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# BASAL LAMINA CHARACTERIZATION IN FROZEN-THAWED AND LONG-TERM GRAFTED HUMAN PREPUBERTAL OVARIAN TISSUE

**Charlotte Philippart[1], Rossella Masciangelo[1], Alessandra Camboni[1], Jacques Donnez[2], Marie-Madeleine Dolmans[1,3*]**

[1]Pôle de Recherche en Gynécologie, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, Avenue Mounier 52, bte. B1.52.02, 1200 Brussels, Belgium

[2]Société de Recherche pour l'Infertilité, Avenue Grandchamp 143, 1150 Brussels, Belgium

[3]Département de Gynécologie, Cliniques Universitaires St. Luc, Avenue Hippocrate 10, 1200 Brussels, Belgium

***Correspondence:**

Professor Marie-Madeleine Dolmans: marie-madeleine.dolmans@uclouvain.be

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## ABSTRACT

**Research question:** Are there differences in the composition and structure of the basal lamina surrounding follicles in prepubertal versus adult human ovarian tissue?

**Design:** Frozen-thawed human ovarian tissue from 6 prepubertal and 7 adult patients was divided into three fragments in each case: two for non-grafted tissue evaluation and one for long-term xenografting to mice. Collagen IV and laminin expression were investigated by immunohistochemistry before and after grafting. The basal lamina was analyzed by transmission electron microscopy on frozen-thawed tissue.

**Results:** In frozen-thawed tissue, collagen IV was significantly less expressed around prepubertal follicles than around adult follicles, while laminin was significantly more expressed. In grafted tissue, collagen IV expression was significantly higher around prepubertal primordial follicles than in non-grafted tissue, reaching similar levels to those in adult tissue. Ultrastructure analysis showed the basal lamina around follicles in prepubertal frozen-thawed tissue to be rather patchy and thinner than around adult follicles (primordial/intermediate, p=0.001; primary, p=0.016).

**Conclusion:** In frozen-thawed tissue, the basal lamina around prepubertal follicles is less mature than around adult follicles, but becomes similar in both prepubertal and adult subjects after grafting. Grafting could therefore induce maturation of the basal lamina around prepubertal follicles.


**Keywords:** Prepubertal ovarian tissue cryopreservation, follicular basal lamina, collagen IV, laminin, immunohistochemistry, transmission electron microscopy.

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## INTRODUCTION

Over recent years, considerable progress in oncological treatments has given cancer patients a much better chance of survival. However, some of these treatments have serious side effects that impact the ovaries (Toloudi et al., 2014), including premature ovarian insufficiency that could lead to infertility (Donnez and Dolmans, 2017). Different techniques have therefore been developed to preserve fertility in women suffering from cancer. One of the most important is ovarian tissue cryopreservation prior to cancer treatment, and subsequent transplantation of the frozen-thawed tissue once the patient is cured of her disease and wishes to be pregnant. The first birth following transplantation of cryopreserved ovarian tissue was achieved in 2004, and since then, the number of births has grown exponentially, reaching 130 by 2017 (Donnez and Dolmans, 2017).

This technique is the only available approach to safeguard fertility in prepubertal girls affected by cancer, but there are only three existing cases of transplantation of ovarian tissue cryopreserved when the subjects were prepubertal. In two cases, the grafted ovarian tissue was intended to induce puberty, while in the third case in 2016, the patient was seeking pregnancy (Poirot et al., 2012; Ernst et al., 2013; Matthews et al., 2018). Due to the lack of published studies, human prepubertal ovarian tissue is poorly understood. However, given the increasing numbers of childhood cancers that could influence the fertility of these young girls, it is vital that we learn more about prepubertal ovarian tissue and how it reacts after grafting back to patients who have now become adults (Wallace et al., 2005).

Ovarian follicles are surrounded by a basal lamina, a specialized form of extracellular matrix that separates granulosa cells from the stroma (Kalluri, 2003). It is a lattice-type network composed of different macromolecules, including collagen IV, laminin, nidogen and perlecan, which are produced by stromal cells and granulosa cells l. Collagen IV is the most abundant component found in a mature basal lamina (Hohenester and Yurchenco, 2013). It is composed of three peptide chains that merge together to form a triple-helix structure, interrupted in some regions to create multiple bends (Kalluri, 2003). Collagen IV is therefore not fibrillar and is more flexible than other types of collagen (Kalluri, 2003). Laminin, the primary organizer of basal lamina structure, is a large and flexible protein formed of three polypeptide chains linked by disulfide bonds (Alberts, 2008). During formation of the basal lamina, laminin deposition occurs first, inducing secondary assembly of the collagen IV network (Poschl et al., 2004; Villacorte et al., 2016). Cross-links between collagen IV and laminin are stabilized by nidogen and perlecan (Arends and Lieleg, 2016). The basal membrane is involved in ovarian follicle maturation (Heeren et al., 2015), so we hypothesize that differences in basal

lamina composition around prepubertal and adult follicles may explain the limited follicle growth activation seen in prepubertal tissue compared to adult tissue after in vitro culture (Anderson et al., 2014). A number of studies have already characterized the basal lamina surrounding follicles in various species, including humans (Berkholtz et al., 2006; Heeren et al., 2015). However, to our knowledge, this is the first study to characterize the basal membrane around follicles from human prepubertal ovarian tissue. Our aim was to establish whether the structure and composition of the basal lamina differ in prepubertal and adult tissue before and after long-term grafting.

## 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 ovarian tissue from deceased patients to be used for scientific purposes.

Frozen-thawed human ovarian tissue from thirteen different patients was collected. Six patients were prepubertal at the time of biopsy (age range 1-10 years, mean 5.5 years) and seven were adults (age range 20-35 years, mean 27.8 years). All of them were suffering from malignant diseases. Ovarian tissue had been cryopreserved prior to cancer treatment as part of the fertility preservation strategy, and cryovials containing this tissue were obtained from all subjects. The fragments were each divided into three pieces, two to be assessed as non-grafted tissue (frozen-thawed tissue) and one to be grafted to severe combined immunodeficient (SCID) mice. Immunohistochemical analyses were performed on one piece of frozen-thawed tissue as well as grafted tissue from all patients. Ultrastructural analyses were conducted on the other piece of frozen-thawed tissue from each patient.

### Ovarian tissue freezing and thawing

The ovarian tissue was cut into strips (dimensions ± 10 mm x 5 mm x 1 mm) and cryopreserved by the slow-freezing technique at Cliniques Universitaires Saint-Luc following the protocol described by Gosden (Gosden et al., 1994). Briefly, the strips were placed in cryovials containing Leibovitz L-15 medium and dimethyl sulfoxide (DMSO), a cryoprotectant. The cryovials were transferred to a programmable freezer, cooled slowly down to -140°C, and stored in liquid nitrogen at -196°C.

Three ovarian strips from each patient were then thawed according to Gosden's protocol (Gosden et al., 1994). Briefly, the tissues were warmed at room temperature for 2 minutes,

before being transferred to a water bath at 37°C for 2 more minutes. Thereafter, the fragments were washed three times in fresh HEPES-MEM (Gibco) medium for 5 minutes to remove the cryoprotectant (Amorim et al., 2012). One strip from each patient was fixed in 4% formaldehyde and embedded in paraffin for immunohistochemical analysis. The second strip was fixed in 2.5% glutaraldehyde and embedded in epoxy resin for ultrastructural analysis. The third strip was grafted to SCID mice for long-term grafting.

**Grafting of ovarian tissue to SCID mice**

Guidelines for animal welfare were fully respected and the study was approved by the Committee on Animal Research of the Université Catholique de Louvain (reference 2018/UCL/MD/40). One tissue fragment from each patient was grafted to an 8-week-old non-ovariectomized female SCID mouse in all cases (Charles River, L'Arbresle, France), as described by Luyckx (Luyckx et al., 2013). After making a medial ventral incision, frozen-thawed ovarian tissue was sutured with one or two stitches (6/0) to the inner side of the peritoneum. The abdominal wall was then closed. The mice were kept in sterile conditions for 5 months, after which they were euthanized by cervical dislocation and the ovarian tissue strips were removed. Finally, the grafted tissue was fixed in 4% formaldehyde and embedded in paraffin for immunohistochemical analysis.

**Histology**

Fixed tissues were embedded in paraffin after different steps of dehydration, inclusion and embedding. Paraffin blocks were cut into 5-µm sections, which were placed on SuperFrost slides.

**Follicle classification**

Follicles were classified based on their stage of development according to Gougeon's criteria (Gougeon, 1996), and only those with a visible nucleus were considered for analysis. Primordial follicles contain an oocyte surrounded by one layer of flattened granulosa cells. In intermediate follicles, the granulosa cells are flattened and cuboid. Oocytes in primary and secondary follicles are surrounded by cuboid granulosa cells, one layer in primary follicles and multiple layers in secondary follicles. In this study, primary and secondary follicles were pooled together for investigation purposes and termed 'growing follicles'.

**Collagen IV and laminin analysis**

*Basal lamina characterization*

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Basal lamina composition was evaluated by immunohistochemistry (IHC) that stained collagen IV and laminin on sections of prepubertal and adult ovarian tissues before and after grafting. Sections of uterine fibroids were used as controls for collagen IV staining, while sections of skin tissue were used as controls for laminin staining, as shown in Supplemental Figure 1. The sections were deparaffinized with Histosafe (Yvsolab SA, Beerse, Belgium), toluene and 2-propanol (Merck, Darmstadt, Germany), then rehydrated in demineralized water. Endogenous peroxidases were inactivated with 3% $H_2O_2$ (Dako). Two antigen retrieval methods were used: for collagen IV, heat epitope retrieval with citrate buffer (concentration 0.01 M, pH 6.0, incubation for 75 minutes at 98°C); and for laminin, enzymatic retrieval with proteinase K and Tris HCl (concentration 20 µg/ml, pH 7.5, incubation for 30 minutes at 37°C). To avoid nonspecific fixation of the primary antibody, the slides were incubated at room temperature for 1 hour in a solution containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA) (Sigma Aldrich, Overijse, Belgium) and Tris-buffered saline (TBS). They were then incubated overnight at 4°C with primary antibodies diluted in a solution of 1% NGS and 0.1% BSA in TBS: mouse monoclonal anti-human collagen IV (Dako, M0785) at a dilution of 1:25; or rabbit polyclonal anti-human laminin (Abcam, Ab11575) at a dilution of 1:1000. According to guidelines, negative controls were conducted with mouse- and rabbit-specific polyclonal antibodies (FLEX ready-to-use, IS600, Dako, North Carolina) instead of the primary antibody (Hewitt et al., 2014). After 12 hours of incubation, the sections were again incubated with goat anti-mouse (for collagen IV) or anti-rabbit (for laminin) secondary antibodies linked to the horseradish peroxidase (HRP) enzyme (K4001 and K4003, Dako Envision+ System HRP). Diaminobenzidine (DAB vector 4100, Dako) was used as a chromogen that reacts with HRP, before counterstaining nuclei with hematoxylin (Merck, Darmstadt, Germany). Finally, the slides were dehydrated in 2-propanol and Histosafe.

Sections were scanned with the Leica SCN400 scanner (Leica Biosystems, Dublin) at a magnification of 40X, and analyzed using TissueIA software (Wetzlar, Germany) to quantify expression of collagen IV and laminin in the basal lamina of follicles. To this end, the basal lamina of follicles containing a visible nucleus was encircled. Computer-assisted quantification of the staining intensity of each marker was then achieved with Leica's proprietary artificial intelligence (TissueIA, Wetzlar, Germany) by applying the following formula (Courtoy et al., 2018):

$$Staining\ intensity = \left[\left(\left(\frac{I_A}{I_{mean}}\right) \times \left(\frac{S_{posA}}{S_{posTot}}\right)\right) + \left(\left(\frac{I_B}{I_{mean}}\right) \times \left(\frac{S_{posB}}{S_{posTot}}\right)\right) + \cdots\right] \times I_{mean}$$

(IA=average intensity annotation A; Imean=mean of average staining intensities of all annotations; SposA=total tissue area of annotation A; SposTot=sum of total tissue area of all annotations). Staining intensity was expressed in arbitrary units, as described by Courtoy (Courtoy et al., 2018).

**Transmission electron microscopy**

Ultrastructural analysis was performed to measure and compare the thickness of the basal lamina around prepubertal and adult follicles in frozen-thawed ovarian tissue. For this purpose, a small portion of frozen-thawed ovarian tissue from each patient was fixed in a solution of 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) for 48 hours at 4°C. The tissues were then washed in PBS at room temperature and postfixed in 1% osmium tetroxide in distilled water for 2 hours. After washing again in PBS, the fragments were dehydrated in a bath containing increasing concentrations of ethyl alcohol. Following overnight immersion in propylene oxide (solvent substitution), the tissue pieces were embedded in epoxy resin (Agar Scientific, Essex, UK). Blocks of resin were then cut into semithin sections (1-µm-thick) using an Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria), stained with toluidine blue, and examined under a light microscope (Zeiss Axioskop, Germany). Thereafter, ultrathin sections (60-nm-thick) were cut with a diamond knife, mounted on copper grids, and contrasted with saturated uranyl acetate and lead citrate. Finally, they were inspected using a Zeiss EM10 electron microscope operating at 80 kV (Zeiss, Germany) and photographed with ProgRes Capture Pro image capture software (version 2.10.0.; Jenoptik, Germany**).** Prepubertal and adult follicles derived from frozen-thawed tissue were analyzed by observers blinded to the origin of the tissue. In each case, the thickness of the basal lamina was measured in 4 different spots around the follicles to calculate the mean.

**Statistics**

Statistical analyses were conducted using GraphPad Prism software (version 8.1.2 for Windows). The unpaired t-test was applied to compare mean values obtained in prepubertal and adult ovarian tissue. Linear regression was done to correlate the staining intensity of collagen IV and laminin around follicles with age. A value of $p<0.05$ was considered significant.

**RESULTS**

**Collagen IV and laminin expression in frozen-thawed prepubertal and adult tissues**

*Collagen IV*

The staining intensity of collagen IV was determined in 176 follicles from prepubertal ovarian tissue and 88 follicles from adult tissue. Exact numbers of each type of follicle are detailed in Supplemental Table 1. Collagen IV expression around follicles from prepubertal (x) and adult (x') tissue pregrafting is shown in Figure 1. The presence of collagen IV around follicles increased during follicular development in both prepubertal and adult subjects (Figure 2A). In addition, collagen IV expression was significantly weaker around prepubertal follicles than adult follicles at all stages (Figure 2A). Linear regression was conducted to correlate expression of collagen IV around follicles with age**.** Collagen IV expression increased significantly with age around primordial follicles ($p=0.032$; $r_2=0.5$) and tend to increase around growing follicles ($p=0.07$; $r_2=0.3$). It was not the case around intermediate follicles ($p=0.29$; $r_2=0.1$) (Figure 2B).

*Laminin*

The staining intensity of laminin was determined in 139 follicles from prepubertal subjects and 100 follicles from adults (exact numbers are detailed in Supplemental Table 1). Laminin staining around follicles from prepubertal (x) and adult (x') tissue pregrafting is shown in Figure 3. Expression of laminin around follicles increased during follicular development in both prepubertal and adult subjects (Figure 2C). Furthermore, primordial follicles (713.8 ± 130.3 prepubertal vs 170.1 ± 27.7 adult; $p=0.03$) and intermediate follicles (888.0 ± 113.0 prepubertal vs 320.1 ± 104.9 adult; $p=0.01$) showed more intense laminin expression in prepubertal tissue than in adult tissue. However, laminin expression in growing follicles was similar between prepubertal and adult subjects (1322.3 ± 132.3 prepubertal vs 1495.0 ± 91.7 adult) (Figure 2C). Linear regression was then conducted to determine the evolution of laminin expression with age. Results presented in Figure 2D show that laminin expression around primordial and intermediate follicles decreased significantly with age ($r^2=0.64$ in primordial follicles, $p=0.003$; $r^2=0.48$ in intermediate follicles, $p=0.02$). On the contrary, laminin expression around growing follicles was found to remain stable with age.

**Collagen IV and laminin expression in prepubertal and adult tissues after transplantation**

*Collagen IV*

The staining intensity of collagen IV was determined in 120 follicles from grafted prepubertal tissue and **13** follicles from grafted adult tissue (exact numbers are shown in Supplemental

Table 1). Collagen IV staining around follicles from prepubertal tissue postgraft (x'') can be seen in Figure 1. After transplantation, our findings (Figure 4A) revealed no significant difference in collagen IV expression between prepubertal and adult follicles at any stage of development (primordial: 111.5 ± 47.0 prepubertal vs 115.3 ± 75.6 adult; intermediate: 265.4 ± 134.4 prepubertal vs 277.2 ± 183.6 adult; growing follicles: 605.5 ± 129.7 prepubertal vs 779.2 ± 367.8 adult).

*Laminin*

The staining intensity of laminin was determined in 71 follicles from grafted prepubertal ovarian tissue and 11 follicles from grafted adult tissue (exact numbers are specified in Supplemental Table 1). Laminin staining around follicles from prepubertal tissue postgraft (x'') is shown in Figure 3. After transplantation, there was no significant difference in staining between prepubertal and adult subjects at any stage of follicle development (primordial: 759.3 ± 143.3 prepubertal vs 1044.7 ± 201.0 adult; intermediate: 1079.5 ± 122.6 prepubertal vs 1554.0 ± 169.7 adult; growing follicles: 1442.5 ± 112.2 prepubertal vs 1553.9 ± 72.0 adult) (Figure 4B).

Comparison **of collagen IV and laminin expression in prepubertal tissue before and after grafting**

*Collagen IV*

In prepubertal tissue, collagen IV expression was significantly stronger around primordial follicles after grafting than in frozen-thawed tissue (21.9 ± 4.9 vs 111.5 ± 47.0 in frozen-thawed and grafted tissue respectively; p=0.03) (Figure 5A). In intermediate follicles, collagen IV expression appeared to increase after grafting, but the difference was not significant (111.0 ± 18.0 [frozen-thawed] vs 267.0 ± 134.4 [postgrafting]). Finally, in growing follicles, collagen IV expression did not significantly change after grafting (600.5 ± 66.3 [frozen-thawed] vs 605.5 ± 129.7 [postgrafting]) (Figure 5A). Regarding adult follicles, we did not observed any significant change in collagen IV expression around any follicle types in grafted tissue compared to frozen tissue (data not shown).

*Laminin*

In prepubertal tissue, if we compare laminin expression before and after grafting, there was no significant difference around any type of prepubertal follicles (primordial: 713.8 ± 130.3 [frozen-thawed] vs 759.28 ± 143.3 [postgrafting]; intermediate: 888.0 ± 113.0 [frozen-

thawed] vs 1079.5 ± 122.6 [postgrafting]; growing follicles: 1322.3 ± 132.3 [frozen-thawed] vs 1442.5 ± 112.2 [postgrafting]) (Figure 5B). In adult tissue, there was a significant increase in laminin expression around primordial and intermediate follicles in grafted tissue compared to frozen tissue. However, laminin expression was similar around growing follicles in grafted and frozen tissues (data not shown). Nevertheless, due to the small numbers of follicles found in adult ovarian tissue after grafting, we should remain cautious with those results.

**Transmission electron microscopy for ultrastructural analysis of frozen-thawed prepubertal and adult tissue**

Healthy-looking primordial/intermediate and primary follicles from frozen-thawed prepubertal (n=12) and adult (n=10) ovarian tissue were analyzed by light and transmission electron microscopy (LM and TEM) (Figure 6A-D). In prepubertal tissue, 9 follicles were at the primordial/intermediate stage and 3 at the primary stage. In adult tissue, 5 follicles were at the primordial/intermediate stage and 5 at the primary stage. No secondary follicles were found in any of the tissue.

By LM and TEM, all analyzed follicles appeared round in shape and each was composed of an oocyte surrounded by a single layer of granulosa cells (Figure 6A and 6C). TEM showed ovarian follicles from both prepubertal and adult samples to contain an oocyte with a large round nucleus and numerous cytoplasmic organelles, including mitochondria, the Golgi apparatus, free ribosomes, membranes of rough and smooth endoplasmic reticulum (RER and SER) and annulate lamellae, as previously described (Nottola et al., 2008; Masciangelo et al., 2020) (Figure 6A and 6C). Close interdigitations were visible between oocyte microvilli and granulosa cell projections. Granulosa cells exhibited an indented nucleus with condensed chromatin, as well as mitochondria with transverse cristae, free ribosomes, RER, SER and scattered lipid droplets (Figure 6B and 6D). At the periphery, all follicles were surrounded by a basal lamina showing different aspects in prepubertal and adult subjects.

In all analyzed prepubertal follicles, regardless of their stage, the basal lamina appeared as a discontinuous and patchy layer, with a somewhat loose aspect (Figure 6A and 6B). Conversely, all adult follicles were surrounded by a continuous, compact and partially laminated basal lamina (Figure 6C and 6D). Moreover, the basal lamina around primordial/intermediate follicles (prepubertal: 0.109 μm ± 0.008 μm; adult: 0.423 μm ± 0.033 μm; p=0.001) and primary follicles (prepubertal: 0.171 μm ± 0.011 μm; adult1.096 μm ±

293 0.119 μm, p=0.0159) was significantly thinner around prepubertal follicles than adult follicles
294 (Figure 6E)

295 **DISCUSSION**

296 A mature basal lamina plays an important role in follicle maturation: structural support for
297 developing follicles, selective filtration for follicular fluid formation, reservoir of hormone
298 and growth factors that affect the development of granulosa cells and oocytes (Rodgers et al.,
299 2003; Sirotkin, 2011; Yue, 2014; Heeren et al., 2015). The basal membrane is mainly
300 composed of laminin and collagen IV. Laminin is able to interact with cells through different
301 cellular receptors, including integrins, that act as a link between components of the
302 extracellular matrix (ECM) and cell cytoskeleton (Khoshnoodi et al., 2008; Yamada and
303 Sekiguchi, 2015). Laminin interacts particularly with integrins α6β1 and α6β4, which are
304 strongly expressed by granulosa cells (Honda et al., 1995). Their interaction induces
305 activation of several signaling pathways, such as the Rho and MAP kinase pathways that
306 influence cell proliferation and differentiation, among other things (Yue, 2014). Collagen IV
307 is also able to interact with granulosa cells, but the precise mechanisms are unknown. Indeed,
308 collagen IV and laminin are both able to execute all of these functions, but studies have
309 demonstrated that collagen IV is more important to maintaining the stability of the basal
310 lamina, while the laminin network is probably more dynamic and interacts with the local
311 environment (Mak and Mei, 2017).

312 Previous studies on frozen-thawed prepubertal tissue have revealed that this tissue contains a
313 majority of quiescent follicles and a small proportion of growing follicles (Luyckx et al.,
314 2013; Anderson et al., 2014), indicating that only few follicles have started their maturation.
315 Furthermore, Anderson et al. have observed that prepubertal tissue shows limited follicle
316 growth activation compared to adult tissue by performing six days of in vitro culture of
317 human prepubertal and adult ovarian tissue (Anderson et al., 2014). On the other hand,
318 Masciangelo et al. detected a large proportion of growing follicles after long-term grafting of
319 prepubertal ovarian tissue to SCID mice (Masciangelo et al., 2020), demonstrating that these
320 follicles are somehow able to activate maturation after grafting. Considering the importance
321 of the basal lamina to follicle growth and maturation, we decided to evaluate the composition
322 of the basal lamina in much greater depth.

323 *Collagen IV and laminin expression in frozen-thawed prepubertal and adult tissue*

324 Collagen IV expression was found to be significantly lower around prepubertal follicles than
325 adult follicles. Conversely, laminin expression around primordial and intermediate follicles

weakens during a woman's lifetime. Heeren et al. studied basal lamina formation in fetal human ovaries (Heeren et al., 2015) and found that at 10.5 and 17.5 weeks of development, germ cells (primordial oocyte precursors) contained granules with collagen IV and laminin protein in their cytoplasm. These authors observed that laminin-rich granules were far more numerous at this stage than collagen IV-rich granules. Then, at 20 weeks of development, emerging primordial follicles were surrounded by both collagen IV and laminin. In our study, the prepubertal follicles are surrounded with more laminin than collagen. Obviously, certain changes occur between the period investigated by Heeren (20 weeks) and the prepubertal state. We may speculate that collagen IV could be needed for compartmentalization to separate germ cells from stromal cells, after which it is degraded, and germ cells liberate their laminin-rich granules to initiate formation of the basal lamina.

To explain the difference in basal lamina composition between prepubertal and adult subjects, we reviewed papers characterizing formation of the basal lamina during embryogenesis. Studies conducted in mice showed that laminin is the first component to shape the basal lamina during early development, and its presence is sufficient for essential functions of the basal lamina, like proliferation and cell survival. However, collagen IV, emerging later in development, is fundamental to maintaining the integrity, stability and functionality of the basal lamina under conditions of increasing mechanical demands (Yurchenco and Cheng, 1993; Poschl et al., 2004; Villacorte et al., 2016). These findings correlate with our results**.** Indeed, prepubertal primordial and intermediate follicles were surrounded almost exclusively by laminin and very little collagen IV protein, and expression of these two markers varied significantly with age (collagen IV increased, laminin decreased). Furthermore, although growing follicles from prepubertal tissue were surrounded by similar amounts of laminin as adult growing follicles, there was still less collagen IV around prepubertal follicles. We hypothesized that the basal lamina surrounding prepubertal follicles is simply not mature enough because of a lack of collagen IV. The presence of laminin should allow granulosa cells to proliferate (Honda et al., 1995; Yue, 2014), but low levels of collagen IV may compromise the stability and structural integrity of the basal lamina, vital to the functioning of this structure (Poschl et al., 2004). This could explain the limited growth activation seen in prepubertal follicles (Anderson et al., 2014), since the basal lamina is not able to support the mechanical stress that arises during follicle activation.

Concerning the low levels of laminin expression encountered around primordial and intermediate follicles in adult compared to prepubertal ovarian tissue, nothing of substance was found in the literature. Our first hypothesis in this regard was that strong laminin

expression in prepubertal tissue could facilitate for collagen IV deposition, required for further basal lamina maturation. Indeed, Matsubayashi et al. showed that an absence of laminin in *Drosophila* strongly affects collagen IV deposition (Matsubayashi et al., 2017). Other authors have suggested that the basal lamina plays a role in development of granulosa cell polarity, particularly laminin, as it is implicated in the polarization of epithelial cells (Rodgers et al., 2003; Plancha et al., 2005). Yet, Gao et al. reported that mice with defects in granulosa cell polarity show aberrant follicle development, indicating that the polarity of granulosa cells is important for follicle development (Gao et al., 2014). We could speculate that in prepubertal ovarian tissue, granulosa cells are not adequately formed, and that strong expression of laminin is needed to help their polarization.

*Collagen IV and laminin expression after long-term grafting*

In our study, transplantation of ovarian tissue to SCID mice was performed to evaluate the impact of grafting on the basal lamina around follicles. There was significantly more collagen IV surrounding primordial follicles in prepubertal tissue after grafting than in frozen-thawed prepubertal fragments. Even in intermediate follicles, which did not yield significant results, we observed that collagen IV expression around prepubertal follicles tended to increase due to grafting. Laminin expression around follicles from prepubertal tissue remained stable in terms of expression before and after grafting.

Because of these changes, there was no longer a significant difference in expression of collagen IV and laminin around prepubertal and adult follicles in grafted tissue. It therefore appears that the basal lamina surrounding prepubertal follicles starts to resemble to basal lamina surrounding adult follicles. If we consider the basal lamina around adult follicles from grafted tissue to be mature (Poschl et al., 2004), it could mean that grafting actually triggers accelerated maturation of the basal lamina around follicles from prepubertal tissue. Several hypotheses may explain this maturation. It could be due to grafting that induces dysregulation of signaling pathways related to ECM remodeling, which could initiate the shift from laminin to collagen. It might also be due to exposure of human ovarian tissue to a new hormonal environment after its grafting to mice. Maturation of the basal lamina could increase its stability, needed to support the mechanical demands arising during follicle activation. It could therefore indicate that follicles from prepubertal tissue are able to activate their development after grafting, as suggested by Masciangelo et al., who observed follicle growth after long-term grafting of prepubertal ovarian tissue to SCID mice (Masciangelo and al., 2020). We must nevertheless remain cautious of these results, because of the limited number of follicles that we were able to analyze in adult ovarian tissue.

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*Transmission electron microscopy in frozen-thawed prepubertal and adult tissue*

All investigated follicles from the two groups were surrounded by a basal lamina that differed in both thickness and appearance. The basal lamina was significantly thinner around prepubertal follicles than adult follicles, and in prepubertal tissue, this structure was patchy, appearing as a loose and much less compact layer than the basal lamina around adult follicles. Our results correlate with those of Poschl et al. who analyzed the ultrastructure of the basal lamina in normal and collagen IV-deficient mice embryos by TEM (Poschl et al., 2004). These authors observed that in embryos with no collagen IV in their basal lamina, the structure was thinner, amorphously deposited, or completely absent in some regions, compared to the basal lamina of control embryos (Poschl et al., 2004). Taken together with other basement membrane protein knockouts, their data suggest that laminin is sufficient to form basement membrane-like matrices during early development, but at later stages, the specific composition of components like collagen IV ensures integrity, stability and functionality. This supports our findings that prepubertal follicles are surrounded particularly by laminin, with a few collagen IV molecules, and that a basal lamina with no collagen IV is abnormal and immature**.** After grafting, histological observations indicated that the basal lamina around prepubertal follicles is more continuous and thicker than in frozen-thawed prepubertal tissue. This is in line with our hypothesis, suggesting that grafting allows maturation of the basal lamina around follicles from prepubertal ovarian tissue.

In conclusion, this study shows that in human frozen-thawed tissue, the basal lamina surrounding prepubertal follicles is less mature than that observed in adult follicles. Considering the key role of this structure in follicle growth, it is possible that immaturity of the basal lamina could explain the poor ability of prepubertal follicles to initiate activation and to have an efficient development. After transplantation, however, the basal lamina around prepubertal follicles is similar to that found in adult follicles, so grafting appears to trigger accelerated maturation of the basal lamina around prepubertal follicles. Maturation of the basal lamina around prepubertal follicles from prepubertal tissue could therefore allow these follicles to have an activation of their development after long-term grafting.

**Key message**

This study shows that the basal lamina surrounding follicles in prepubertal tissue is less mature than in adult tissue. Nevertheless, it appears that transplantation of ovarian tissue that was cryopreserved when the patient was still prepubertal could induce adequate levels of maturation of the basal lamina for follicle activation. Considering the importance of a mature

basal lamina in follicle activation, these results could well be relevant in the context of prepubertal ovarian tissue cryopreservation and transplantation.

**Declarations of interest**

Nothing to declare

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**Figures**

**Figure 1.** Collagen IV around follicles in frozen-thawed prepubertal and adult tissue and prepubertal tissue after grafting. Immunohistochemical labeling of collagen IV expression in the basal lamina of primordial (a, a', a''), intermediate (b, b', b''), primary (c, c', c'') and secondary (d, d', d'') follicles in frozen-thawed prepubertal (x) and frozen-thawed adult (x') ovarian tissue and in prepubertal tissue after transplantation (x'').

**Figure 2.** Intensity of collagen IV and laminin expression in frozen-thawed ovarian tissue (prepubertal vs adult). a: Intensity of collagen IV expression in frozen-thawed prepubertal and adult ovarian tissue. There was a significant difference in collagen IV expression around primordial (prepubertal: 21.9 ± 4.9 vs adult: 107.0 ± 27.5; p=0.02), intermediate (prepubertal: 111.0 ± 18 vs adult: 181.9 ± 24.1; p=0.03) and growing follicles (prepubertal: 600.5 ± 66.3 vs adult 1031.8 ± 110.6; p=0.03) between prepubertal and adult tissue. b: Linear regression showing the evolution of collagen IV expression with age around primordial (red circle), intermediate (green square), and growing follicles (blue triangle). Around primordial follicles, expression of collagen IV increased significantly with age (p=0.04, $r^2$=0.5). c: Intensity of laminin expression in frozen-thawed prepubertal and adult ovarian tissue. There was a significant decrease in laminin expression around primordial (prepubertal: 713.8 ± 130.3 vs adult: 170.1 ± 27.7; p=0.03) and intermediate (prepubertal: 888.0 ± 113.0 vs adult: 320.0 ± 104.9; p=0.01) follicles. Results are reported as mean ± SEM. d: Linear regression showing the evolution of laminin expression with age around primordial (red circle), intermediate (green square), and growing follicles (blue triangle). Expression of laminin decreased

significantly with age around primordial (p=0.003, $r^2$=0.6) and intermediate (p=0.03, $r^2$=0.5) follicles.

**Figure 3.** Laminin around follicles in frozen-thawed prepubertal and adult tissue and prepubertal tissue after grafting. Immunohistochemical labeling of laminin expression in the basal lamina of primordial (a, a', a''), intermediate (b, b', b''), primary (c, c', c'') and secondary (d, d', d'') follicles in frozen-thawed prepubertal (x) and frozen-thawed adult (x') ovarian tissue and prepubertal tissue after transplantation (x'').

**Figure 4.** Intensity of collagen IV and laminin expression in ovarian tissue after transplantation (prepubertal vs adult). a: Intensity of collagen IV expression in prepubertal and adult ovarian tissue after grafting, showing no significant difference. b: Intensity of laminin expression in prepubertal and adult ovarian tissue after transplantation, showing no significant difference. Results are reported as mean ± SEM.

**Figure 5.** Intensity of collagen IV and laminin expression in prepubertal ovarian tissue before and after grafting. a: Intensity of collagen IV expression in frozen-thawed prepubertal ovarian tissue and after transplantation. Expression of collagen IV was significantly stronger around primordial follicles (p=0.03) after grafting. b: Intensity of laminin expression in frozen-thawed prepubertal ovarian tissue and after transplantation, showing no significant difference. Results are reported as mean ± SEM.

**Figure 6.** Ultrastructural analysis of the basal lamina around follicles in frozen-thawed prepubertal and adult tissue. Ultrastructure of an intermediate prepubertal ovarian follicle at a magnification of 700x (a) and 7000x (b). Ultrastructure of an intermediate adult ovarian follicle at a magnification of 250x (c) and 4000x (d). Legend: BL: basal lamina; GC: granulosa cells; SC: stromal cells; O: oocyte; N: nucleus; M: mitochondria. Tissues were inspected using a Zeiss EM10 electron microscope (Zeiss, Germany) and photographed with ProgRes Capture Pro image capture software (version 2.10.0.1; Jenoptik, Germany). e: Thickness (μm) of the basal lamina around prepubertal and adult follicles. The basal lamina was significantly thinner around prepubertal follicles (primordial/intermediate: p=0.001; primary: p=0.0159). Results are reported as mean ± SEM.

**Supplemental Figure 1.** Positive and negative controls for collagen IV and laminin immunohistochemistry.

**Supplemental Table 1.** Numbers of follicle types considered for each analysis (primo: primordial; interm: intermediate; prima: primary; second: secondary).

Charlotte Philippart obtained her biomedical degree in 2020 from UCLouvain (Brussels).

During her last year of study, she completed a 1-year internship in the gynecology laboratory

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599 at UCLouvain under the supervision of Professor Dolmans. Her research focused on the

600 characterization of frozen-thawed and transplanted prepubertal human ovarian tissue.


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Figure 1

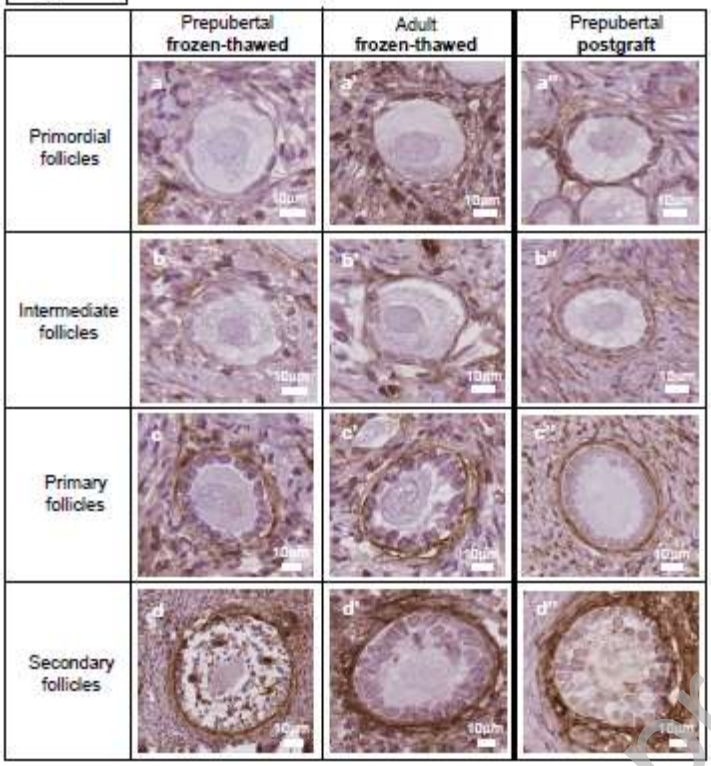
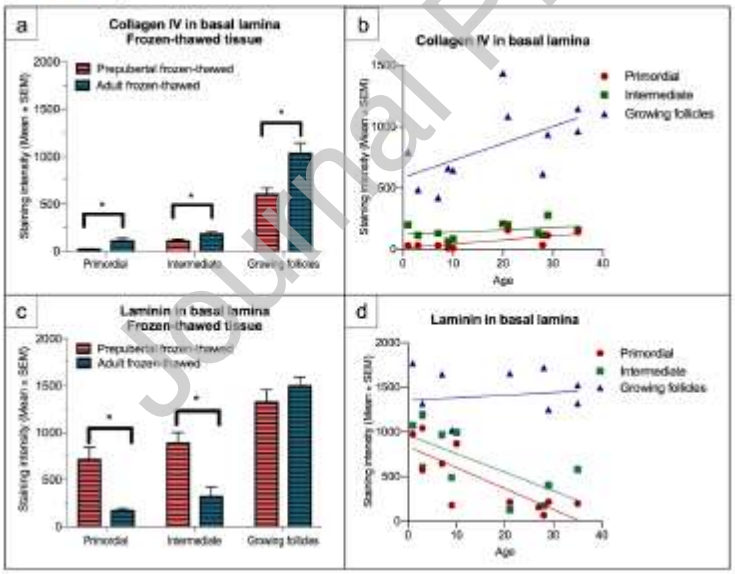


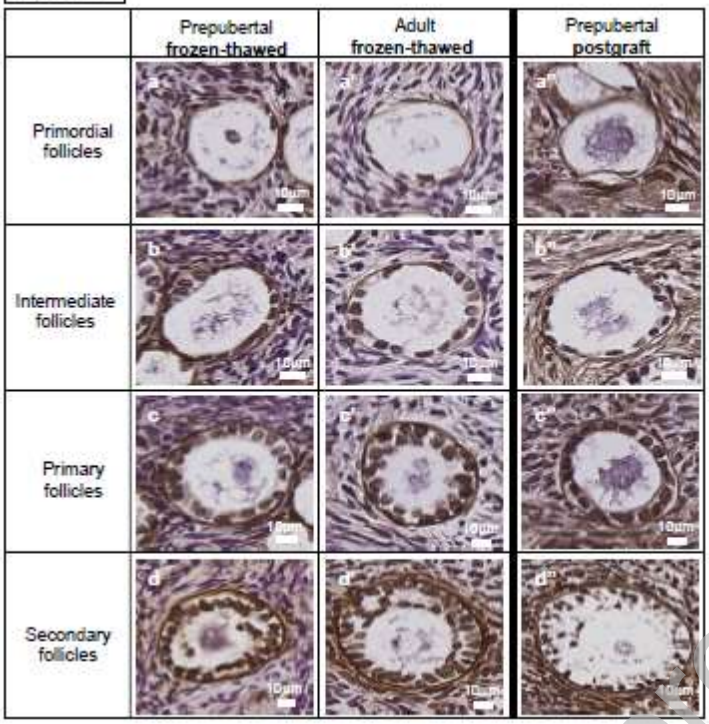

Figure 2




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Figure 3




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Figure 4

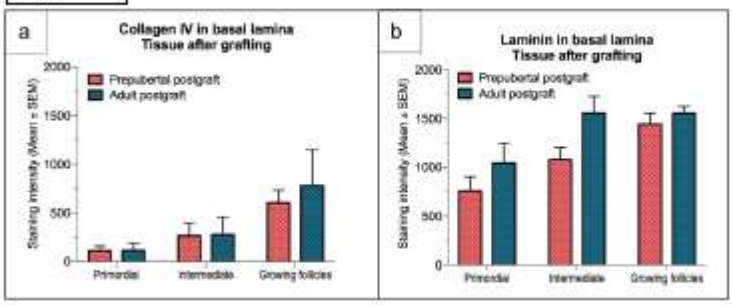
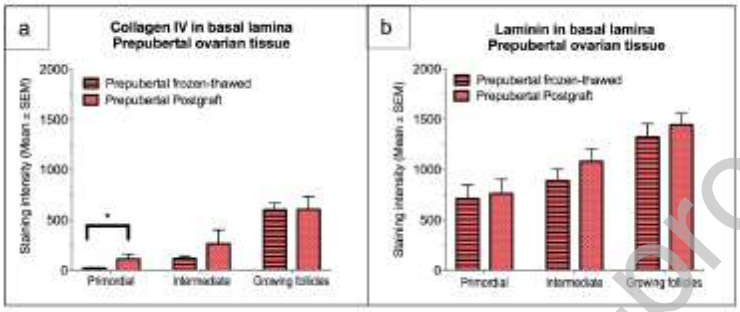


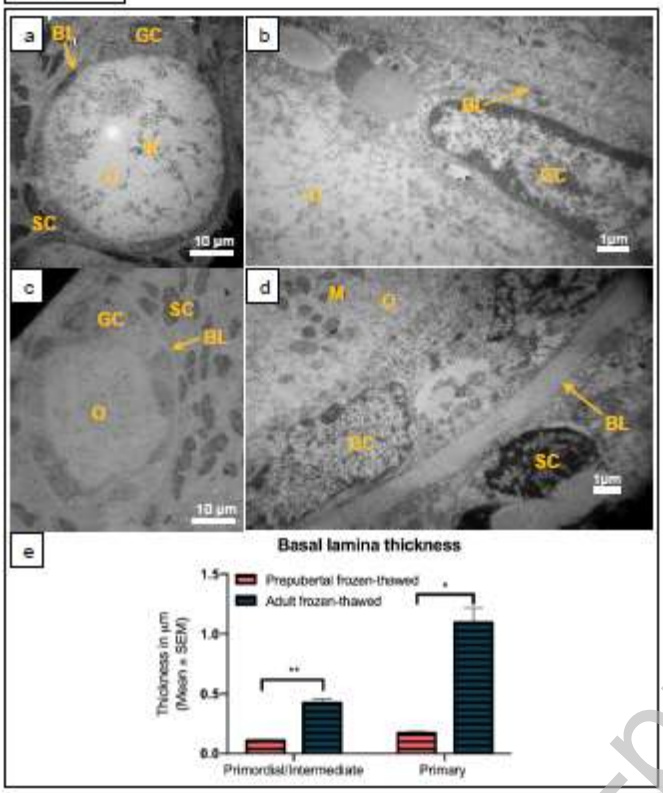

Figure 5




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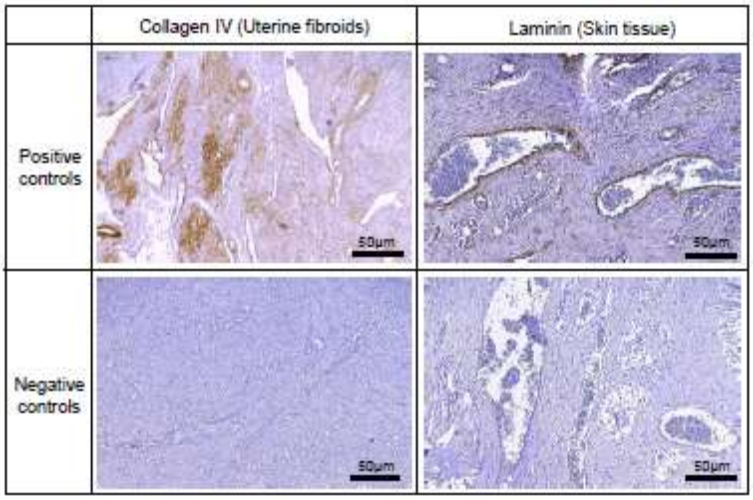
Figure 6




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Supplemental Figure 1




Supplemental Table 1

| Collagen IV | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Frozen-thawed | | | | Postgraft | | | |
| | Primo | Interm | Prima | Second | Primo | Interm | Prima | Second |
| Prepubertal | 53 | 103 | 13 | 7 | 77 | 26 | 4 | 13 |
| Adult | 22 | 43 | 13 | 10 | 4 | 3 | 4 | 2 |
| **Laminin** | | | | | | | | |
| | Frozen-thawed | | | | Postgraft | | | |
| | Primo | Interm | Prima | Second | Primo | Interm | Prima | Second |
| Prepubertal | 60 | 58 | 14 | 7 | 22 | 18 | 10 | 21 |
| Adult | 33 | 31 | 21 | 15 | 4 | 4 | 2 | 1 |

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