Spatial and temporal changes in follicle distribution in the human ovarian cortex

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ABSTRACT

Research question: How does follicle distribution evolve in the human ovarian cortex between the ages of 20 and 35?

Design: Fragments of ovarian cortex from women who underwent unilateral oophorectomy for fertility preservation were obtained for quantitative histological assessment, including recording of the two-dimensional coordinates of follicles. Data were analysed using spatial statistical methods.

Results: From 14 patients aged 20-35 years, 53 ovarian cortex tissue samples were obtained, containing 1-803 follicles. Primordial and transitory follicles lay in a clustered manner in the human ovarian cortex, with an average cluster radius of $\sim 270 \mu\text{m}$ (95% CI range 154 – 377 μm ; $n = 49$). Follicle density declined with age ($p = 0.006$, $n = 13$) and the distance to the nearest neighbouring follicle increased ($p = 0.004$, $n = 13$). The cluster radius decreased with age ($p = 0.02$, $n = 13$), but the degree of clustering tended to increase ($p = 0.11$, $n = 13$). In the majority of the samples, follicles of different stages lay in different clusters ($p < 0.05$, $n = 13$).

Conclusions: This study shows that primordial and transitory follicles lie in different clusters in the human ovarian cortex. Spatiotemporal computer simulation suggests that interfollicular signals may hinder follicle loss and therefore may drive clustered follicle distribution. In clinical practice, the patient's age should be taken into account when assessing follicle density, as follicle distribution is increasingly clustered with advancing age.

Keywords: Fertility preservation, human ovarian cortex, follicle distribution, follicle dynamics, reproductive ageing

INTRODUCTION

The human ovaries contain a stock of non-growing primordial follicles, which is established during the first trimester *in utero* (Parker and Schimmer 2006, Robboy 2009). Directly after establishment of this pool, follicle number starts to decline in a process that will continue throughout life (Wallace and Kelsey 2010). Estimations of follicle numbers at birth range from 266,000 to 2,000,000 (Block 1953, Baker 1963, Forabosco *et al.* 1991, Faddy *et al.* 1992, Wallace and Kelsey 2010, Pangas and Rajkovic 2015) and drop to 10,000 before a woman reaches the age of 35 (Broekmans *et al.* 2009, Wallace and Kelsey 2010). After this age, an even more rapid decrease in numbers is described, and when the stock declines under the threshold of 1,000 follicles, a woman enters menopause (Faddy *et al.* 1992, Faddy and Gosden 1996, Broekmans *et al.* 2009, Wallace and Kelsey 2010).

Prevailing models for the dynamics of reproductive ageing suggest that the rate of reproductive decline is related to the size of the remaining follicle cohort, so that the loss is accelerated when fewer follicles are present (Faddy *et al.* 1992). Surprisingly, in women who have lost half of the ovarian reserve due to unilateral oophorectomy, the rate of decline was lower than predicted by the Faddy model (Wilkosz *et al.* 2014). This indicates that local intra-ovarian factors may modulate the rate of decline rather than the global size of the follicle pool *per se* (Wilkosz *et al.* 2014). As primordial follicles are in close contact with each other in murine ovaries, presence of a growth-suppressing substance that is released by the primordial follicles has been proposed (Da Silva-Buttkus *et al.* 2009). Such factor may affect the global distribution of follicles during reproductive ageing. Indeed, in ovaries of aged mice, a clustered distribution of follicles has been reported (Faire *et al.* 2015, Feng *et al.* 2017).

Several studies have investigated the spatial architecture of the murine ovary, aiming to reveal the dynamic environment of ovarian tissue (Faire *et al.* 2015, Skodras and Marcelli 2015, Feng *et al.* 2017, Hu *et al.* 2017). Using nuclear volume to distinguish resting and growing follicles, Faire *et al.* assessed the dynamics of follicle growth in mice (Faire *et al.* 2015). Feng *et al.* and Hu *et al.* applied the CLARITY method to render the whole murine ovary transparent and to assess follicle growth dynamics (Feng *et al.* 2017, Hu *et al.* 2017). Skodras *et al.* developed computer simulations to model the three-dimensional follicle distribution and test the precision of tissue sampling techniques (Skodras and Marcelli 2015). In contrast, few studies have addressed the distribution of follicles in human ovaries (Qu *et al.* 2000, Poirot *et al.* 2002, Schmidt *et al.* 2003). Poirot *et al.* and Qu *et al.* reported that follicles in the human ovarian cortex are not distributed homogeneously without further characterising the distribution pattern (Qu *et al.* 2000, Poirot *et al.* 2002). Schmidt *et al.* also

86 described non-randomness of follicle distribution, but also mentioned clustering of the
87 follicles (Schmidt *et al.* 2003).

88

89 Intrigued by the challenges and controversy regarding ovarian ageing and follicle dynamics
90 in women, we aimed to assess the spatial distribution of resting and early growing follicles in
91 the human ovary during 20-35 years of age, when optimal fertility declines (Broekmans *et al.*
92 2009). There is no consensus on the distribution pattern, if any, of follicles in the human
93 ovarian cortex but it may be of importance in clinical practice regarding ovarian tissue
94 cryopreservation and transplantation.

MATERIAL AND METHODS

Experimental design

This was an observational study.

Ethical approval

Approvals to use clinical data and biological samples were obtained from the Data Protection Officer (No. 19/6526 of 13-MAR-2019) and from the Department of Pathology, Oslo University Hospital (No. 2019-09 of 04-MAR-2020). This project was included within the framework of quality assurance prior to every ovarian tissue auto transplantation, and therefore it was exempt from obtaining separate informed consent and/or additional review from the research ethics committee.

Tissue source

For this study, ovarian tissue was obtained from women ($n = 14$) who underwent unilateral oophorectomy for tissue cryopreservation. During surgery, one ovary was removed, collected in transport medium (Dulbecco's phosphate-buffered saline, DPBS (D8662) Sigma-Aldrich, Merck KGaA, Germany) and shipped on ice to the laboratory at the Department of Reproductive Medicine. The organ was dissected under sterile conditions. Incision was made through the mesovarium and the medullar tissue was carefully removed using a sharp scalpel or fine scissors. The cortex cleared from the medulla was cut into fragments of approximately $5 \times 5 \times 1 \text{ mm}^3$. The number of fragments collected varied (range 3 – 60, mean 28). The majority of the fragments was cryopreserved according to the protocol (Tanbo *et al.* 2015), and a few representative fragments (range 1 – 8, mean 4) were used for histopathological assessment.

Sample preparation

The fragments that were assigned for histopathological assessment were fixed in 4% formalin. The tissue was dehydrated using ethanol, embedded in paraffin while oriented parallel with the ovarian surface, and then sectioned in microtome. The tissue sections were fixed on a glass plate, deparaffinised, and stained with haematoxylin and eosin. Formalin, ethanol, paraffin, glass plates and haematoxylin-eosin staining were sourced from Sigma-Aldrich, Merck KGaA, Germany.

For quantitative assessment of follicle distribution, paraffin-embedded tissue samples were sectioned again at $4 \mu\text{m}$ thickness at two different levels, exactly $250 \mu\text{m}$ apart. We argued that this distance will prevent counting the same resting follicle in both sections, while would still allow assessment of comparatively large cortical area before depletion of the paraffin blocks. This is in accordance with the findings of Charleston *et al.* (Charleston *et al.* 2007).

Data collection

The samples were scanned using the NanoZoomer XR scanner (Hamamatsu Photonics, Japan) and imported into the NanoZoomer Digital Pathology software, version 2.6.13 (Hamamatsu Photonics). This program was used for viewing and annotating of the tissue samples as follows. First, the cortex was outlined using the freehand region annotation tool. All discontinuous fragments of cortex were outlined separately; for example, the tissue sample of patient No. 9 at level 1 yielded 5 separate cortical fragments. If present, the medulla was also outlined.

The image was then thoroughly searched for follicles, which were manually annotated using the pin tool that planted a pin in the centre of the follicle. The stage of the follicle was annotated according to histological features. Follicles that were surrounded by flattened pre-granulosa cells were considered primordial follicles. Follicles surrounded by a single layer of mixed flattened and cuboidal granulosa cells were considered 'intermediate follicles'. Follicles surrounded by a single layer of cuboidal granulosa cells were considered to be in the primary stage. A follicle was assigned in the secondary stage if there were multiple layers of cuboidal granulosa cells and fluid collections present. All objects of uncertain assignment were marked and reviewed by a trained pathologist (M.R.). After this review, structures that remained subject of discussion were further assessed by a specialist in gynaecological pathology (G.H.G.), who made a final judgement about whether to assign a structure as a follicle.

Data analysis and statistics

The outline of the cortex and coordinates of follicles were imported into R (R Core Team 2017), version 3.6.1 for spatial data analysis with the *spatstat* package (version 1.59) (Baddeley and Turner 2005). Centres of ovarian follicles were considered as a point pattern within a frame defined by the outline of the cortex.

Density of the follicles and distance to nearest neighbour were calculated with appropriate functions. To avoid bias because of the number of samples per patient, the data were analysed so that average follicle density for each patient was calculated. The individual data points were weighted with total cortical area to take the sample size into consideration, as larger sample area may mean more precise and reliable data.

Spatially random distribution of follicles was tested using two methods, including the quadrat test, which compares quadrat counts to random Poisson distribution, and the Clark Evans

test of aggregation, which considers clustered point pattern as the alternative hypothesis. In addition, the aggregation index R was calculated for each sample, where $R < 1$ indicated clustered point pattern. Clusters are defined by Knox as ‘a geographically bounded group of occurrences of sufficient size and concentration to be unlikely to have occurred by chance’ (Elliott *et al.* 1995), a definition that has been criticised (Olsen *et al.* 1996). We define clusters as a group of follicles which are positioned closely together by visual assessment, surrounded by an area of lower follicle density.

The radius of point clusters was estimated using Ripley’s L function, and the nadir of the first derivate was defined as cluster radius (Kiskowski *et al.* 2009). Ripley’s L function represents the probability of finding another follicle at distance r from a given follicle in a homogeneous random distribution.

Weighted linear regression analysis was used to test the association between point pattern statistics and clinical data.

Follicle distribution model

We used computer simulation to model spatial and temporal dynamics of primordial and transitory follicle patterns during the period 20 – 36 years of age, corresponding to the range in the observed data. This was done to test different hypotheses of the follicle dynamics. As a model for ovarian cortex, we considered a two-dimensional rectangular frame of 50×50 mm. The initial placement of points was determined by the simple sequential inhibition algorithm, which provides a random point distribution while never placing points closer than $45 \mu\text{m}$. We reasoned that the mean diameter of primordial follicles ($44.6 \pm 12.4 \mu\text{m}$, $n = 2005$) would be the minimal allowed distance between centres of two follicle-sized objects in the two-dimensional space.

Simulations consisted of 200 consecutive steps, where each step would correspond to one month in time. At the start of a step, we assigned a probability of loss to each available point. If the probability of loss exceeded a set threshold (see below), the point would be eliminated and unavailable during subsequent steps. We created 100 simulations per scenario on a high-performance computer and analysed the average spatial point pattern across simulations.

The simulated scenarios were as follows. In scenario 1, the probability of loss of a point was linearly increasing with time, and was calculated as a random number drawn from the uniform distribution multiplied by time. This scenario simulated an accelerating follicle loss model where neighbouring follicles do not interfere with each other. In scenario 2, the probability of loss was linearly increasing with time and also linearly increasing with distance larger than $256 \mu\text{m}$ (standard deviation: $16 \mu\text{m}$) to the nearest neighbouring point. In this scenario, the probability of loss was also dependent whether or not a point was in close

206 vicinity to another point. This mimics the situation where the proximity of another follicle is
207 protective to loss of the follicle, e.g. if there was a survival factor or a growth-inhibiting factor
208 released by the follicles. The parameters of simulation (the slope of time-related follicle loss
209 probability curve and the distance threshold to neighbour with increased probability of loss)
210 were extensively optimized across a wide range of possible values using the *optim* function
211 of R, in order to maximize the fit of the model, i.e. minimize the weighted sum of squared
212 deviations from the observed age versus follicle density data.

RESULTS

Fifty-six samples of ovarian cortex derived from 14 women (1 - 6 samples per woman) undergoing ovarian tissue cryopreservation were analysed. From one patient, none of the three tissue samples contained follicles and therefore this patient was excluded from analysis.

The mean age of the included patients ($n = 13$) at the time of tissue collection was 27.9 years (range 20 – 35 years). None of the patients had ovarian pathology as an indication for ovarian tissue cryopreservation or received chemotherapy before tissue collection. None of the patients had received radiotherapy in the pelvic region. The fifty-three assessed cortical fragments contained 1 to 803 follicles with an average of 47 follicles per sample.

Characteristics of the patients and the samples are given in **Table 1**. An overview of all tissue samples and its details can be found in Supplementary Figure 1.

Spatial distribution of primordial and transitory follicles

By visually analysing fixed ovarian tissue ($n = 53$), we could identify follicles from the cortex and identify potential follicle clusters in some samples (**Fig. 1A**). Four samples contained only one follicle and therefore were excluded from further analysis, as no pattern can be defined from a single point. In total, schematic representations from 49 tissues were obtained, including the extracted coordinates of follicles and the traces outlining the cortex (**Fig. 1B**).

We tested the point patterns defined by the follicles for the presence of complete spatial randomness by the quadrat test and the Clark Evans test. According to quadrat test, 31 out of the 49 samples (63%) presented significantly non-random distribution of follicles ($p < 0.05$, **Fig. 1D**), whereas Clark Evans test for non-randomness indicated that 39 (80%) samples had a clustered follicle distribution ($p < 0.05$, **Fig. 1D**).

The cumulative density of points within distance r from a given point was estimated by the Ripley's L function. In case of a clustered point pattern, L follows a characteristic shape with initially increasing values indicating congestion of points in the cluster followed by decreasing values indicating the edge of the cluster (**Fig. 1C**). The radius of the cluster can therefore be obtained from the derivate of L (Kiskowski *et al.* 2009). Using this method for all the samples, we estimated a mean cluster radius of 266 μm (95% CI, range 154 – 377 μm ; $n = 49$ samples).

Distribution of primordial and transitory follicles

In order to compare the spatial distribution of the primordial and transitory follicles, we examined 20 tissue samples with at least 5 follicles in each category in 6 patients (Supplementary table 1, **Fig. 2A-B**), and derived quadrat counts that were compared with the

Pearson's χ^2 test. The minimum of five per stage was chosen for statistical robustness. Thirteen (65%) samples had demonstrated significantly different ($p < 0.05$) quadrat counts and difference of $L(r)$ from theoretical random distribution (**Fig. 2C**), indicating that groups of primordial and transitory follicles did not overlap and occupied distinct areas in the cortex.

Follicle distribution and density in relation to age

Ovarian follicles are progressively lost to apoptosis or growth during the reproductive lifespan (Faddy *et al.* 1992, Faddy and Gosden 1996, Broekmans *et al.* 2009, Wallace and Kelsey 2010). We aimed to analyse the evolution of follicle distribution according to age. Using weighted linear regression, we observed a decreasing density of follicles between 20 to 36 years of age ($p = 0.006$, $n = 13$, **Fig. 3A-D**). In addition, increased age was associated with reduced cluster radius ($p = 0.02$, linear regression), increased distance to nearest neighbour ($p = 0.004$, linear regression), and a tendency towards reduced Clarke Evans R score ($p = 0.11$), indicating that loss of follicles during reproductive ageing was associated with clustering (**Fig. 3D**).

Spatiotemporal simulation of follicle dynamics

Emergence of follicle clusters during reproductive ageing may be a result of intra-ovarian paracrine factors that regulate the loss of follicles over a close distance. We modelled the effect of these factors by simulating follicle dynamics in a 50×50 mm two-dimensional space. Initially, this area was populated with 3000 points achieving a final point density of $1.2 \times 10^{-5} \mu\text{m}^{-2}$. Notably, the maximal density of follicles observed in women of 20 years of age was $\sim 10^{-5} \mu\text{m}^{-2}$. The starting parameters of the simulation were derived from observed data, including the average radius of primordial follicles from all patients ($44.6 \pm 12.4 \mu\text{m}$, $n = 2005$) and the density of primordial follicles ($1.2 \times 10^{-5} \mu\text{m}^{-2}$) at 20 years of age, which was the youngest age included in our study. We defined the probability of follicle loss to depend on the closeness to other follicles and increase progressively with time. Optimal fit with observed follicle density data was achieved when the survival of follicles depended on closeness to other follicles within $256 \mu\text{m}$ (standard deviation: $16 \mu\text{m}$) distance. The simulations were continued over 200 steps, representing time between 20 – 36 years of age. We created 100 realizations of the simulated data.

The simulations affirmed that clustering increases with increased age, as indicated by the shape of $L(r)$ functions and decreasing aggregation index R (**Fig. 4A-C**). The simulations estimated the average values of cluster radius, distance to nearest neighbour, and Clark Evans aggregation index R within a reasonable range of the observed data (**Fig. 4C**). The dynamics of cluster radius and nearest neighbour distance differed from the observed data, indicating an inferior fit of the simple model (**Fig. 4C**). Simulations ($n = 100$) that only

287 accounted for time and ignored the effect of closeness to other follicles consistently failed to
288 yield a clustered point pattern distribution (Clark Evans aggregation index $R = 1$;
289 Supplementary Table 1).

DISCUSSION

Ovarian ageing and follicle dynamics in the ovarian cortex are of considerable clinical interest. Insight into the complex physiological processes that take place in the ovary throughout reproductive life may be useful to improve fertility preservation treatment. Although the distribution of follicles in the ovarian cortex has been the subject of several studies, there was no consensus on the distribution pattern, if any, of follicles in the human ovarian cortex. In this study, we assessed the relationship between follicle distribution and age in the human ovarian cortex by analysing follicle location in ovarian tissue samples from women undergoing ovarian tissue cryopreservation. Our results provide insight into follicle dynamics and follicle distribution during the reproductive period (20 – 35 years).

We found that ovarian follicles were localized in defined clusters with a radius of approximately 270 μm in most samples, with primordial and transitory follicles often occupying distinct clusters. Advanced age was associated with reduced follicle density and higher distance to nearest neighbouring follicle. Moreover, there was a positive relation between age and the degree of clustering, while the radius of follicle clusters decreased with increasing age.

We found that primordial and transitory follicles follow a non-random distribution in the ovarian cortex, in agreement with several previous reports (Qu *et al.* 2000, Poirot *et al.* 2002, Schmidt *et al.* 2003). Poirot *et al.* concluded from ovarian tissue samples from 31 women that follicles distribute in a non-homogenous manner (Poirot *et al.* 2002). Qu *et al.* described an 'extremely uneven' distribution of follicles (Qu *et al.* 2000). Furthermore, Schmidt *et al.* analysed three whole human ovaries and reported an uneven distribution of follicles in all three cases, where follicles tended to lie in clusters (Schmidt *et al.* 2003).

We carried out two different statistical assessments to evaluate the distribution of follicles. The majority of our samples demonstrated non-random follicle distribution (31/49, 63%) and follicle clustering by Clark Evans test (39/49, 80%). Clustering of primordial follicles may contribute to sustained cyclical ovarian function during the reproductive transition (30-40 years), when follicle density is rapidly declining (Faddy *et al.* 1992, Faddy and Gosden 1996, Broekmans *et al.* 2009, Wallace and Kelsey 2010). Clusters may allow improved paracrine signalling compared to random follicle distribution, and thus may promote survival of primordial follicles and coordinated follicle growth. Indeed, we observed in 13 of 20 (65%) samples that follicles in the growth phase are spatially separated from the resting cohort. Coordinated activation of follicles may amplify a spatially clustered distribution, as entire clusters of follicles may remain dormant while others enter growth, and, as a result, clusters become increasingly recognizable. Feng *et al.* described that follicle clusters predominantly

consist of follicles of the same stage in mice, and that coordinated entry into the growth phase takes place in these clusters (Feng *et al.* 2017). Furthermore, Da Silva-Buttkus *et al.* found that the chance of growth in a given primordial follicle in a murine ovary is lower within 10 μ m vicinity of another primordial follicle, suggesting that primordial follicles release an inhibiting signal which prevents growth in neighbouring follicles (Da Silva-Buttkus *et al.* 2009).

We affirmed the long-established view that follicle density declines with age (Schmidt *et al.* 2003, Broekmans *et al.* 2009, Faire *et al.* 2015, Feng *et al.* 2017). Moreover, alterations in follicle distribution during reproductive ageing are described. For example, Faire *et al.* observed a homogenous distribution of follicles in neonatal aged mice (Faire *et al.* 2015). At more advanced age, an increased degree of clustering was observed. Feng *et al.* assessed follicle distribution in young and aged mice, and affirmed increased degree of clustering in aged animals (Feng *et al.* 2017). Our findings tend to support this observation by a correlation between age and increased clustering, as assessed by the Clark-Evans index R ($p = 0.11$).

Follicle-to-follicle inhibition may promote maintenance of follicle clusters during a general depletion of ovarian reserve. Hypothetically, paracrine inhibitory signals released by resting follicles may prevent other primordial follicles from entering the growth phase. Such inhibitory signals could easily be transmitted to closely situated follicles in the cluster, thereby prolonging cluster survival and ovarian function. Previous reports suggest that cytokines from the TGF- β superfamily may play a role in the maintenance of the dormant pool of follicles (Wang *et al.* 2014, Granados-Aparici *et al.* 2019).

To further assess whether follicle-to-follicle signalling could contribute to spatially clustered distribution in human ovaries, we created a computer simulation of ovarian follicle loss. In every successive step of simulation, we assigned probability of survival to each remaining follicle based on time and the distance to the nearest follicle. The characteristics and dynamics of simulated tissue were similar to observed data, indicating that signals among primordial follicles, with an effective range of a few hundred micrometres, do affect the rate of loss and spatial distribution of follicles.

The uneven clustered distribution of the follicles in the ovarian cortex may have multiple clinical consequences. Firstly, as suggested in previous studies, the estimation of total follicle count may be increasingly imprecise and require more tissue samples when the distribution of follicles becomes clustered, which may be already notable at ~ 30 years of age. Secondly,

the number of cortical strips that are re-implanted during ovarian tissue autotransplantation should be adjusted to age (Schmidt *et al.* 2003). In the presence of clustering, the risk of grafting follicle-poor strips may increase, which may require transplantation of multiple fragments. Thirdly, the optimal size and thickness of tissue fragments may need to be considered, as the follicles are mostly clustered in ~ 500 μm large area. Fragments that are exceedingly thick may hinder diffusion of cryoprotectants into the tissue (Zhang *et al.* 2011) and result in follicle loss. On the contrary, exceedingly thin fragments may disrupt clusters and prematurely deplete graft function, as seen by increased follicle loss in thin fragments of bovine ovarian tissue (Gavish *et al.* 2014). Moreover, excessive dissection of ovarian tissue into small fragments may disrupt clusters that measure approximately ~ 0.5 mm across, which may lead to loss of inhibitory signals causing rapid successive waves of follicle activation and exhaustion of graft function. Therefore, careful assessment of the optimal biopsy and graft size should be carried out, making use of new knowledge regarding follicles distribution in order to optimize the chances of fertility preservation. Moreover, the existence of a paracrine factor between follicles that inhibits growth could be of interest in the development of future techniques in the field of reproductive medicine. Targeting of such a factor could increase the number of growing oocytes and therefore, the number of oocytes available for IVF treatment. Therefore, further research into the role of paracrine signalling of the TGF- β superfamily could be of clinical importance.

We recognize several limitations of our analyses. We analysed small tissue fragments that were retained for diagnostic purposes, and the dimension, number, and orientation of the samples was according to clinical diagnostic routines. In particular, the small size of available fragments hinders us from validating some basic assumptions of the spatial statistical analysis, including that the samples were representative of the whole cortex with identical distribution outside and inside the sample. Moreover, all our samples were sectioned in the same orientation, i.e. parallel to the ovarian surface. The distance between sections (250 μm) allowed us to avoid overlapping follicles and overestimating follicle density, but hindered us from obtaining a complete three-dimensional view of follicle distribution. Most previous studies sectioned the tissue perpendicularly to the ovarian surface (which is the standard pathology practice) (Richardson *et al.* 1987), but perpendicular sectioning would capture a small cross-section of the cortex and hinder assessment of higher-level distribution, including clustering. Moreover, there is a large variation in number of fragments analyzed in different patients.

We also acknowledge that the choice to only exclude samples with one follicle is arbitrary. One can argue that the minimal count should be set at a higher number. However, if two follicles positioned very closely together, when there are no other follicles in the sample, this

could still be considered a 'cluster'. Therefore, it was chosen to exclude all the samples that have only one follicle, but to include all other samples.

Our computer model of follicle dynamics was based on a two-dimensional view of the ovary, which did not allow us to achieve sufficiently tight packing of follicles in three dimensions. Indeed, the simulation failed to estimate the observed nearest neighbour distance and cluster size, and it handled fewer follicles than would be possible by a 3D model. Furthermore, our simplified simulation failed to account for more complex intra-ovarian effects, such as longer-range stimulatory or inhibitory signals, as well as integrated effects of multiple neighbouring follicles. Collectively, this simple simulation may be ineffective to make predictions about trajectories of ovarian ageing beyond the observed data.

In conclusion, the distribution of primordial and transitory follicles in the ovarian cortex varies throughout the reproductive age. We found that in the majority of patients, follicles lie in the ovarian cortex in a clustered manner. As the mean radius is approximately 270 μm , the mean diameter of the clusters at young age is approximately ~ 0.5 mm and shows a correlation with age, with a decreasing diameter at increasing age. In addition, there is a positive relationship between age and the degree of clustering. Therefore, we hypothesize the presence of inhibitory factors acting among follicles within the same cluster and protecting the ovarian reserve. The presence of such a factor is conjectural based on the results of this study. Future research could focus on immunohistochemical staining experiments, to provide more understanding of the intrafollicular environment.

As the majority of primordial follicles cluster together, the woman's age should always be considered when deciding on the number of samples to retrieve in order to get an accurate follicle count. Moreover, this study adds to the knowledge of follicle distribution in the human ovarian cortex and especially to the suggestion that these follicles lie in clusters. Lastly, during auto transplantation of ovarian cortex, the number, size, and thickness of the tissue samples should be carefully adjusted, according to the age and follicle count.

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Conflict of interests

None to declare.

Notes

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LEGEND TO FIGURES

Figure 1. Clustered distribution of primordial and transitory follicles in the human ovarian cortex. **(A)** Sample number 16: ovarian tissue from a 24 years-old woman stained with haematoxylin and eosin; inset shows a potential cluster of follicles. The right image is a 10x zoom in from the inset. **(B)** Graphical representation of the ovarian tissue sample shown in panel (A). Individual follicles are indicated with black crosses. Follicle density is represented by a colour code: red colour represents high follicle density and white colour low density. **(C)** Ripley's L function chart (upper graph), which represents the probability of finding another follicle at distance r from a given follicle in a homogeneous random distribution (dotted red line) and for the ovarian tissue sample from panel (A) (black curve). Size of the cluster can be estimated by the derivate of L (lower graph), where the first nadir indicates the cluster size. **(D)** Log P values for hypothesis tests of spatial distribution of follicles for all tissue samples ($n = 49$). Clustered distribution as alternative hypothesis was assessed by Clark Evans test, spatially random Poisson distribution as alternative hypothesis was assessed by the quadrat test.

Figure 2. Primordial and transitory follicles tend to occupy different clusters. **(A)** Sample number 31: ovarian tissue of a 27 years old woman. **(B)** Representations of the sample shown in (A), crosses indicate individual follicles. Follicle density is indicated by the colour code. **(C)** Ripley's L function chart, which represents the probability of finding another follicle at distance r from a given follicle in a homogeneous random distribution (dotted red line) and for the ovarian tissue sample from panel (A). The black line indicates significantly increased distance between primordial and transitory follicles (shaded area shows 95% CI for no difference), suggesting that these occupy different clusters.

Figure 3. Association between follicle distribution and age. **(A-C)** Ovarian tissue fragments and follicle distribution representations from a 21-year-old woman, sample 1 (A), a 28-year-old woman, sample 29 (B), and a 35-year-old woman, sample 52 (C). Tissue fragments were stained with haematoxylin and eosin (top panels). Down panels include graphical representation of the same samples. Individual follicles are indicated with black crosses and follicle density is represented by a colour code: red colour represents high follicle density and white colour low density. **(D)** Scatterplots indicating the association of age with, respectively, follicle density ($p = 0.006$), cluster radius ($p = 0.02$), nearest-neighbour distance ($p = 0.004$), and Clark Evans aggregation index R ($p = 0.11$). The size of the markers indicates the total area (mm^2) of a tissue sample.

567 **Figure 4.** Modelling spatiotemporal dynamics of primordial follicles in a simulated rectangular
 568 50 × 50 mm tissue fragment. **(A)** Initial random follicle distribution at 20 years of age,
 569 representative simulated distributions at 24, 28, 32, and 36 years of age. Red circles
 570 illustrate respective cluster size, Clark Evans aggregation index R and cluster radius r are
 571 given under the plots. **(B)** Ripley's L function chart, which represents the probability of finding
 572 another follicle at distance r from a given follicle. In a homogeneous random distribution, the
 573 result (black curve) is very close to the completely dotted red line, which indicates complete
 574 randomness. With increase of age, the black line divides further away from the red dotted
 575 line, indicating that distribution becomes less random at higher age.
 576 **(C)** Association between age and follicle density, cluster radius, nearest neighbour distance,
 577 and Clark Evans aggregation index R . Black circles indicate observed data, where the size of
 578 the markers indicates the total area (mm^2) of a tissue sample. Mean of simulated data are
 579 indicated by continuous red line and shaded area indicates 95% CI.

A

B

C

D

Table I – Characteristics of patients and ovarian tissue samples.

Patient ID	Diagnosis	Age (years) at time of freezing	Number of samples	Total number of follicles
1	Diffuse large B-cell lymphoma	20	2	76
2	Hodgkin's lymphoma	23	2	15
3	Multiple sclerosis	24	2	58
4	Sarcoma	24	16	775
5	Hodgkin's lymphoma	25	3	111
6	Ewing sarcoma	25	2	23
7	Ewing sarcoma	27	8	535
8	Sarcoma	28	2	817
9	Hodgkin's lymphoma	32	7	35
10	Hodgkin's lymphoma	32	2	14
11	Breast carcinoma	34	4	6
12	Breast carcinoma	34	3	8
13	Colon carcinoma	35	2	20
14	Large cell anaplastic lymphoma*	25	1	0

*This patient was excluded from analysis as the tissue did not contain follicles.

Supplementary table A – Characteristics of analysed tissue samples.

Patient	Sample number	Number of follicles			Cortical tissue area (mm ²)	Follicle density (follicles/mm ²)	Complete spatial randomness (p-value, quadrant test)	Clark Evans aggregation index R	Mean cluster radius (µm)	Distribution of resting vs growing follicles (p-value, Pearson's X ² test)	
		Total	Primordial	Transitory							Other/unknown stage
1	1	35	28	6	1	5.7	6.14	0.43	0.63	55.6	p < 0.05
	2	41	31	4	6	4.4	9.31	p < 0.05	0.46	N.A.	N.A.
2	3	15	14	1	0	4.3	3.49	p < 0.05	0.25	320.5	N.A.
3	4	58	48	8	2	9.7	5.98	p < 0.05	0.42	729.8	p < 0.05
4	5	23	19	4	0	9.2	2.50	p < 0.05	0.58	76.9	N.A.
	6	34	28	6	0	12.1	2.81	p < 0.05	0.66	45.6	0.43
	7	9	4	4	1	13.4	0.67	0.32	0.76	275.0	N.A.
	8	18	13	4	1	7.3	2.47	p < 0.05	0.21	357.7	N.A.
	9	19	11	7	1	29.4	0.65	0.59	0.56	857.5	0.11
	10	41	28	11	2	22.7	1.81	p < 0.05	0.53	965.6	p < 0.05
	11	54	43	11	0	21.9	2.47	p < 0.05	0.51	N.A.	0.07
	12	56	43	11	2	30.1	1.86	p < 0.05	0.41	675.2	p < 0.05
	13	122	103	16	3	16.2	7.53	p < 0.05	0.66	1005.0	0.26
	14	185	145	31	9	28.2	6.56	p < 0.05	0.74	580.3	p < 0.05
	15	44	37	6	1	4.8	9.17	p < 0.05	0.59	105.5	p < 0.05

	16	43	35	7	1	14.3	3.00	p < 0.05	0.44	N.A.	p < 0.05
	17	12	8	3	1	2.3	5.22	0.35	0.65	126.4	N.A.
	18	21	19	2	0	15.9	1.32	p < 0.05	0.40	214.2	N.A.
	19	12	10	1	1	10.3	1.17	p < 0.05	0.31	180.8	N.A.
	20	82	59	18	5	26.5	3.09	p < 0.05	0.61	941.2	0.10
	21	92	75	13	4	9.3	9.89	p < 0.05	0.48	734.4	p < 0.05
	22	7	6	0	1	0.5	14.0	0.16	0.49	63.1	N.A.
	23	12	10	2	0	3.0	4.00	p < 0.05	0.51	122.1	N.A.
5	24	10	8	1	1	18.1	0.55	p < 0.05	0.13	713.3	N.A.
	25	13	9	3	1	15.3	0.85	p < 0.05	0.26	623.2	N.A.
7	26	4	1	2	1	23.6	0.17	p < 0.05	N.A.	424.1	N.A.
	27	114	103	9	2	19.3	5.91	p < 0.05	0.56	482.5	p < 0.05
	28	8	6	2	0	11.1	0.72	0.69	N.A.	286.4	N.A.
	29	75	69	5	1	15.4	4.87	p < 0.05	0.55	527.3	0.20
	30	66	46	8	12	18.2	3.63	p < 0.05	0.36	750.1	p < 0.05
	31	232	179	33	20	29.6	7.84	p < 0.05	0.62	1666.4	p < 0.05
	32	17	11	5	1	10.2	1.67	p < 0.05	0.34	293.0	p < 0.05
	33	19	8	9	2	27.9	0.68	p < 0.05	0.48	404.2	p < 0.05
8	34	803	679	104	20	5.1	157.5	p < 0.05	0.86	69.3	0.25
	35	14	8	3	3	2.5	5.60	p < 0.05	0.47	142.7	N.A.

9	36	9	7	2	0	5.2	1.73	0.14	0.39	141.0	N.A.
	37	2	1	1	0	4.7	0.43	0.15	N.A.	N.A.	N.A.
	38	1	1	0	0	3.4	0.29	N.A.	N.A.	N.A.	N.A.
	39	4	2	0	2	11.4	0.35	0.18	0.57	157.3	N.A.
	40	5	4	1	0	17.9	0.28	0.41	0.42	439.9	N.A.
	41	10	7	0	3	26.3	0.38	p < 0.05	0.35	78.0	N.A.
	42	4	0	3	1	22.4	0.18	0.59	0.42	292.0	N.A.
	43	6	5	1	0	4.8	1.25	0.10	0.37	143.5	N.A.
10	44	8	5	1	2	6.6	1.21	0.08	0.47	195.4	N.A.
	45	2	1	1	0	6.2	0.32	0.21	N.A.	N.A.	N.A.
	46	1	1	0	0	9.9	0.10	N.A.	N.A.	N.A.	N.A.
	47	1	0	1	0	1.6	0.63	N.A.	N.A.	N.A.	N.A.
	48	2	1	1	0	9.5	0.21	0.90	N.A.	N.A.	N.A.
	49	5	3	1	1	17.0	0.29	0.12	0.21	141.9	N.A.
12	50	1	1	0	0	9.9	0.10	N.A.	N.A.	N.A.	N.A.
	51	2	2	0	0	9.9	0.20	0.13	N.A.	N.A.	N.A.
	52	15	15	0	0	24.7	0.61	p < 0.05	0.36	573.0	N.A.
13	53	5	5	0	0	14.3	0.35	0.06	0.42	69.2	N.A.
Total		2493	2005	373	115	703.5					

N.A. = not applicable.

* Tissue from this patient was excluded from analysis as there were no follicles in the tissue samples.

Supplementary Figure A – Overview of all assessed tissue samples including number of cortical fragment and total number of follicles.

Patient 1, level 1	Patient 1, level 2	Patient 2, level 1	Patient 2, level 2

Number of cortical fragments: 1 Total number of follicles: 35 Sample number: 1	Number of cortical fragments: 1 Total number of follicles: 41 Sample number: 2	Number of cortical fragments: 1 Total number of follicles: 15 Sample number: 3	Number of cortical fragments: 1 Total number of follicles: 0 <i>Excluded from analysis</i>
Patient 3, level 1	Patient 3, level 2	Patient 4, fresh tissue, level 1	Patient 4, fresh tissue, level 2

Number of cortical fragments: 1 Total number of follicles: 58 Sample number: 4	Number of cortical fragments: 1 Total number of follicles: 0 <i>Excluded from analysis</i>	Number of cortical fragments: 2 Total number of follicles: 57 Sample numbers: 5, 6	Number of cortical fragments: 2 Total number of follicles: 27 Sample numbers: 7,8
Patient 4, thawed, level 1	Patient 4, thawed, level 2	Patient 5, level 1	Patient 5, level 2

Number of cortical fragments: 6 Total number of follicles: 477 Sample numbers: 9-14	Number of cortical fragments: 6 Total number of follicles: 214 Sample numbers: 15-20	Number of cortical fragments: 1 Total number of follicles: 92 Sample number: 21	Number of cortical fragments: 2 Total number of follicles: 19 Sample numbers: 22, 23
Patient 6, level 1	Patient 6, level 2	Patient 7, fresh tissue, level 1	Patient 7, fresh tissue, level 2

Number of cortical fragments: 1 Total number of follicles: 10 Sample numbers: 24	Number of cortical fragments: 1 Total number of follicles: 13 Sample number: 25	Number of cortical fragments: 2 Total number of follicles: 118 Sample numbers: 26, 27	Number of cortical fragments: 2 Total number of follicles: 83 Sample numbers: 28, 29
Patient 7, thawed, level 1	Patient 7, thawed tissue, level 2	Patient 8, level 1	Patient 8, level 2

Number of cortical fragments: 2 Total number of follicles: 298 Sample numbers: 30, 31	Number of cortical fragments: 2 Total number of follicles: 36 Sample numbers: 32, 33	Number of cortical fragments: 1 Total number of follicles: 803 Sample number: 34	Number of cortical fragments: 1 Total number of follicles: 14 Sample number: 35
Patient 9, level 1	Patient 9, level 2	Patient 10, level 1	Patient 10, level 2

Number of cortical fragments: 5 Total number of follicles: 21 Sample numbers: 36-40	Number of cortical fragments: 2 Total number of follicles: 14 Sample numbers: 41, 42	Number of cortical fragments: 1 Total number of follicles: 6 Sample number: 43	Number of cortical fragments: 1 Total number of follicles: 8 Sample number: 44
Patient 11, level 1	Patient 11, level 2	Patient 12, level 1	Patient 12, level 2

Number of cortical fragments: 2 Total number of follicles: 3 Sample number: 45, 46	Number of cortical fragments: 2 Total number of follicles: 3 Sample numbers: 47, 48	Number of cortical fragments: 2 Total number of follicles: 6 Sample numbers: 49, 50	Number of cortical fragments: 1 Total number of follicles: 2 Sample number: 51
Patient 13, level 1	Patient 13, level 2	Patient 14, level 1

Number of cortical fragments: 1 Total number of follicles: 15 Sample number: 52	Number of cortical fragments: 1 Total number of follicles: 5 Sample number: 53	Number of cortical fragments: 1 Total number of follicles: 0 <i>Excluded from analysis</i>	