Morphometric characteristics of the primordial to primary follicle transition in the human ovary in relation to age

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BACKGROUND: The growth pattern of the smallest ovarian follicles in humans is still incompletely documented. Using follicle hemispheres in thick histological sections, morphometric changes of primordial to primary follicles and possible age-related effects were evaluated. METHODS: In ovarian sections from 25 females aged 4–39 years a total of 1122 primordial, transitory or primary follicles were assessed for the diameters of follicles, oocytes and oocyte nuclei and for number of granulosa cells (GCs). RESULTS: The number of GCs in primordial, transitory and primary follicles were \( \leq 30 \), 50 and 100, respectively. The diameters of primordial follicles and oocytes increased with the woman’s age until the mid-30’s, after which time they decreased in size. The number of GCs in primordial follicles showed a moderate increase with age, whereas the number of GCs in transitory follicles showed a clear increase with age. The oocyte nucleus diameter (14–23 \( \mu m \)) showed significant linear correlations with the oocyte and follicular diameters and number of GCs in the follicle, while the number of GCs in the whole follicle and in the largest cross-section were closely related to the oocyte diameter. CONCLUSIONS: The number of GCs in small follicles is accurately estimated and shows an increase with age, indicating that the starting point of follicular development alters with female age. The age-related changes observed may be linked to the poorer reproductive performance of older women.

Keywords: morphometry; human ovary; follicles; age

Introduction

Primordial follicles are small non-growing ovarian follicles, so-called ‘resting follicles’, and constitute at any one moment in time the follicular reserve or fertility potential of a female. In humans, primordial follicle formation is initiated during fetal life and is almost completed at birth. Already before birth, cohorts of primordial follicles leave the resting pool by initiating growth and antral follicles are continuously formed until the primordial pool is exhausted at menopause (Peters et al., 1975). While the molecular mechanisms regulating initiation of follicular growth remain elusive in most mammals, previous studies have focused on morphological descriptions of the relationship between granulosa cells (GCs) and the oocyte in the early stages of ovarian follicular development (Block, 1951, Gougeon and Chainy, 1987, Gougeon, 1996). Transition of just one or a few flattened GCs to a cuboidal form indicates early proliferation. Such follicles are usually called transitory or intermediary follicles, foregoing primary follicles, which are characterized by one single layer of cuboidal GC cells surrounding the oocyte. The oocyte has been reported to enlarge at the time the number of GCs in the largest cross-section (LCS) reaches 40 in the cow (Braw-Tal and Yossefi, 1997), 14 in the sheep (Cahill and Mauleon, 1981) and 9 in mice (Lintern-Moore and Moore, 1979). In the human ovary, it has been estimated that the oocytes of primordial follicles are surrounded by \( \leq 13 \) flattened GC’s, increasing to \( \leq 28 \) in transitory/intermediary follicles and 76 in primary follicles (Gougeon and Chainy, 1987). Further, morphometrical studies in primate ovaries have suggested that the smallest follicles are characterized by an oocyte nucleus diameter of \( <19 \mu m \), irrespective of whether they are primordial, intermediary or primary type follicles (Gougeon and Chainy, 1987; Gougeon et al., 1992). These data were based on histological sections in which counting and measurements were performed on the LCS of the follicle. With each section having a thickness of just a few micrometers, only a small portion of each of the follicles were evaluated (Lintern-Moore and Moore, 1979; Koering, 1983; Gougeon and Chainy, 1987; Braw-Tal and Yossefi, 1997). In contrast, the present study used sections with a thickness of around 30 \( \mu m \), allowing evaluation of individual follicle hemispheres. The aim of this present study was to estimate the number of GCs and to measure the follicle and oocyte diameter in hemispheres of primordial, intermediary and primary follicles and...
thereby determine the number GCs characterizing initiation of follicular growth. In addition, this study aimed to demonstrate possible age-related effects and to evaluate whether the number of GCs in small human follicles increased with age, as studies in rats have suggested (Hirshfield, 1989).

Material and Methods
Ovarian cortex was obtained from a total of 25 patients with an age ranging 4–39 years. All patients underwent unilateral oophorectomy for ovarian cryopreservation prior to gonadotoxic treatment of a malignant disease with the aim of preserving future fertility potential. The ovarian cryopreservation programme was conducted according to the Declaration of Helsinki and approved by the Ethical Committee of municipalities of Copenhagen and Frederiksberg, Denmark. None of the included patients had experienced any prior chemo- and/or radiotherapy and the diagnosis was in all cases unrelated to any ovarian malignancies. The present study used a random collected small fragment of ovarian cortex that was routinely taken for histology to evaluate the presence of follicles. The cortical fragment was fixed with Bouin’s solution, dehydrated and embedded in paraffin and sections from 30 to 35 μm of thickness was prepared and stained with hematoxylin-PAS using standard procedures.

Measurements were performed with 400× magnification and recordings were performed using the Visiopharm Integrator System version 2.10.3.0 (Visiopharm A/S, Copenhagen, Denmark) that allowed measurements with a precision of 0.1 μm. The patients were divided into five age groups each containing five patients: <13, 13–20, 20–27, 27–36 and >36 years, respectively.

Follicle hemispheres
The thickness of the sections (30–35 μm) allowed a follicle hemisphere to be determined in follicles with the double diameter (i.e. 60–70 μm). For a follicle to qualify for the present study a clear well-defined hemisphere needed to be present. A follicle hemisphere was defined by the visibility of the LCS of the follicle containing a clear basement membrane forming the largest ring surrounding the GCs (i.e. follicle ‘equator’) and by zooming, the follicle top or bottom should be visible within the section. Atretic follicles, defined as those with pycnotic granulosa cell nuclei or distorted oocytes, were excluded. Follicles containing two oocytes or oocytes with two nuclei were also excluded. No secondary follicles or follicles from later stages of growth were included because one section was unable to encompass a hemisphere. The ovarian cortex from each patient contributed at least 25 primordial, 10 transitory and 10 primary follicles.

Counting GCs in follicle hemispheres
GCs were counted by their nuclei. At the follicular equator, GC nuclei were marked on the screen via the computer system, and the number of nuclei was recorded. Zooming from this cross-section to the top or the bottom of the follicle provided gradual visibility of all GC nuclei, which were also computer marked, resulting in the total number of GCs in the follicle hemisphere being marked to ensure that cells were not counted twice and that no nuclei remained unaccounted for. The total number of GCs in a follicle was found by doubling the number present in a hemisphere.

Measurement of follicle and oocyte diameters
Using the LCS, follicle and oocyte diameters were monitored by measuring the largest diameter and perpendicular diameter, with the mean of these two measures giving the result (Koering, 1983; Wandji et al., 1997). Measurements of the nucleus were done in the section where the nucleus membrane formed the largest ring around the nuclear chromatin. This section could deviate from the follicle LCS.

Morphological criteria for activated/resting follicles
The follicles were classified according to Lintern-Moore et al. (1974) in: (i) Type B, primordial follicles with only squamous GC surrounding the oocyte, (ii) Type B/C, transitory or intermediate follicles with a mixture of squamous and cuboidal GC surrounding the oocyte and (iii) Type C, primary follicles showing one layer of cuboidal GC surrounding the oocyte. Examples of the three classes of follicles are shown in Fig. 1.

Statistics
Comparison of subgroups (n > 2) was analysed using ANOVA test. Comparison of two independent groups was done using Student’s t-test. Least-square linear regression analyses using SPSS 12 (SPSS Inc., Chicago, Illinois, USA) was also used.

Results
Diameter of follicles and oocytes related to developmental stage
Based on the measurements of a total of 1122 follicle hemispheres, morphometric characteristics of human primordial, intermediate and primary follicles are shown in Table 1. The follicular diameter shows a significant gradual increase from around 40 μm in primordial follicles to 54 μm in primary follicles. Similarly, oocyte diameter and oocyte nuclear diameter increased significantly with most of the growth apparently occurring between the intermediate and the primary stages.

Number of GCs related to developmental stage
The total number of GCs in the follicle based on follicle hemispheres showed that primordial follicles contain a total of 30 cells an average increasing to an average of 105 cells in primary follicles. In comparison, the number of GCs in LCS increased from 8 in primordial follicles to almost 27 in primary follicles.

Morphometric characteristics related to age
The follicular diameter and the oocyte and oocyte nuclear diameters and the total number of GCs in the three classes of follicles in relation to age is shown in Table 2. Taking all the variables into account for the primordial follicles, the overall picture showed an increase in the first four age groups, whereas the parameters were somewhat reduced in the oldest age group. The intermediary follicles showed a less pronounced picture with the follicular diameter and the oocyte and oocyte nuclear diameters showing a very limited age-related effect. In contrast, the total number of GC in intermediary follicles showed a clear age-related increase. Characteristics of the primary follicles showed for all parameters no obvious age relations.

Correlations amongst morphometric data
A highly significant correlation was found between the number of GCs in LCS and the total number of GCs as determined by using follicle hemispheres (Fig. 2). A slope close to four shows
that the LCS an average represented 25% of the total GC number in a follicle.

The distribution of follicular diameter within each class of follicle is depicted in Fig. 3. The number of follicles in each size grouping was expressed as the percentage of the total number of follicles in each class (Class B, B/C and C: 628, 252 and 241 follicles, respectively).

Figure 4 shows the oocyte nuclear diameter plotted against the mean oocyte diameter, mean number of GCs and mean follicular diameter, respectively. For all three parameters, there were highly significant positive, linear correlations with very high correlation coefficients.

A correlation between the numbers of GC in a follicle in relation to the oocyte diameter irrespective of age is shown in Fig. 5. The oocyte diameter showed a biphasic increase in relation to the number of GCs present in the follicle. In follicles with <30 GC the oocyte diameter increased from around 31 to around 37 μm, whereas almost no increase in oocyte diameter was observed in follicles containing between 30 and 70 GC. The oocyte started to enlarge further when follicles acquired more than around 70 GC per follicle.

**Histological and morphological descriptions**

Irrespective of age, primordial and intermediary follicles were often seen in clusters, whereas primary follicles were often found as a single follicle or in smaller clusters away from areas containing primordial and intermediary follicles.

The follicular morphology showed some variance between patients in terms of follicular/oocyte shape (spherical or ovoid), size and in the distribution of GCs. In primordial and intermediary follicles from some patients, the GCs were clustered in one, or two opposite, poles of the follicles; in others they were evenly distributed around the oocyte. The GCs were either flat or rectangular in shape. The proportion of primordial follicles seemed to decrease with age in contrast to a relative increase in the percentage of primary follicles.

**Discussion**

To our knowledge, this study is the first to use computer-aided measurements of follicle hemispheres to estimate the number of GCs in early human follicles. Based on an evaluation of more than one thousand follicles from 25 women with an age range from 4 to 39 years, it was shown that human resting primordial follicles on average contain around 30 GC increasing to almost 50 GC in transitory follicles and reaching an average of 105 GC in primary follicles. This increase in the number of GC was paralleled by an increase in the follicular diameter from 40 μm in primordial to 54 μm in primary follicles. Whereas the oocyte of primordial follicles comprises the vast majority of the follicle making up 68% of the follicular volume, this figure is already reduced to 39% in primary follicles as determined by this study. Thus, although the diameter of the oocyte increased from an average diameter of 36 μm to almost 40 μm during the primordial to primary transition, early human follicular growth is characterized by a pronounced reduction in the relative volume of the oocyte in the follicle.

Based on counting of GCs in the LCS an earlier study found 13 GC in primordial, 28 in intermediate and 76 in primary follicles (Gougeon and Chainy, 1987). These figures are somewhat lower than those of the present study. Except for the fact that more accurate data is likely to be obtained by counting a
The total number of GCs in primordial follicles shows a gradual increase with age, resulting in a >20% increase of GC in follicles from the youngest to oldest age group. This shows that the pool of primordial follicles is dynamic with higher fraction of the GC in each follicle (and ultimately an entire follicle), the former study may have achieved a lower number of GC by using the LCS defined as the section with the clearest oocyte nucleolus, since not all nucleoli are situated centrally in the oocyte nucleus, and, in addition, the nucleus is often not centred in the oocyte. This could potentially cause overall systematically lower measurements for all of the parameters, which indeed is the case when follicular diameter, oocyte diameter and oocyte nuclear diameter is considered. Although this difference in diameters only accounts for few micrometers it is likely to have a more pronounced impact when used to calculate volumes.

The present study is also the first to include morphometric studies on early human follicles from ovaries of young girls and the broad age span from 4 to 39 years allowed evaluation of possible age-related effects. Especially in the group of primordial follicles, the average follicular and the oocyte diameter increased with age in the first four age groups, i.e. from childhood to the mid 30’s. In the oldest age group above 36 years of age a reduction was observed as compared with the groups of women aged 20–27 and 27–36 years. A similar but less pronounced pattern was also observed in the transitory follicles and in the class of primary follicles, there were only modest effects and suggests that women in the late 30’s experience a reduction in size of their follicles and their oocytes when compared with those earlier in life. This may reflect the fact that recruitment of follicles and their oocytes when compared with younger years. This may reflect the fact that recruitment of follicles and their oocytes when compared with younger years. This may reflect the fact that recruitment of follicles and their oocytes when compared with younger years. This may reflect the fact that recruitment of follicles and their oocytes when compared with younger years. This may reflect the fact that recruitment of follicles and their oocytes when compared with younger years.

### Table 1: Morphometric data on primordial, intermediate and primary follicles (mean ± SEM). Corresponding superscripts represent Student’s t-test of the two mean values. ANOVA test of each parameter was performed with P-values shown at the bottom.

<table>
<thead>
<tr>
<th>Follicle class</th>
<th>Follicular diameter (µm ± SEM)</th>
<th>Oocyte diameter (µm ± SEM)</th>
<th>Oocyte nuclear diameter (µm ± SEM)</th>
<th>Number of GCs in LCS ± SEM</th>
<th>Total number of GCs in each follicle ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>B (n = 628)</td>
<td>40.4 ± 0.2^c</td>
<td>35.5 ± 0.2^c</td>
<td>18.3 ± 0.1^c</td>
<td>7.9 ± 0.1</td>
<td>29.5 ± 0.3</td>
</tr>
<tr>
<td>B/C (n = 252)</td>
<td>44.1 ± 0.3^a,b</td>
<td>36.4 ± 0.3^a,d</td>
<td>18.5 ± 0.1^c</td>
<td>13.0 ± 0.4</td>
<td>48.0 ± 0.9</td>
</tr>
<tr>
<td>C (n = 241)</td>
<td>54.4 ± 0.6^b</td>
<td>39.7 ± 0.4^d</td>
<td>19.8 ± 0.1^e</td>
<td>26.6 ± 1.2</td>
<td>105.0 ± 2.4</td>
</tr>
<tr>
<td>ANOVA</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

Student t-test: ^aP < 0.0001; ^bP < 0.0001; ^cP = 0.015; ^dP < 0.0001; ^eP = 0.24; ^fP < 0.0001.

### Table 2: Morphometric data on primordial, intermediate and primary follicles (mean ± SEM) in relation to age. Corresponding superscripts represent Student’s t-test of the two mean values. Results of ANOVA are shown in a separate column.

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Class</th>
<th>Tool (n = 125)</th>
<th>13–20 (n = 241)</th>
<th>20–27 (n = 222)</th>
<th>27–36 (n = 218)</th>
<th>&gt;36 (n = 215)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocyte diameter (µm ± SEM) (range)</td>
<td>B/C</td>
<td>36.4 ± 0.6</td>
<td>(28.6–43.0)</td>
<td>(31.0–49.0)</td>
<td>(29.0–53.1)</td>
<td>(32.9–53.9)</td>
<td>(29.0–55.1)</td>
</tr>
<tr>
<td>C</td>
<td>40.8 ± 0.8</td>
<td>39.0 ± 0.4</td>
<td>(23.7–50.7)</td>
<td>(33.1–49.6)</td>
<td>(35.9–54.4)</td>
<td>(31.5–52.4)</td>
<td>(30.4–51.7)</td>
</tr>
<tr>
<td>B</td>
<td>17.8 ± 0.2</td>
<td>18.5 ± 0.2</td>
<td>(11.9–23.6)</td>
<td>(14.4–23.3)</td>
<td>(12.7–22.9)</td>
<td>(14.0–22.5)</td>
<td>(12.2–23.2)</td>
</tr>
<tr>
<td>C</td>
<td>20.0 ± 0.3</td>
<td>18.8 ± 0.3</td>
<td>(15.2–23.1)</td>
<td>(14.2–23.3)</td>
<td>(15.8–24.2)</td>
<td>(14.7–22.0)</td>
<td>(13.9–23.9)</td>
</tr>
<tr>
<td>B</td>
<td>25.6 ± 0.6^b</td>
<td>18.8 ± 0.3</td>
<td>(14–48)</td>
<td>(16–42)</td>
<td>(20–54)</td>
<td>(16–52)</td>
<td>(16–54)</td>
</tr>
<tr>
<td>Total number of GCs in each follicle (SEM) (range)</td>
<td>B/C</td>
<td>43.4 ± 2.0</td>
<td>(26–86)</td>
<td>(24–74)</td>
<td>(32–70)</td>
<td>(28–88)</td>
<td>(36–98)</td>
</tr>
<tr>
<td>C</td>
<td>113.6 ± 4.6</td>
<td>92.3 ± 4.0</td>
<td>(70–210)</td>
<td>(38–164)</td>
<td>(62–212)</td>
<td>(60–230)</td>
<td>(66–248)</td>
</tr>
</tbody>
</table>

Student t-test: ^aP < 0.0001; ^bP = 0.0286; ^cP = 0.2590; ^dP = 0.0016; ^eP = 0.0037; ^fP = 0.1603; ^gP = 0.0096; ^hP < 0.0001; ^iP < 0.0001; ^jP < 0.04.
The mean oocyte diameter increased significantly, although modestly, from the primordial to the primary follicular stage, 2.5% from the primordial to intermediary phase and 9.1% from intermediary to primary phase. This suggests that the oocyte is not entirely resting until the ongoing growth from the primary stage. These results are consistent with results from baboons (Wandji et al., 1997) and sheep (Lundy et al., 1999), whereas other studies have found little or no oocyte growth during the primordial to primary follicular transition in humans (Lintern-Moore et al., 1974; Gougeon and Chainy, 1987; de Bruin et al., 2002) and bovines (Braw-Tal and Yossefi, 1997; Braw-Tal, 2002).

Further, previous studies have found that oocyte nuclear growth seems to occur in one of two distinct phases: when the oocyte nucleus exceeds 19 μm in diameter a faster growth takes place. This two-phase growth pattern of the oocyte nucleus was not observed in the present study, as evidenced by high linear correlation coefficients. However, there was a tendency for an altered growth pattern when the oocyte nucleus diameter exceeded 20–21 μm and since we did not include secondary follicles [the upper limit in this study was 23 μm, whereas Gougeon and Chainy (1987) included nuclear diameters of 27 μm], we may have missed the follicles with the most pronounced effect. Actually, a previous study from our laboratory described a monophasic, linear growth pattern between the oocyte and nucleus at nucleus diameter values below 26 μm in young human ovaries (Lintern-Moore et al., 1974). Further, the present study actually found diameters around 2 μm smaller than those reported by Gougeon and Chainy (1987). The present study only included follicles found in the ovarian cortex, whereas previous studies used the whole ovary and it is presently unknown whether that would have any impact on the results. Also the use of different fixation procedures may influence measurements. Whereas the study of Gougeon and Chainy (1987) used either Bouin’s fluid or alcohol–formaldehyde–acetic acid, the present study only used Bouin’s fluid. It is, however, difficult to evaluate the precise effects of this, and further studies including larger follicles will be needed to approach the precise growth pattern of the oocyte nucleus.

Previous studies have reported around 20–21 GCs in the LCS at time when the oocytes starts to grow (Lintern-Moore et al., 1974; Gougeon and Chainy, 1987). Results of the present study correspond to this figure and taking the entire follicle into account, we found 70 GC to be present when oocyte growth starts. Applying our calculated GC-total/GC-LCS ratio of 4.0 further confirms data previously reported.

The results of the present study suggested a three-phase oocyte growth model as determined by the GC number. In the first phase, between 10 and 40 GCs, the oocyte diameter increased with increasing GC number, suggesting oocyte growth along with GC proliferation in primordial follicles. Hereafter, oocyte diameter remains almost constant in follicles with 40 up to 70 GCs, implying GC proliferation without oocyte growth. Finally, in the third phase, in follicles with >70 GCs, the oocyte diameter increases again, in agreement with all previous results.
A highly significant linear correlation between the total number of GCs and GCs in the LCS was found with a slope of 4.0. Thus, the total number of GCs in a small follicle can be accurately estimated directly from the GC number in the LCS.

The lower number of total GCs in early follicles as estimated previously (Gougeon and Chainy, 1987, Gougeon and Busso, 2000) as compared with the present study may be a reflection of the nucleus and thereby nucleolus not necessarily being placed in the LCS and hence there was a risk of fewer GCs being visible. However, results of the present study may be slightly overestimated since a follicle hemisphere may also include granulosa cell nuclei counted in LCS (slightly overlapping the follicular equator). This may be especially pronounced in primordial follicles since the granulosa cell nuclei, because of their flatness and the smaller sphere area, make up for a larger percentage of the follicular circumference. This explanation is supported by the relative differences between the two studies. We found primordial follicles to contain 127% more GC, 74% more for intermediate and 38% more for primary follicles as compared with Gougeon and Chainy (1987). Although the present study is likely to have enhanced precision of morphometric data of early human follicles precise data, will only be available when entire follicles can be monitored.

In conclusion, the present study is likely to have enhanced the precision of morphometric data describing small human follicles, and shows that the number of GC within each follicle is likely to be higher than previously reported. Furthermore, the pool of small follicles is dynamic with clear age-related changes. It remains a possibility that the age-related changes observed reflect a residual pool of small follicles that result in a poorer reproductive performance in older women.
References

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Submitted on March 7, 2007; resubmitted on April 16, 2007; accepted on April 20, 2007