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# Revascularization of human ovarian grafts is equally efficient from both sides of the cortex tissue

## BIOGRAPHY

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## ABSTRACT

**Research question:** Does revascularization of human ovarian grafts in a mouse model occur with equal efficiency from both sides of the cortex tissue?

**Design:** Twenty-four frozen-thawed ovarian cortex pieces from 12 women were transplanted to immunodeficient mice, for 8 days to analyse graft revascularization using immunohistochemical detection of murine CD31, or for 8 weeks to evaluate follicle density (follicles/mm$^3$). The CD31-positive vessel area and density were quantified using a custom-designed application. Three regions of interest (ROI) were defined in each tissue section: the cortical side, the centre and the medullary side. Vessels were subdivided into three categories according to size: microvessels (<300 μm$^2$), small vessels (300–1000 μm$^2$) and large vessels (>1000–3000 μm$^2$).

**Results:** No significant difference in the mean percentage of the CD31-positive vessel area was found between the three ROI (cortical side: 3.9% ± 0.2%; centre: 3.5% ± 0.2%; medullary side: 4.0% ± 0.3%; $P = 0.17$), but a significantly lower density of vessels was found in the centre of the human ovarian grafts compared with the cortical and medullary sides (cortical side: 323 ± 14 vessels/mm$^2$; centre: 240 ± 12 vessels/mm$^2$; medullary side: 301 ± 18 vessels/mm$^2$; $P < 0.001$). Microvessels comprised 89–91% of all vessels in the three ROI. Follicle density in ungrafted cortex pieces was 51.8 ± 17.3 and 14.7 ± 3.7 follicles/mm$^3$ after 8 weeks of xenografting, resulting in a follicle survival rate of 28%.

**Conclusions:** Host revascularization was established equally efficiently from both sides of transplanted human ovarian cortex, suggesting that transplantation techniques ensuring revascularization from both sides of the ovarian graft could potentially facilitate faster graft revascularization.

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## KEYWORDS

Fertility preservation
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## INTRODUCTION

The efficacy of ovarian tissue cryopreservation and ovarian tissue transplantation (OTT) is hampered by the massive loss of ovarian follicles after OTT (*Demeestere et al., 2009*). The lack of vascular anastomosis causes hypoxia and ischaemia/reperfusion injury in the ovarian grafts and up to 70–90% of the follicles may be lost, thereby limiting the functionality and lifespan of the ovarian grafts (*Demeestere et al., 2009*; *Mamsen et al., 2021*). Orthotopic transplantation in which the frozen-thawed ovarian tissue is transplanted to either the pelvic wall or into/on the ovary is the preferred transplantation site compared with heterotopic transplantation (*Demeestere et al., 2009*). However, similar reproductive outcomes were recently reported for sub-peritoneal abdominal, ovarian and pelvic sites at one centre (*Gook et al., 2021*). Currently, no consensus exists regarding the surgical approach for transplanting ovarian tissue and numerous techniques are being used clinically worldwide (*Donnez et al., 2018*).

Structurally the avascular cortex harbouring the resting follicle pool is covered by a capsule of condensed fibrous tissue, the tunica albuginea, and the ovarian surface epithelium. It is unknown whether revascularization occurs equally efficiently from both sides of human ovarian grafts. The aim of the current study was to evaluate the spatial distribution of neovascularization in human ovarian xenografts and discuss the potential clinical implications.

## MATERIALS AND METHODS

### Human ovarian tissue

Cryopreserved human ovarian cortex was donated from 12 women aged 27–31 years who had had their ovarian tissue frozen for fertility preservation. The diagnoses of the women were breast cancer ($n$ = 5), Hodgkin lymphoma ($n$ = 4), non-Hodgkin lymphoma ($n$ = 1), molar pregnancy ($n$ = 1) and aplastic anaemia ($n$ = 1). The study was approved by the Scientific Ethics Committee of the Capital Region on 9 June 2020 (J. no. H-2-2011-044).

### Experimental design

This study comprised the untreated control group used in the study by Mamsen and colleagues (*Mamsen et al., 2021*). Two pieces of thawed ovarian cortex (approximately 5 mm × 5 mm × 1 mm in size) from each patient was transplanted to a subcutaneous pocket on the dorsal side of an ovariectomized female immunodeficient Naval Medical Research Institute (NMRI)-NUDE mouse. Grafts were retrieved after 8 days and 8 weeks and processed for histology. Evaluation of follicle density has been described by Mamsen and colleagues (*Mamsen et al., 2021*). Animal experiments were approved by the Danish Animal Experiments Inspectorate on 8 June 2020 (case file 2015-15-0201-00505) under Danish legislation and conducted as previously described (*Mamsen et al., 2021*).

### Immunohistochemical analysis

Retrieved ovarian grafts were fixed in 4% paraformaldehyde overnight, dehydrated, embedded in paraffin and sectioned at 5 µm for immunohistochemical analysis using a rabbit anti-mouse CD31 (Pecam-1 [platelet endothelial cell adhesion molecule-1]) monoclonal antibody (catalogue number 77699; Cell Signaling Technology, Denmark; dilution: 1:100) as previously described (*Mamsen et al., 2021*).

An application (APP) was developed to quantify murine CD31-positive endothelial cell area and the number of vessels per $mm^2$ using whole-slide imaging technology and the Visiopharm Author module (Visiopharm, Denmark). The NanoZoomer S360 digital slide scanner (Hamamatsu Phototonics K.K., Japan) was used to scan the slides with magnification × 40 and one focal point. The whole-slide imaging files were analysed using the Visiopharm Integrator System (VIS) program (version: 2020.08.4 Production Release). Three regions of interest (ROI) were defined in each section: cortical side, centre and medullary side. The 'cortical side' refers to the outer part of the ovarian cortex orientated outwards, and the 'medullary side' refers to the inner part of the ovarian cortex orientated towards the medulla. Murine host tissue attached to the graft was excluded from the analysis by manually adjusting the ROI. Heatmaps were generated for each section based on the CD31-positive area. Vessels were subdivided into three categories according to size: microvessels (<300 $µm^2$), small vessels (300–1000 $µm^2$) and large vessels (>1000–3000 $µm^2$).

### Statistical analyses

Statistical analysis was performed using R version 3.5.1 (R Foundation for Statistical Computing, Austria). A linear mixed model was used to analyse the overall differences between groups, and a Tukey's test was used to analyse differences between individual groups. $P$-values <0.05 were considered significant.

## RESULTS

### Graft revascularization

FIGURE 1A shows the CD31 immunostaining in a human ovarian xenograft after 8 days. FIGURE 1B, C shows the three predefined sizes of vessel and the three ROI identified by the APP. FIGURE 1D shows a representative heatmap from each participant ($n$ = 12). No significant difference in the mean percentage of CD31-positive vessel area was found between the three ROI (cortical side: 3.9% ± 0.2%; centre: 3.5% ± 0.2%; medullary side: 4.0% ± 0.3%; $P$ = 0.17; FIGURE 1E); however, a significantly lower density of vessels was found in the centre of the human ovarian grafts compared with the cortical and medullary sides (cortical side: 323 ± 14 vessels/$mm^2$; centre: 240 ± 12 vessels/$mm^2$; medullary side: 301 ± 18 vessels/$mm^2$; $P$ < 0.001; FIGURE 1F). Most of the quantified vessels were microvessels, which comprised 89–91% of all vessels in the three ROI (FIGURE 1G).

### Follicle density

The mean follicle density in ungrafted ovarian cortex ($n$ = 12) was 51.8 ± 17.3 follicles/$mm^3$, and after 8 weeks of xenografting the mean follicle density in the grafted controls ($n$ = 12) was 14.7 ± 3.7 follicles/$mm^3$, resulting in a follicle survival rate of 28%.

## DISCUSSION

Various surgical transplantation techniques are used clinically worldwide, but it is still unknown which techniques and transplantation sites provide the most efficient revascularization of the ovarian grafts. The current findings demonstrate that the fibrous outer cortex does not act as barrier for the neovascularization process after transplantation since revascularization in the form of murine vessels was established equally efficiently from both sides of transplanted human ovarian cortex. In Belgium (Donnez technique) and the USA (Silber technique) ovarian tissue is secured with stitches or Interceed® on top of the decorticated remaining ovary with the medullary graft side adhering to the remaining

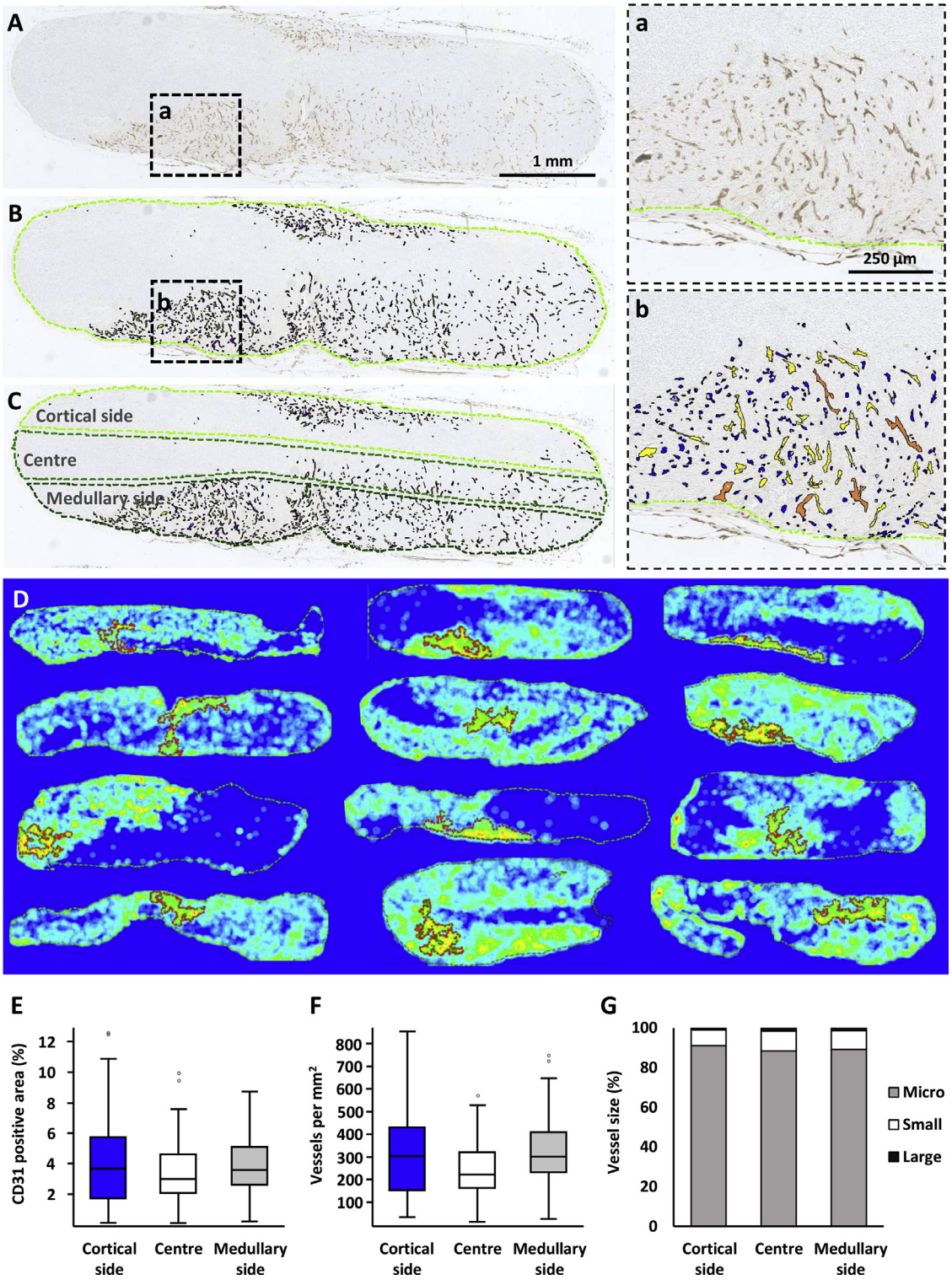


**FIGURE 1** Quantification of murine CD31-positive vessel area and density in human ovarian xenografts. (A) CD31 immunostaining in a human ovarian graft after 8 days of xenotransplantation. Insert (a) shows the high magnification of a selected area. (B) Vessels identified by the application (APP) according to the three size categories: microvessels (<300 µm$^2$; blue), small vessels (300–1000 µm$^2$; yellow) and large vessels (>1000–3000 µm$^2$; orange). Insert (b) shows the high magnification of a selected area. (C) Image from the APP showing the three defined ROI (cortical side, centre and medullary side). (D) A panel of representative heatmaps from transplanted ovarian grafts from the 12 participants. (E) No significant difference in the mean percentage of CD31-positive vessel area was found between the three ROI. (F) A significantly ($P < 0.001$) lower number of vessels per mm$^2$ was found in the centre of the human ovarian grafts compared with the cortical and medullary sides. (G) Distribution of vessels according to size given as a percentage of the total number of vessels. The boxplots show the median, interquartile range, maximum and minimum and outliers. Blue boxes: cortical side; white boxes: centre; grey boxes: medullary side.

ovary to mimic the normal ovarian architecture (*Donnez et al., 2018*). In Denmark (Andersen technique) and Israel (Meirow technique) ovarian tissue is transplanted to subcortical pockets made by longitudinal or transverse incisions in the remaining intact ovary to facilitate angiogenesis from both the cortical and medullary sides of the graft (*Donnez et al., 2018*).

The current authors suggest that ensuring an ingrowth of vessels from both sides of the ovarian grafts, for example transplantation to subcortical or sub-peritoneal pockets, would potentially facilitate faster revascularization compared with transplantation to a decorticated ovary (allowing an ingrowth of vessels from only the medullary side). Faster revascularization would shorten the ischaemic period and potentially increase follicle survival; however, further studies are needed to directly compare transplantation techniques.

Despite differences in cryoprotectant regimens, transplantation sites and surgical techniques, experienced centres appear to report comparable reproductive outcomes for women who have undergone OTT (*Dolmans et al., 2021*; *Gook et al., 2021*). Thus, the efficiency of OTT is not notably affected by either surgical technique or transplantation site when comparing experienced centres. Moving forward, improving graft viability and follicle survival before and after OTT will be key to increase success rates.

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