in this study.

Hormone assays

For measurements of concentrations of FSH and inhibin B, serum was stored at -20°C until processing. Serum FSH levels were measured by fluorimmunometric determination on the AutoDelfia (Wallac/Perkin Elmer, Turku, Finland). For FSH, the inter-assay coefficient of variation was 3.7%, the sensitivity <0.05 IU/l. The lower limit of detection was 0.03 IU/l. The standard of the FSH assay was calibrated against the World Health Organization Second International Reference Preparation for human FSH (78/549). Inhibin B concentrations were assayed with an enzyme-linked immunosorbent assay from Serotec (Kidlington, Oxford, UK). The inter-assay coefficient of variation for inhibin B was 11% and the sensitivity was <10 pg/ml. The lower limit of detection was 5 pg/ml.

Statistical analysis

Data were analysed with the Statistical Package for Social Sciences (SPSS 12.0 Inc., Chicago, IL, USA). Patient characteristics and results of the ORTs are presented as median values with 10^{th}–90^{th} percentiles or as numbers with percentages. The correlation between the various parameters is expressed as Spearman's correlation coefficient (*r*). Regression analysis was used to study the influence of age and various sizes of antral follicles on the endocrine variables. Statistical significance was considered to be reached at a *P*-value <0.05.

RESULTS

The study population consisted of 474 women. Patient characteristics are shown in Table 1. At the time of the CCCT median female age was 32.5 years, median duration of subfertility was over 2 years and about two-third of the women had a primary subfertility. Most couples were diagnosed with unexplained subfertility (51.3%) or male factor (44.9%). In Table 2, the results of the various ORTs are shown.

Table 1. Patient characteristics

	n = 474	
Age (years)	32.5	(26.6-38.8)
Duration of subfertility (months)	26.5	(16.8-50.4)
Primary subfertility	324	(68.4 %)
Cycle length (days)	28	(25-32)
Semen analysis (TMC, x10^6)	34.5	(4.0-175.6)
Diagnostic category of subfertility		
Unexplained	243	(51.3 %)
Male factor	213	(44.9 %)
Cervical factor	18	(3.8 %)

Values are median (10^{th} – 90^{th} percentiles) or numbers (%); TMC = total motile count (volume x concentration x motility)

Table 2. Outcome of the ovarian reserve tests

	Median	(10^{th} - 90^{th} percentiles)	*n*
Antral follicle count (n)	12	(5 - 24)	399
Total ovarian volume (cm^3)	10.6	(6.0 - 18.3)	388
Basal inhibin B (ng/l)	89.0	(37.7 - 140.0)	466
Stimulated inhibin B (ng/l)	226.0	(104.0 - 414.4)	462
Basal FSH (IU/l) (bFSH)	6.6	(4.6 - 10.6)	465
CCCT (bFSH+sFSH) (IU/l)	13.2	(9.1 - 21.8)	460

FSH = follicle stimulating hormone; CCCT = clomiphene citrate challenge test; sFSH = stimulated FSH

The various endocrine ORTs were not determined at one exact point in the cycle, but within a period of three cycle days. We analysed the differences between the results of the tests performed on cycle days 2, 3 and 4 and found two significant differences. Basal InhB values on cycle day 2 were significantly lower than bInhB values on cycle days 3 and 4 (*P*-value =0.001). It is known that serum levels of inhibin B rise in the early follicular phase up to a peak level around cycle day 5 or 6 [194]. Therefore, it is not surprising that inhibin B levels on cycle day 2 are lower than on cycle days 3 and 4. We then analysed the correlation between the results of the endocrine ORTs in groups per cycle day with age, AFC, follicle size and with each other. The correlation coefficients within the groups per cycle day were comparable to those in the total group, also for bInhB. We therefore analysed the group as a whole despite the differences mentioned.

Table 3 shows a correlation matrix of the ORTs and age. Age is significantly negatively correlated with AFC (r = -0.298; P < 0.01) and total ovarian volume (r = -0.183; P < 0.01) and positively correlated with bFSH (r = 0.201; P < 0.01) and CCCT (r = 0.219; P < 0.01). Age is not significantly related to bInhB and sInhB in the CCCT. AFC is significantly correlated with all ORTs performed.

Table 3. Correlation matrix of age and ovarian reserve tests (Spearman's coefficients)

	Age	AFC	TOV	bInhB	sInhB	bFSH
Antral follicle count (AFC)	- .298**					
Total ovarian volume (TOV)	- .183**	.486**				
Basal inhibin B (bInhB)	- .007	.142**	.139**			
Stimulated inhB (sInhB)	- .020	.200**	.182**	.291**		
Basal FSH (bFSH)	.201**	- .288**	- .332**	- .103*	- .135**	
CCCT (bFSH+sFSH)	.219**	- .296**	- .305**	- .138**	- .188**	(.871**)

* P-value <0.05, ** P-value <0.01
FSH = follicle stimulating hormone; CCCT = clomiphene citrate challenge test; sFSH = stimulated FSH

To answer the question of which size of antral follicles is most closely associated with age, the number of antral follicles was correlated with age for each size separately. The number of follicles measuring 2, 3, 4, 5 and 6 mm all declined with age, whereas the number of follicles measuring 7 mm or more did not (data not shown). These results were combined to two categories, i.e. small antral follicles (2–6 mm) and larger antral follicles (7–10 mm). Figure 2 shows that total AFC and the number of small follicles decline with age, whereas the number of larger follicles remains quite constant with increasing age up to 45 years. The curves of AFC and number of small antral follicles versus age mimic each other; they are not linear but show a steeper decline when female age reaches the second half of the fourth decade. Non-linear spline regression analysis was used to detect transition points between the horizontal part and the steeper part of the curve for follicles 2–6 mm in size. However, no statistically significant change in slope was found, neither for true nor for log transformed values.

In view of the findings in Fig. 2, the correlation between the same two categories of small and larger antral follicles and the endocrine ORTs and age was explored (Table 4). The number of small antral follicles (2–6 mm) is significantly correlated with all endocrine ORTs. The larger follicles (7–10 mm) correlate only with total ovarian volume and bInhB.

Since age is related to number and size of the antral follicles as well as the outcome of various ORTs, regression analysis was performed to determine which size of antral follicles is most closely correlated with the outcome of endocrine ORTs, independent of age. In Table 5, the results of the regression analysis are shown, depicting the non-standardized correlation coefficient B and its 95% confidence interval (CI) for each individual variable. The total number of small antral follicles (2–6 mm) is significantly related to all endocrine ORTs, whereas the number of larger follicles (7–10 mm)

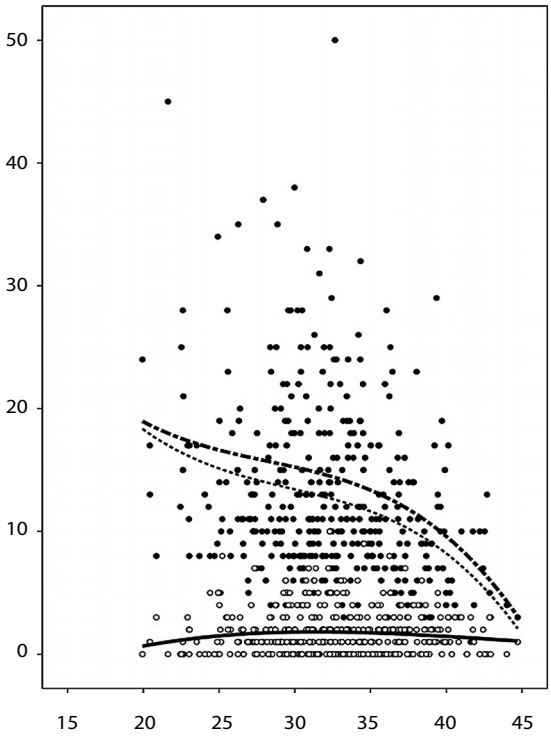

Figure 2. Relation of total antral follicle count, number of small (2-6 mm) and larger (7-10 mm) antral follicles with female age (in years) (n = 399)

- ● Number of small follicles (2–6 mm)
- ○ Number of larger follicles (7–10 mm)
- –·–· Relation of total antral follicle count (2–10 mm) and age
- ····· Relation of number of small follicles (2–6 mm) and age
- ——— Relation of number of larger follicles (7–10 mm) and age

Table 4. Correlations of small (2-6 mm) and larger (7-10 mm) antral follicles with age and ovarian reserve tests (Spearman's coefficients)

	Total number of small follicles (2-6 mm)	Total number of larger follicles (7-10 mm)
Age	- .304**	.005
Total ovarian volume	.418**	.316**
Basal inhibin B	.112*	.151**
Stimulated inhibin B	.184**	.051
Basal FSH (bFSH)	- .280**	- .029
CCCT (bFSH + sFSH)	- .310**	.010

* P-value <0.05, ** P-value <0.01

FSH = follicle stimulating hormone; CCCT = clomiphene citrate challenge test; sFSH = stimulated FSH

Table 5. Regression analysis of small (2-6 mm) and larger (7-10 mm) antral follicles, age and endocrine ovarian reserve tests.

	Basal FSH (n = 395) 95% CI				CCCT (n = 391) 95% CI			
	B	Lower	Upper	*P*	B	Lower	Upper	*P*
Age	0.128	0.061	0.196	<0.001	0.297	0.149	0.446	<0.001
2-6 mm	-0.088	-0.130	-0.047	<0.001	-0.201	-0.293	-0.110	<0.001
7-10 mm	-0.121	-0.287	0.045	NS	-0.175	-0.540	0.191	NS
	Basal inhibin B (n = 395) 95% CI				Stimulated inhibin B (n = 391) 95% CI			
	B	Lower	Upper	*P*	B	Lower	Upper	*P*
Age	0.170	-0.732	1.071	NS	3.403	-0.292	7.098	NS
2-6 mm	0.799	0.242	1.356	0.005	5.780	3.497	8.064	<0.001
7-10mm	2.957	0.733	5.181	0.009	3.729	-5.388	12.846	NS

FSH = follicle stimulating hormone; CCCT = clomiphene citrate challenge test; CI = confidence interval

is only significantly related to bInhB. Repeated analyses with single 1 mm follicle size categories as independent variables instead of grouped categories confirmed the influence of smaller size follicles, but this could not be narrowed to a specific size category (data not shown).

Since both age and number of small antral follicles are significantly correlated with bFSH and CCCT (Table 5), standardized correlation coefficients (ß) were computed to compare the strength of these correlations (not shown in table). The relation of the number of small antral follicles with bFSH is slightly stronger than the relation of age with bFSH (respectively ß = -0.209 and ß = 0.188). Similarly, the number of small antral follicles is slightly more strongly related to the CCCT than age (respectively ß = -0.217 and ß = 0.198).

DISCUSSION

This study demonstrates that the number of small antral follicles (2–6 mm) declines with age, whereas the number of larger follicles (7–10 mm) remains nearly constant. Irrespective of female age, the number of small antral follicles is strongly correlated with the outcome of various basal and dynamic endocrine ORTs, whereas the number of larger antral follicles is not. The relations with both female age and endocrine ORTs suggest that the number of small antral follicles represents the functional ovarian reserve.

The number of antral follicles as seen on ultrasound is assumed to depend on the size of the primordial follicle pool from which they are recruited. This phenomenon has been described in rodents by Krohn[195]

and Krarup *et al.*[196]: the more primordial follicles are available, the more follicles will grow. In humans, this may explain why the AFC is an adequate ORT. Research in various fertile and IVF-treated populations demonstrates a close correlation of AFC with age[105;106;188;189]. In our subfertile population, the number of follicles measuring 2 up to 6 mm all decline with age; the number of larger follicles (7–10 mm) per woman is low and remains relatively constant with age. In line with our results, Scheffer *et al.*[189] described in a fertile population a steeper yearly decline in the number of small antral follicles (2–5 mm) than for larger follicles (6–10 mm).

As a result of our findings, the fitted curve of the number of small follicles versus age mimics the fitted curve of the total AFC versus age. The pattern of decline of follicle number and AFC with age has been studied extensively. From autopsy studies of ovarian tissue a model of follicle disappearance from birth to menopause was obtained, showing a biphasic pattern with a steeper decline of follicles after the age of 37.5 years[21]. Scheffer *et al.*[189] found the same pattern of decline of AFC with age, assessed by ultrasound in a proven fertile population. Thus, AFC was considered to be a reflection of the total follicle pool. The biphasic pattern of the decline of follicles has been debated strongly, among others by Leidy *et al.*[197]. They state that the biphasic pattern is an artefact of the statistical methods used. Reuss *et al.*[106] and Ng *et al.*[184] did not find a biphasic pattern either in the decline of AFC with age in different populations. Broekmans *et al.*[104] reanalysed the data from the earlier study of their group as described above[189] and proposed a linear decline of antral follicles with age. Our data show a decline of total AFC with age with a steeper decline starting in the years preceding the age of 40. However, spline regression did not reveal a statistically significant change in slope. These results subscribe to the findings of Tufan *et al.*[198] that there is no single point after which the decline in follicles increases, but that indeed there is a general trend of an accelerating decline of follicles when female age reaches the second half of the fourth decade. The shape of the curve may partly explain why the correlation coefficient of AFC with age in the fertile population of Scheffer *et al.*[185] ($r = 0.68$) is higher than in our population ($r = 0.298$). Since median age in their group is 38.0 years, many participants are represented in the steeper part of the curve. Since correlation assumes a linear relation between two variables, it is expected that the correlation coefficient in the group of Scheffer is higher than in our younger population (median age of 32.5 years), because of the difference in age distribution. Moreover, the findings of Kline *et al.*[199] show that the correlation of AFC and age is stronger with advancing age. This may be explained by the fact that the reproducibility of the AFC declines when higher follicle counts are observed[193], which is more often the case in younger women. However, a stronger correlation of AFC and age with advancing age might also be a biological phenomenon.

Our data show that the smaller antral follicles (2–6 mm) correlate not only with age, but also independently correlate with the results of the various endocrine ORTs. Age and the endocrine ORTs used are no gold standard for ovarian reserve. However, since the various tests used in our study are all - independently of each other - related to the number of small follicles, we believe that our

data support the conclusion that the number of small follicles represents the ovarian reserve. The larger follicles only correlate with ovarian volume, which is biologically plausible, and bInhB. The relation of bInhB with both small and larger antral follicles corresponds with the fact that inhibin B is produced by pre-antral and antral follicles, with concentrations in the follicular fluid rising with the size of the follicle from 4 up to 13 mm[190;200]. The fact that the number of larger follicles does not relate to age and most ORTs may be explained by the limited variation in number of larger follicles per woman, precluding statistical relations with any parameter. The lack of a clear correlation with ovarian reserve may also be explained biologically, if the larger follicles represent follicles on their way to dominance. The process of selection of the dominant follicle may well be independent of the size of the antral follicle pool it is chosen from. This may be true especially for our study population, since the participants were selected on having an ovulatory cycle, proven by sonographic cycle analysis and sufficient midluteal progesterone. Women with cycle disturbances caused by a profound diminished ovarian reserve were excluded from our study beforehand. Selection of the dominant follicle is assumed to take place between cycle days 1 and 5[201] or between cycle days 3 and 10[192]. This variation in moment of selection may explain why in 31% of the (ovulatory) women no larger follicle was seen when the AFC was performed (cycle days 2–4).

Our data suggest that, when performing an AFC, follicles up to 6 instead of 10 mm should be counted. It is unlikely that this will considerably change the clinical accuracy of the AFC, since total AFC (2–10 mm) is mainly defined by the number of small antral follicles (2–6 mm). The AFC of 2–6 mm may be especially useful for research purposes or when a clinical cut-off point is used, e.g. when the start of treatment depends on a minimum number of follicles available.

All endocrine variables are more strongly associated with the number of small antral follicles than with age. This finding corresponds to the biological function of the antral follicles: the granulosa cells of the antral follicle pool produce E2 and inhibin B and, via feedback mechanisms, indirectly determine pituitary FSH secretion.

A promising new endocrine marker for ovarian reserve, AMH, was unfortunately not measured in our study. It has been described that AMH is closely related to both female age and AFC[85;86;93] and that AMH is especially secreted by smaller follicles[88;90]. These findings subscribe to our hypothesis that the smaller antral follicles actually represent the functional ovarian reserve.

A notable additional finding in our subfertile population concerns bFSH and CCCT. In line with other studies, bFSH and CCCT correlate with both female age and AFC[81;82;184;185]. Interestingly, there is no substantial difference in the correlations of bFSH and CCCT with female age (respectively, $r = 0.201$ and $r = 0.219$). Similarly, the correlations of bFSH and CCCT with AFC are comparable (respectively $r = -0.288$ and $r = -0.296$). These results suggest that the CCCT has no additional value as an ORT over bFSH alone. This issue has been addressed in several clinical studies examining the value of CCCT compared with bFSH in predicting ovarian response to hyperstimulation or pregnancy chances, spontaneously or after IVF treatment. Some studies state that the CCCT does provide extra clinical

information over bFSH alone[202-204], but other studies cannot confirm these findings[205-208]. In a future evaluation, we will assess the predictive value of these tests for the occurrence of spontaneous and assisted reproduction technique-related pregnancy in our study population of subfertile ovulatory women. It is known that both bFSH and CCCT can vary substantially per cycle in the same woman[83;84], which might hamper the interpretation of these tests.

In conclusion, the results of our study suggest that, independent of age, the number of small antral follicles measuring 2–6 mm represents the functional quantitative ovarian reserve. Therefore, we propose that if an AFC is performed, only follicles sized 2–6 mm could be counted and used for the interpretation of the outcome of endocrine ORTs. Furthermore, our data suggest that bFSH might have the same clinical value as CCCT, since there is no substantial difference between their correlations with, respectively, female age and AFC. Only clinical use of these tests can confirm or reject this assumption. Research in different populations, e.g. fertile women or women indicated for IVF, is needed to assess whether our results are valid outside our population of subfertile ovulatory women.

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