Check for updates

Follicle populations and vascularization in ovarian tissue of pediatric patients before and after long-term grafting

Rossella Masciangelo, M.D., Ph.D.,[a] Maria Costanza Chiti, Ph.D.,[a] Charlotte Philippart, M.Sc.,[a] Christiani Andrade Amorim, D.M.V., Ph.D.,[a] Jacques Donnez, M.D., Ph.D.,[b] Alessandra Camboni, M.D., Ph.D.,[a] and Marie-Madeleine Dolmans, M.D., Ph.D.[a,c]

[a] Pôle de Recherche en Gynécologie, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain; [b] Société de Recherche pour l'Infertilité; [c] Département de Gynécologie, Cliniques Universitaires St. Luc, Brussels, Belgium

Objective: To characterize ovarian tissue from pediatric patients by evaluating development and vascularization in follicle populations and comparing it with adult tissue after xenografting.
Design: Prospective experimental study.
Setting: Academic research center.
Patient(s): Five children (median age 3 years) and seven women (median age 28 years).
Intervention(s): Hematoxylin and eosin staining, immunofluorescence, and transmission electron microscopy (TEM) evaluation before and after grafting.
Main Outcome Measure(s): Follicle density, morphology, classification, and size, ovarian tissue vascularization, follicle ultrastructure.
Result(s): Frozen-thawed ovarian tissue was divided into three fragments: nongrafted controls, TEM, and xenografting for 20 weeks. Follicle density was statistically significantly higher in pediatric than adult patients; even though it decreased in both groups after transplantation, it remained higher in pediatric patients. In the pediatric group, quiescent-stage follicles were the majority of the follicle pool before and after grafting, while growing follicles statistically significantly increased in both groups after grafting. Abnormal and atretic follicles were also observed in pediatric tissue and declined with age and after grafting. Pediatric ovarian tissue contained more and larger immature vessels, while mature vessels were larger in adults. The TEM analysis of abnormal pediatric follicles showed loss of shape and vacuolization of the cytoplasm without organelle damage.
Conclusion(s): Statistically significant differences in follicle density were observed between pediatric and adult patients, but the follicle proportions were similar before and after grafting, with the exception of atretic and abnormal follicles. Pediatric tissue contains more and larger immature vessels than adult tissue, and the posttransplantation revascularization process is accelerated in this group. (Fertil Steril® 2020;114:1330–8. ©2020 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Abnormal follicles, ovarian tissue vascularization, pediatric ovarian tissue

Discuss: You can discuss this article with its authors and other readers at https://www.fertstertdialog.com/users/16110-fertility-and-sterility/posts/29631

In recent years, progress in cancer treatment has yielded greatly improved survival rates in many cancer patients, but most still experience the long-term side effects of chemotherapy and radiotherapy, such as premature ovarian insufficiency (1, 2). Fertility preservation can be offered before anticancer treatments, but the only option currently available for prepubertal patients is ovarian tissue cryopreservation and transplantation (2). Transplantation of ovarian tissue frozen before puberty has been performed in three patients to date. Evidence of puberty induction was reported in two cases (3, 4), as was the live birth of a healthy baby (5). Previous studies (6, 7) demonstrated the capacity of frozen-thawed ovarian tissue from prepubertal girls to become activated, with follicles developing to advanced

Received January 7, 2020; revised June 4, 2020; accepted June 23, 2020; published online August 19, 2020.
R.M. has nothing to disclose. M.C.C. has nothing to disclose. C.P. has nothing to disclose. C.A.A. has nothing to disclose. J.D. has nothing to disclose. A.C. has nothing to disclose. M.-M.D. has received grants and personal fees from Preglem Gedeon Richter, and personal fees from Obseva outside the submitted work.
Supported by grants from the Fonds National de la Recherche Scientifique de Belgique (FNRS-PDR Convention T.0077.14, Télévie grant 7.4590.16 (to R.M.)), EOS grant 30443682 (to M.C.C.), and grant 5/4/150/5 (to M.-M.D.); C.A.A. is an FSR-FNRS research associate), the Fonds Speciaux de Recherche, and the Foundation Against Cancer.
Reprint requests: Marie-Madeleine Dolmans, M.D., Ph.D., Département de Gynécologie, Cliniques Universitaires St. Luc, Avenue Hippocrate 10, 1200 Brussels, Belgium (E-mail: marie-madeleine.dolmans@uclouvain.be).

Fertility and Sterility® Vol. 114, No. 6, December 2020 0015-0282/$36.00
Copyright ©2020 American Society for Reproductive Medicine, Published by Elsevier Inc.
https://doi.org/10.1016/j.fertnstert.2020.06.031

stages both in vivo after hormone stimulation and in vitro. Differences were found in the rates of activation compared with adult ovarian tissue, specifically with respect to the large pool of quiescent primordial follicles in prepubertal tissue that was well preserved after transplantation. Nevertheless, our understanding of ovarian tissue from very young children is currently lacking.

The objective of the present study was to characterize ovarian tissue from very young pediatric patients (median age 3 years) by evaluating follicle density, classification, and size before and after long-term grafting to elucidate its growth dynamics as well as vascularization and ultrastructural changes. Comparisons were then made with adult ovarian tissue.

MATERIALS AND METHODS

Experimental Design

Use of human ovarian tissue was approved by the institutional review board of the Université Catholique de Louvain (reference 2012/23MAR/125). Informed consent allows cryopreserved tissue from deceased patients to be used for scientific purposes so frozen-thawed human ovarian tissue from five deceased children (age 1–7 years; median: 3 years) was used for the study. All five patients had had malignant diseases: medulloblastoma (n = 2; ages 3 years and 3 years), sarcoma (n = 2; ages 1 year and 7 years), and leukemia (n = 1; age 3 years). Cryopreservation was performed before cancer treatment in all cases.

Frozen-thawed ovarian tissue from seven adult women (ages 20–35 years; median: 28 years) was used as the controls. All had undergone cryopreservation of ovarian tissue for cancer pathologies: breast cancer (n = 4), Hodgkin's lymphoma (n = 2), and sarcoma (n = 1). All had provided written informed consent to donate their frozen ovarian tissue to research.

Ovarian Tissue Freezing and Thawing

Cryopreservation of ovarian tissue was undertaken according to the slow-freezing protocol, as described elsewhere (8, 9). The protocol is detailed in the supplemental material.

Transplantation to Immunodeficient Mice

Guidelines for animal welfare were fully respected, and the study was authorized by the Committee on Animal Research of the Université Catholique de Louvain (Reference 2018/UCL/MD/40). Fourteen female severe combined immunodeficient (SCID) mice (Charles River) were used for transplantation. A median incision was made in the abdomen and peritoneum of the animals, and one human ovarian tissue fragment per patient ($5 \times 3 \times 1$ mm^3 for pediatric patients; $10 \times 5 \times 1$ mm^3 for adult patients) was fixed to the inner sides of the peritoneum with one or two stitches of 6-0 Prolene (Ethicon; Johnson & Johnson International). The abdominal wall and skin were then closed with 6-0 Prolene. The mice were kept in sterile conditions for 20 weeks and subsequently were euthanized by cervical dislocation. Upon retrieval, the ovarian grafts were assigned to histologic analysis (formol).

Histology

After fixation in 4% formaldehyde, frozen-thawed ovarian tissue and ovarian tissue grafts were embedded in paraffin and serially sectioned (5-μm-thick sections). Every fourth slide was stained with hematoxylin and eosin (Merck) for histologic evaluation.

Follicle Density and Classification

Follicle quality was assessed on the basis of basement membrane integrity, cellular density, presence or absence of pyknotic bodies, and integrity of the oocyte (10). Additional information is provided in the supplemental material.

Follicle Diameter Measurements

Follicle diameter, oocyte diameter, and oocyte nucleus diameter were measured by calculating the average between the largest diameter and perpendicular diameter (11). Further data are reported in the supplemental material.

Ovarian Tissue and Graft Vascularization

Vascularization in frozen-thawed ovarian tissue was investigated by double immunofluorescence staining for new human blood vessels using anti-human von Willebrand factor antibody and mature blood vessels using anti-smooth muscle actin polyclonal antibody. The complete protocol can be found in the supplemental material.

Transmission Electron Microscopy

For transmission electron microscopy (TEM) evaluation, the morphological characteristics of the stroma, granulosa cells (GCs), and oocytes as well as their organelles, basal and plasmatic membranes, and nuclear envelope were taken into consideration. Shape, morphology, distribution, and the electron density of chromatin were also investigated. The detailed protocol is described in the supplemental material.

Statistical Analysis

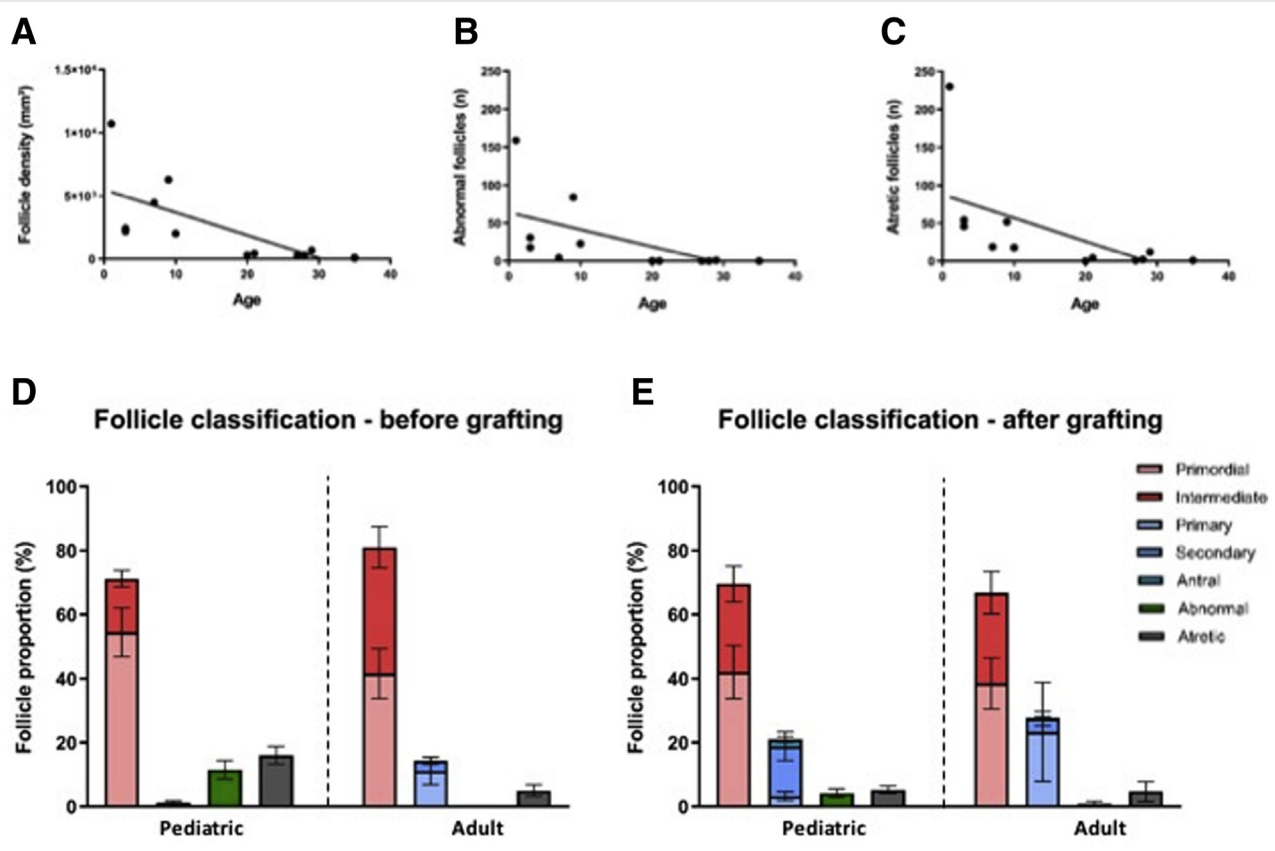
GraphPad Prism, version 8.1.2 for Windows (GraphPad Software), was used for statistical analyses. To evaluate follicle density, size, and vascularization, an unpaired *t*-test was conducted to compare results between pediatric and adult patients, and a paired *t*-test to compare results before and after grafting in the same groups. Linear regression was applied to correlate age with the number of abnormal or atretic follicles as well as with follicle dimensions and the proportion of vessels. $P<.05$ was considered statistically significant.

RESULTS

Follicle Density and Classification

Before grafting. Follicle density ranged between 3,281.1 and 16,414.9 follicles/mm^3 in pediatric ovarian tissue and between 53.3 and 738.7 follicles/mm^3 in adult ovarian tissue, so it was statistically significantly higher in pediatric patients than adult patients ($P=.01$) (Supplemental Fig. 1A and B,

FIGURE 1



Linear regression analysis comparing age and (**A**) follicle density (P=.005, r=−0.7), (**B**) abnormal follicles (P=.02, r=−0.6), and (**C**) atretic follicles (P=.0, r=−0.39). Follicle classification in (**D**) nongrafted pediatric and adult ovarian tissue and (**E**) after long-term xenografting. Results are reported as mean ± standard error of the mean.

Masciangelo. Ovarian tissue characterization in children. Fertil Steril 2020.

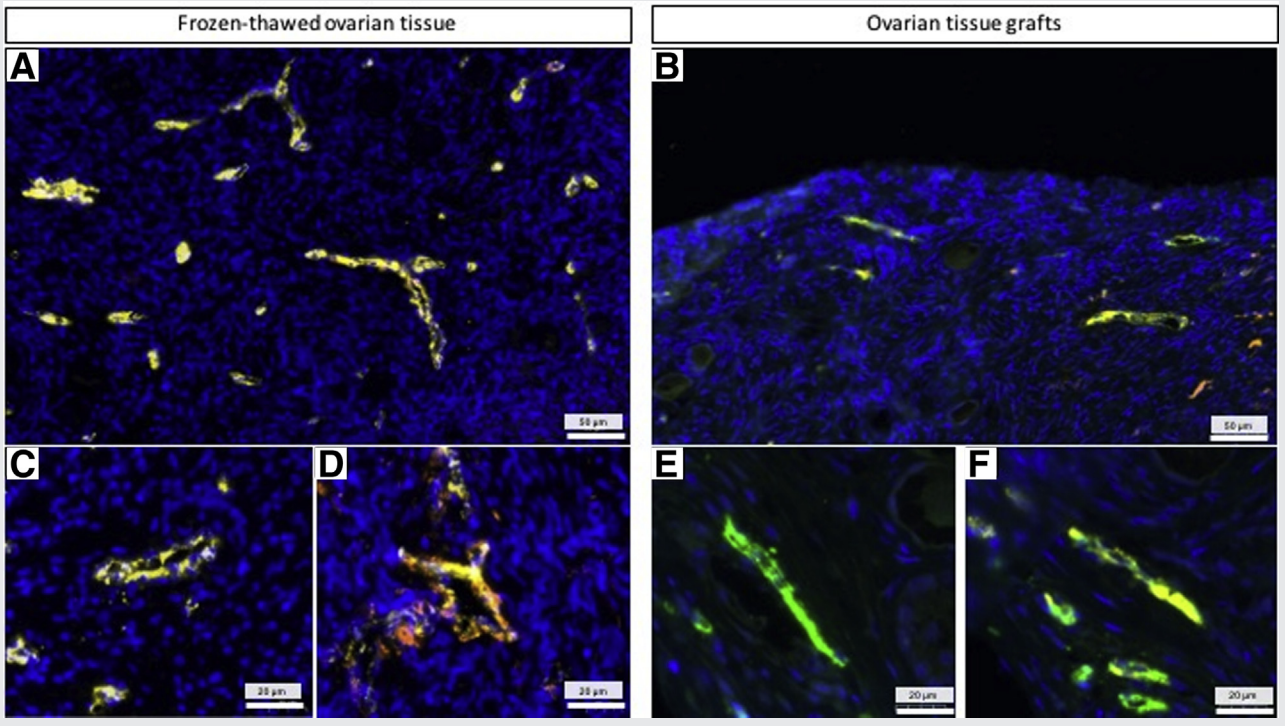
available online). Linear regression analysis conducted to assess the correlation between patient age and follicle density revealed a statistically significant negative correlation (P=.005, r= −0.7) (Fig. 1A). Follicles with abnormal features (Supplemental Fig. 1E) were observed in pediatric ovarian tissue (9.5%) but not in adult patients. Atretic follicles were also detected in frozen-thawed pediatric ovarian tissue (18.2% vs. 5% in adults).

Classification of healthy follicles at different growth stages as well as abnormal and atretic follicles is presented in Figure 2D. Quiescent-stage (primordial and intermediate) follicles constituted the largest proportion of the follicle pool (71.5% in young girls and 80.9% in adults). The rates of growing follicles at the primary stage were 1% in pediatric subjects and 11.1% in adults; at the secondary stage, 0 in pediatric subjects and 3.1% in adults; and at the antral stage, 0 in both groups. When we excluded nonviable follicles (abnormal and atretic), quiescent-stage follicle rates increased to 98% in pediatric patients and 85% in adult patients. A correlation coefficient (r) used to assess the relationship between patient age and the number of abnormal and atretic follicles (Fig. 1B–C) showed a statistically significant negative correlation between age and the number of abnormal (P=.03, r= −0.60) and atretic (P=.02, r=−0.39) follicles.

After grafting. In grafted tissue, follicle density ranged between 1,825.2 and 2824.8 follicles/mm^3 in pediatric patients and between 0 and 1,381.9 follicles/mm^3 in adult patients. Follicle density was statistically significantly higher in pediatric than adult subjects (P=.0005) (Supplemental Fig. 1C–D). Classification of healthy follicles at different growth stages, as well as abnormal and atretic follicles, is shown in Figure 1E.

In pediatric patients, the rates of quiescent-stage (primordial and intermediate) follicles remained high after grafting (64.3% in young girls vs. 66.8% in adults). The proportions of growing follicles at the primary stage were 3% in pediatric patients and 23.3% in adults; at the secondary stage, 21.3% in pediatric subjects and 4.1% in adults; and at the antral stage, 3% in pediatric subjects and 0.3% in adults. After transplantation, growing follicles at the last stage of development were observed in statistically significantly higher numbers in the pediatric group (secondary follicles, P=.0005; antral follicles, P=.02). No statistically significant difference was evidenced in the numbers of primordial, intermediate, or primary follicles. In pediatric patients, abnormal follicles appeared to

FIGURE 2



Immunofluorescence staining for (**A**) human von Willebrand factor (vWF) (yellow) and anti-smooth muscle actin (aSMA) (orange) in nongrafted ovarian tissue, and (**B**) human vWF (yellow), murine CD31 (green), and aSMA (orange) in ovarian tissue grafts. Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). (**C**) Human immature vessels, (**D**) human mature vessels, (**E**) murine immature vessel, and (**F**) chimeric human and murine immature vessel.

Masciangelo. Ovarian tissue characterization in children. Fertil Steril 2020.

decline after long-term grafting (2.6%), as did atretic follicles (6.3%, $P=.03$).

Follicle Diameter Measurements

Supplemental Table 1 (available online) reports the number of analyzed follicles and mean values for follicle diameter, oocyte diameter, oocyte nucleus diameter, and GC layer thickness in both pediatric and adult subjects before (top) and after (bottom) grafting. In all measured dimensions and in both groups, gradual increments were observed from the primordial to the primary stage. Moreover, pediatric follicles were systematically smaller than their adult counterparts at all follicle stages before and after grafting, although not statistically significantly.

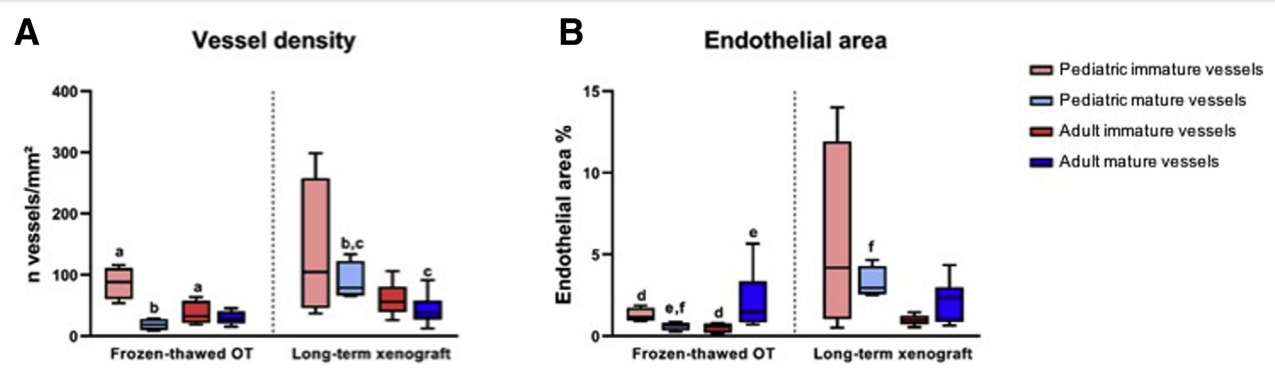
Vascularization

Frozen-thawed ovarian tissue. Figure 2 shows immunofluorescent staining for vascularization before (Fig. 2A) and after (Fig. 2B) grafting. Vessels in nongrafted tissue were identified as either human immature (Fig. 2C) or human mature (Fig. 2D), and vessels after grafting were identified as human immature, murine immature (Fig. 2E), or chimeric immature (Fig. 2F), or human or chimeric mature vessels. Negative and positive controls are shown in Supplemental Figure 2. The results of vessel density and endothelial area for human, murine, or chimeric immature and mature vessels are reported in Supplemental Table 2 (available online) before and after grafting. No difference was observed between human and murine vessels (data not shown), so human, murine, and chimeric immature vessels were pooled into "immature vessels," and human and chimeric mature vessels into "mature vessels."

Figure 3 reports the results of the vessel density (Fig. 3A) and endothelial area (Fig. 3B) analyses. Evaluation of vessel density revealed the presence of more immature vessels in pediatric ovarian tissue than in adult ovarian tissue (86.6 ± 13.1 vs. 37.4 ± 7.4 immature vessels/mm^2, respectively; $P=.007$). With regard to mature vessel density, no difference was encountered (18.7 ± 4.8 vs. 29.7 ± 4.4 mature vessels/mm^2, respectively). Analysis of endothelial area found that the endothelial area of immature vessels was greater in pediatric subjects than in adults (1.2 ± 0.2% vs. 0.5 ± 0.1%; $P=.009$), whereas the endothelial area of mature vessels was statistically significantly larger in adults than in young girls (0.6 ± 0.1% vs. 2.1 ± 0.7% in children and adults, respectively; $P=.01$).

Grafts. Analysis of vessel density after grafting revealed no statistically significant difference in the number of immature vessels (136.1 ± 57.8 vs. 60.01 ± 11.1 in pediatric and adult patients, respectively), but a statistically significant increase was found in the number of mature vessels in pediatric versus adult subjects (89 ± 15.7 vs. 42.7 ± 10.7 in children and adults, respectively). Moreover, no statistically significant difference

FIGURE 3



(**A**) Vessel density and (**B**) endothelial area in pediatric and adult patients before and after grafting. [a]P=.007; [b]P=.009; [c]P=.03; [d]P=.09; [e]P=.01; [f]P=.009.

Masciangelo. Ovarian tissue characterization in children. Fertil Steril 2020.

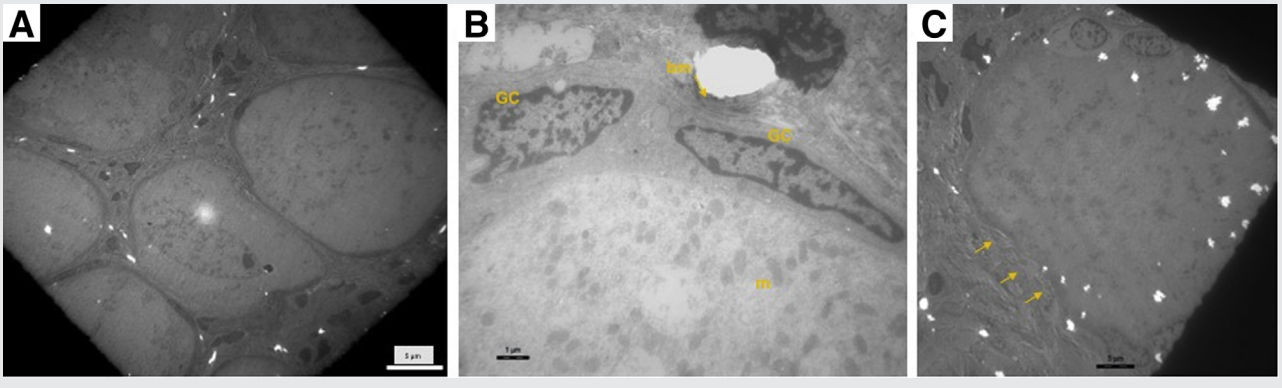
was found in the immature vessel endothelial area between pediatric (5.7 ± 2.9%) and adult (0.9 ± 0.1%) patients, nor in the mature vessel endothelial area (3.2 ± 0.4% vs. 2.2 ± 0.5% in pediatric and adult subjects respectively). Specifically in pediatric patients, a statistically significant increase was encountered in both vessel density and endothelial area of mature vessels after grafting (vessel density: P=.009; endothelial area: P=.009); no difference was observed in adult patients.

TEM

Over 110 follicles were analyzed in the pediatric group and more than 30 follicles in the adult group. In both groups, follicles were either at the primordial/intermediate or primary stage, and their size was approximately 30–50 μm. Follicles from adult patients were composed of a complete layer of flattened or cuboidal GCs, depending on growth stage, surrounding a round-shaped oocyte. Some follicles were closely associated with only slight cytoplasmic expansion of GCs interposed in between (nests). The oocyte exhibited a large, eccentrically placed, spherical nucleus. Cytoplasmic organelles were observed around the nucleus, constituting Balbiani's vitelline body (12).

Figure 4A shows a TEM image of pediatric ovarian tissue with several follicles at the primordial stage. Follicles from young girls were surrounded by a thin basal membrane, and GCs showed a large nucleus containing irregularly distributed chromatin and one or two nucleoli. The GCs also contained numerous mitochondria, with transverse cristae, free ribosomes, endoplasmic reticulum, and scattered vesicles. Microvillus interdigitations were present between GCs and the oocyte (Fig. 4B). Oocytes in pediatric follicles contained a large round vesicular nucleus, with finely granular dispersed chromatin and at least one nucleolus. Abnormal follicles were also evidenced (Fig. 4C). Loss of the round shape was visible, and the cytoplasm was entirely filled with vacuoles, but its organelles appeared to be preserved.

FIGURE 4



(**A**) Ultrathin section of frozen-thawed human pediatric follicles in a 3-year-old patient. (**B**) A portion of one pediatric follicle: a single layer of granulosa cells (GCs) along a thin basal membrane (bm) encloses the oocyte (o). The oocyte cytoplasm shows scattered mitochondria (m). (**C**) Abnormal follicle with an irregular shape (arrows).

Masciangelo. Ovarian tissue characterization in children. Fertil Steril 2020.

DISCUSSION

Since the first successful ovarian tissue transplantation resulting in a live birth back in 2004 (9), considerable progress has been made in an attempt to improve the efficacy of the procedure, and various studies looking to further enhance the technique are currently ongoing (2, 13–15). Nevertheless, the majority of these studies are conducted on frozen-thawed ovarian tissue from adult patients, so at present our understanding of ovarian tissue from young pediatric patients is limited.

Our study evaluated follicle density, stage, and size, and the vasculature of ovarian tissue from pediatric patients (median age 3 years) before and after long-term grafting because data on the dynamics of ovarian follicle growth in these patients are obviously scarce. A previous study (6) investigated ovarian tissue from girls approaching puberty (median age 11 years), but we decided to focus on a cohort of very young pediatric patients. Moreover, unlike in the study by Luyckx et al. (6), we did not administer hormone stimulation to our mice. Frozen-thawed ovarian tissue showed greater follicle density in pediatric (mean 7,054.8 follicles/mm^3) than adult (mean 335.3 follicles/mm^3) patients, and a statistically significant decline was noted with age. Furthermore, when data from our study were compared with those of Luyckx et al. (6), follicle density in pediatric patients was indeed found to be higher in older prepubertal patients: 3,281.1–16,414.9 follicles/mm^3 in our study versus 210–9578 follicles/mm^3 in the study by Luyckx et al. (6). These results confirm and reinforce earlier studies reporting a statistically significant decrease in the follicle population related to age (6, 16–19).

With regard to follicle classification, the majority of follicles (71.5%) were at the quiescent stage, and around 28% were either abnormal or atretic. After exclusion of these nonviable follicles, the proportion of quiescent follicles shifted to almost 100%. These findings are in line with the literature, as it was previously reported that ovaries at birth contain around 300,000 primordial follicles, with only a small percentage at more advanced growth stages (19–21). Indeed, massive germ cell loss occurs in the fetal ovary during oogenesis, which governs the perinatal supply of primordial follicles (20) and continues into postnatal life with a substantial loss of primordial follicles (19, 22, 23). Such loss involves not only healthy viable follicles, but also those with abnormal morphology, as demonstrated by the strong decline in the prevalence of these follicles.

The high rate of atretic and abnormal follicles observed in our study actually contributes to the decrease in follicle density observed from birth to puberty, as their rate statistically significantly diminishes with age. Our hypothesis is supported by the results of Sadeu et al. (24), who found that human fetal ovarian tissue contains predominantly primordial follicles, often with abnormalities, as well as morphological signs of atresia in 18% of follicles. Although ovaries at birth contain thousands of follicles, the true ovarian reserve, composed of follicles potentially able to grow and give rise to pregnancy, is substantially lower. The mechanisms of follicle death are still poorly understood (25), and further studies are needed to elucidate the fate of atretic and abnormal follicles.

It has been proposed that a quality-control mechanism exists in fetal ovaries, extending into the early pediatric period, whereby deficient follicles act as a source of cytoplasmic and nuclear material for healthy viable follicles. These nonviable follicles may be recycled to support the growth of neighboring healthy follicles that possess distinct but still unknown characteristics, allowing them to be selected to survive (7, 26). This theory may be supported by our TEM findings on abnormal follicles. Indeed, even though we evidenced loss of the round shape, with the cytoplasm completely filled with vacuoles, the cytoplasmic organelles looked well preserved. We could therefore speculate about the existence of a recycling mechanism that leads to preservation of the cytoplasmic organelles of these "ancillary" follicles and their transmission to viable follicles to sustain their survival. However, investigations into this theory need to continue.

In our study, pediatric ovarian tissue was also transplanted to SCID mice to elucidate whether pediatric follicles are able to grow and, if so, to better understand follicle growth dynamics by evaluating follicle density and stage. Our results demonstrated that follicle density in pediatric patients remains elevated after grafting, despite a drop in follicle numbers (mean 7,054.8 follicles/mm^3 before grafting vs. mean 2,219.6 follicles/mm^3 after grafting). This outcome suggests that most follicles can survive until transplantation, but a large proportion are subsequently lost, probably due to hypoxia and ischemia mechanisms (27–29). The majority of our follicles were at the primordial stage (64.3% and 66.8% in pediatric and adult tissue, respectively), and statistically significant follicle growth was observed after long-term transplantation in both groups. Indeed, we found more than 27% of follicles to be at the primary, secondary or antral stage in both the pediatric and adult groups.

Although enhanced follicle growth could also be the consequence of hypoxia and ischemia, this finding is at odds with earlier data from our and other teams (6, 7). Luyckx et al. (6) detected less than 5% of growing follicles after 5 months of xenografting and hormone stimulation in older prepubertal patients (median age: 11 years). Our hypothesis to explain this difference is that the patient's age at the time of biopsy could have an impact. We could speculate that establishment of the hypothalamic-pituitary-gonadal axis in the prepubertal period has a priming effect on follicles that have managed to survive massive follicle attrition, rendering them susceptible to the hormone fluctuations of the postpubertal period. On the other hand, very young pediatric ovarian tissue may respond to external stimuli, such as transplantation, in a more uncontrolled way.

We also assessed follicle diameter to shed light on the morphometric characteristics of pediatric and adult follicles and how they change with age. We observed that pediatric follicles were systematically smaller than adult follicles in the dimensions taken into account, confirming the findings of Westergard et al. (11) in 2007, who reported an increase in average follicle and nucleus diameter from childhood to the mid-30s. The impact of these differences is still unknown, but an interesting parallel can be drawn with results from in vitro maturation of oocytes.

Indeed, oocytes from prepubertal animals appear to have reduced ability to progress to mature stages and are therefore considered to be of low quality. This limited ability has been attributed to a discrepancy between meiotic competence and ooplasm maturation caused by impaired cytoplasmic and metabolic maturation (30–33). The diminished capacity of prepubertal oocytes to reach adequate maturity could arise when the oocyte is still at the preantral follicle stage, and may be the consequence of deficient or insufficient acquisition of nuclear and cytoplasmic components required to support physiologic growth.

Ours is the first detailed report on pediatric ovarian tissue vascularization. Established vasculature consists of an inner lining of endothelial cells, surrounded by pericytes and vascular smooth muscle cells for vessel maturity and stabilization. Upon stimulation by an angiogenic event like hypoxia, vessels are destabilized, and endothelial cells migrate toward the angiogenic stimulus. Endothelial cells then reorganize themselves to form a lumen, followed by stabilization by pericytes and vascular smooth muscle cells (34–35). Among angiogenic factors required for new vessel formation are vascular endothelial growth factor, fibroblast growth factor-2, and angiopoietin. We previously demonstrated that these factors are up-regulated in hypoxic conditions (36), but their heightened production in grafted ovarian tissue from adult patients could also be related to the physiological angiogenic characteristics of the ovary (37–38).

Our results show that pediatric ovarian tissue contains more and larger immature vessels than adult tissue. Immature pediatric vessels might have the ability to migrate towards one another, forming more structured vessels that have acquired the ability to perfuse the tissue properly. The greater number of immature vessels in pediatric ovarian tissue may also lead to enhanced proliferation of new blood vessels. Indeed, when we compared the vessel density and endothelial area of pediatric immature vessels with adult tissue after grafting, no difference was observed. We may therefore hypothesize that the pediatric ovary is able to undergo the process of revascularization after transplantation in a physiologic but accelerated manner, finally reaching the same vessel number and size as adult ovarian tissue.

Concerning mature vessels, we observed statistically significantly larger mature vessels in adult tissue than in pediatric tissue. The presence of such large vessels may be attributed to the continual cyclic changes of folliculogenesis and ovulation to which the adult ovary is subjected monthly (39). When evaluating mature vessel density and endothelial area before and after grafting, statistically significantly more and larger mature vessels were reported in the pediatric group, suggesting that recruitment of vascular smooth muscle cells is enhanced in these patients. This may be the consequence of a different response of pediatric tissue to angiogenic factors in hypoxic conditions, so further studies specifically focusing on this hypoxia-induced vascular response are required to provide greater insights into this process.

In conclusion, this is the first time that pediatric ovarian tissue from very young patients (median age 3 years) has been evaluated and transplanted to SCID mice without any hormone stimulation. Our results suggest that ovarian tissue from very young patients behaves differently, not only compared with ovarian tissue of adult patients but also that of older prepubertal subjects. It also appears to respond to external stimuli in a more uncontrolled way. By analyzing the ovarian vasculature of pediatric patients, we found that pediatric ovarian tissue—which has more abundant immature vessels that are also larger—can undergo the posttransplantation revascularization process in an accelerated way, reaching a higher level of vessel stability than in adults.

Acknowledgments: The authors thank Mira Hryniuk, B.A., for reviewing the English language of the article; and Dolores Gonzalez, Olivier Van Kerk, and Sarah Storder for their technical assistance.

REFERENCES

1. Wallace WHB, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? Lancet Oncol 2005;6:209–18.
2. Donnez J, Dolmans MM. Fertility preservation in women. N Engl J Med 2017;377:1657–65.
3. Poirot C, Abirached F, Prades M, Coussieu C, Bernaudin F, Piver P. Induction of puberty by autograft of cryopreserved ovarian tissue. Lancet 2012;379:588.
4. Ernst E, Kjærsgaard M, Birkebæk NH, Clausen N, Andersen CY. Case report: stimulation of puberty in a girl with chemo- and radiation therapy induced ovarian failure by transplantation of a small part of her frozen/thawed ovarian tissue. Eur J Cancer 2013;49:911–4.
5. Matthews SJ, Picton H, Ernst E, Andersen CY. Successful pregnancy in a woman previously suffering from β-thalassemia following transplantation of ovarian tissue cryopreserved before puberty. Minerva Ginecol 2018;70:432–5.
6. Luyckx V, Scalercio S, Jadoul P, Amorim CA, Soares M, Donnez J, et al. Evaluation of cryopreserved ovarian tissue from prepubertal patients after long-term xenografting and exogenous stimulation. Fertil Steril 2013;100:1350–7.
7. Anderson RA, McLaughlin M, Wallace WH, Albertini DF, Telfer EE. The immature human ovary shows loss of abnormal follicles and increasing follicle developmental competence through childhood and adolescence. Hum Reprod 2014;29:97–106.
8. Gosden RG, Baird DT, Wade JC, Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at −196 degrees C. Hum Reprod 1994;9:597–603.
9. Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, et al. Live-birth after orthotopic transplantation of cryopreserved ovarian tissue. Lancet 2004;364:1405–10.
10. David A, Dolmans MM, Van Langendonckt A, Donnez J, Amorim CA. Immunohistochemical localization of growth factors after cryopreservation and 3 weeks' xenotransplantation of human ovarian tissue. Fertil Steril 2011;95:1241–6.
11. Westergaard CG, Byskov AG, Andersen CY. Morphometric characteristics of the primordial to primary follicle transition in the human ovary in relation to age. Hum Reprod 2007;22:2225–31.
12. Nottola SA, Camboni A, Van Langendonckt A, Demylle D, Macchiarelli G, Dolmans MM, et al. Cryopreservation and xenotransplantation of human ovarian tissue: an ultrastructural study. Fertil Steril 2008;90:23–32.
13. Manavella DD, Cacciottola L, Desmet CM, Jordan BF, Donnez J, Amorim CA, et al. Adipose tissue-derived stem cells in a fibrin implant enhance neovascularization in a peritoneal grafting site: a potential way to improve ovarian tissue transplantation. Hum Reprod 2018;33:270–9.
14. Manavella DD, Cacciottola L, Pommé S, Desmet CM, Jordan BF, Donnez J, et al. Two-step transplantation with adipose tissue-derived stem cells increases follicle survival by enhancing vascularization in xenografted frozen-thawed human ovarian tissue. Hum Reprod 2018;33:1107–16.
15. Manavella DD, Cacciottola L, Payen VL, Amorim CA, Donnez J, Dolmans MM. Adipose tissue-derived stem cells boost vascularization in

grafted ovarian tissue by growth factor secretion and differentiation into endothelial cell lineages. Mol Hum Reprod 2019;25:184–93.
16. Faddy MJ. Follicle dynamics during ovarian ageing. Mol Cell Endocrinol 2000;163:43–8.
17. Qu J, Godin PA, Nisolle M, Donnez J. Distribution and epidermal growth factor receptor expression of primordial follicles in human ovarian tissue before and after cryopreservation. Hum Reprod 2000;15:302–10.
18. Schmidt KLT, Byskov AG, Nyboe Andersen A, Müller J, Andersen CY. Density and distribution of primordial follicles in single pieces of cortex from 21 patients and in individual pieces of cortex from three entire human ovaries. Hum Reprod 2003;18:1158–64.
19. Wallace WH, Kelsey TW. Human ovarian reserve from conception to the menopause. PLoS One 2010;5:e8772.
20. Baker TG. A quantitative and cytological study of germ cells in human ovaries. Proc Roy Soc Lond B Biol Sci 1963;158:417–33.
21. Forabosco A, Sforza C, De Pol A, Vizzotto L, Marzona L, Ferrario VF. Morphometric study of the human neonatal ovary. Anat Rec 1991;231:201–8.
22. Himelstein-Braw R, Byskov AG, Peters H, Faber M. Follicular atresia in the infant human ovary. J Reprod Fertil 1976;46:55–9.
23. Faddy MJ, Telfer E, Gosden RG. The kinetics of pre-antral follicle development in ovaries of CBA/Ca mice during the first 14 weeks of life. Cell Tissue Kinet 1987;20:551–60.
24. Sadeu JC, Cortvrindt R, Ron-El R, Kasterstein E, Smitz J. Morphological and ultrastructural evaluation of cultured frozen-thawed human fetal ovarian tissue. Fertil Steril 2006;85:1130–41.
25. Albamonte MI, Albamonte MS, Stella I, Zuccardi L, Vitullo AD. The infant and pubertal human ovary: Balbiani's body-associated VASA expression, immunohistochemical detection of apoptosis-related BCL2 and BAX proteins, and DNA fragmentation. Hum Reprod 2013;28:698–706.
26. Tingen C, Kim A, Woodruff TK. The primordial pool of follicles and nest breakdown in mammalian ovaries. Mol Hum Reprod 2009;15:795–803.
27. Van Eyck AS, Jordan BF, Gallez B, Heilier JF, Van Langendonckt A, Donnez J. Electron paramagnetic resonance as a tool to evaluate human ovarian tissue reoxygenation after xenografting. Fertil Steril 2009;92:374–81.
28. Amorim CA, David A, Dolmans MM, Camboni A, Donnez J, Van Langendonckt A. Impact of freezing and thawing of human ovarian tissue on follicular growth after long-term xenotransplantation. J Assist Reprod Genet 2011;28:1157–65.
29. Masciangelo R, Hossay C, Donnez J, Dolmans MM. Does the Akt pathway play a role in follicle activation after grafting of human ovarian tissue? Reprod Biomed Online 2019;39:196–8.
30. Sánchez F, Adriaenssens T, Romero S, Smitz J. Quantification of oocyte-specific transcripts in follicle-enclosed oocytes during antral development and maturation in vitro. Mol Hum Reprod 2009;15:539–50.
31. Pawlak P, Cieslak A, Warzych E, Zejden Z, Szumacher-Strabel M, Molinska-Glura M, et al. No single way to explain cytoplasmic maturation of oocytes from prepubertal and cyclic gilts. Theriogenology 2012;78:2020–30.
32. Paczkowski M, Yuan Y, Fleming-Waddell J, Bidwell CA, Spurlock D, Krisher RL. Alterations in the transcriptome of porcine oocytes derived from prepubertal and cyclic females is associated with developmental potential. J Anim Sci 2011;89:3561–71.
33. Leoni GG, Palmerini MG, Satta V, Succu S, Pasciu V, Zinellu A, et al. Differences in the kinetic of the first meiotic division and in active mitochondrial distribution between prepubertal and adult oocytes mirror differences in their developmental competence in a sheep model. PLoS One 2015;10:e0124911.
34. Gerhardt H, Betsholtz C. Endothelial–pericyte interactions in angiogenesis. Cell Tissue Res 2003;314:15–23.
35. Robinson RS, Woad KJ, Hammond AJ, Laird M, Hunter MG, Mann GE. Angiogenesis and vascular function in the ovary. Reproduction 2009;138:869–81.
36. Van Langendonckt A, Romeu L, Ambroise J, Amorim C, Bearzatto B, Gala JL, et al. Gene expression in human ovarian tissue after xenografting. Mol Hum Reprod 2014;20:514–25.
37. Redmer DA, Reynolds LP. Angiogenesis in the ovary. Rev Reprod 1996;1:182–92.
38. Semenza GL. Regulation of hypoxia-induced angiogenesis: a chaperone escorts VEGF to the dance. J Clin Invest 2001;108:39–40.
39. Suzuki T, Sasano H, Takaya R, Fukaya T, Yajima A, Nagura H. Cyclic changes of vasculature and vascular phenotypes in normal human ovaries. Hum Reprod 1998;13:953–9.

Poblaciones foliculares y vascularización en el tejido ovárico de pacientes pediátricos antes y después de injertos a largo plazo.

Objetivo: Caracterizar el tejido ovárico de pacientes pediátricos evaluando el desarrollo y la vascularización en las poblaciones de folículos y comparándolo con el tejido adulto después del injerto.

Diseño: Estudio experimental prospectivo.

Entorno: Centro de investigación académica.

Paciente(s): Cinco niñas (edad media de 3 años) y siete mujeres (edad media de 28 años).

Intervención(es): Tinción de hematoxilina y eosina, inmunofluorescencia y evaluación de microscopía electrónica de transmisión (TEM) antes de y después del injerto.

Principales medidas de resultado: Densidad, morfología, clasificación y tamaño de los folículos, vascularización del tejido ovárico, y ultraestructura de los folículos.

Resultado(s): El tejido ovárico congelado-descongelado fue dividido en tres fragmentos: controles no injertados, TEM, e injertos durante 20 semanas. La densidad de folículos fue significativamente mayor en los pacientes pediátricos que en los adultos, aunque disminuyó en ambos grupos después del trasplante, se mantuvo más alto en los pacientes pediátricos. En el grupo pediátrico, los folículos en etapa de reposo eran la mayoría del conjunto de folículos antes y después del injerto, mientras que los folículos en crecimiento aumentaron estadísticamente de manera significativa en ambos grupos después del injerto. Se observaron también folículos anormales y atrésicos en el tejido pediátrico y disminuyeron con la edad y después del injerto. El tejido ovárico pediátrico contenía más vasos grandes inmaduros, mientras que los vasos maduros eran más grandes en los adultos. El análisis de la TEM de los folículos pediátricos anormales mostró la pérdida de forma y vacuolización del citoplasma sin daño de los orgánulos.

Conclusión(es): Se observaron diferencias estadísticamente significativas en la densidad folicular entre los pacientes pediátricos y los adultos, pero la distribución de los folículos fueron similares antes y después del injerto, con la excepción de los folículos atrésicos y anormales. El tejido pediátrico contiene más y mayores vasos inmaduros que el tejido adulto, y el proceso de revascularización post-trasplante se acelera en este grupo.