Evidence in Support for the Progressive Nature of Ovarian Endometriomas

Ding Ding,[1,*] Xi Wang,[1,*] Yishan Chen,[1,*] Giuseppe Benagiano,[3] Xishi Liu,[1,2,*] and Sun-Wei Guo[1,2]

[1]Shanghai OB/GYN Hospital, Fudan University, Shanghai 200090, China; [2]Shanghai Key Laboratory of Female Reproductive Endocrine-Related Diseases, Fudan University, Shanghai 200011, China; and [3]Faculty of Medicine and Dentistry, Sapienza, University of Rome, 00161 Rome, Italy

ORCiD number: 0000-0002-8511-7624 (S.-W. Guo).

Context: Whether endometriosis is a progressive disease is a highly contentious issue. While progression is reported to be unlikely in asymptomatic deep endometriosis, progression in symptomatic deep endometriosis has recently been reported, especially in menstruating women. However, pathophysiological reasons for these differences are unclear.

Objective: This study was designed to investigate whether ovarian endometrioma (OE) is progressive or not.

Setting, Design, Patients, Intervention and Main Outcome Measures: Thirty adolescent patients, aged 15 to 19 years, and 32 adult patients, aged 35 to 39 years, all laparoscopically and histologically diagnosed with OE, were recruited into this study after informed consent. Their demographic and clinical information were collected. Their OE tissue samples were collected and subjected to immunohistochemical analysis for E-cadherin, α-smooth muscle actin (α-SMA), desmin, and adrenergic receptor β2 (ADRB2), as well as quantification of lesional fibrosis by Masson trichrome staining.

Results: OE lesions from the adolescent and adult patients are markedly different, with the latter exhibiting more extensive and thorough progression and more extensive fibrosis, suggesting that lesions in adults progressed to a more advanced stage. Adult lesions and higher staining level of α-SMA and ADRB2 are positively associated with the extent of lesional fibrosis, while the lesion size and the E-cadherin staining are negatively associated.

Conclusions: Our data provide a more definitive piece of evidence suggesting that OE is a progressive disease, since the adult lesions have had a longer time to progress. In addition, the pace of progression depends on lesional age as well as the severity of endometriosis-associated dysmenorrhea, if any. **(*J Clin Endocrinol Metab* 105: 2189–2202, 2020)**

Freeform/Key Words: adrenergic receptor β2, endometriosis, epithelial-mesenchymal transition, fibroblast-to-myofibroblast transdifferentiation, fibrogenesis, progression

One highly contentious issue regarding endometriosis is whether it is a progressive disease or not, especially early-onset endometriosis (1-7). Indeed, the effective management of endometriosis requires proper knowledge of how the disease progresses, how it responds to different treatment modalities, and whether

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*These 4 authors contributed equally to this work.

Abbreviations: ADRB2, adrenergic receptor β2;BMI, body mass index; DE, deep endometriosis;EAP, endometriosis-associated pain;EMT, epithelial-mesenchymal transition;FMT, fibroblast-to-myofibroblast transdifferentiation;HPA, hypothalamic-pituitary-adrenal;IHC, immunohistochemistry;NK1R, neurokinin receptor 1;OE, ovarian endometrioma;rASRM, revised American Society for Reproductive Medicine (rASRM);ReTIAR, repeated tissue injury and repair;α-SMA, α-smooth muscle actin;SMC, smooth muscle cells;SMM, smooth muscle metaplasia;VAS, visual analogue scale.

and how it may respond to changes in diet and lifestyle. This is especially true for younger women with endometriosis, who are often reluctant or unwilling to undergo more aggressive treatment, such as surgery, and who have yet to achieve pregnancy.

Various studies have attempted to characterize the evolution of endometriotic lesions. So far it has been proposed that the natural history of individual lesions may be established by their color (8-11), size (6), and to a much lesser extent, by the depth of invasion (10). Serial, repeated laparoscopic evaluations in nonhuman primates also have been employed to document the lesional kinetics (11-13). While these studies suggest a progressive nature of endometriotic lesions, the reported lesional evolutionary course should be viewed with caution, since surgery, in and by itself, is a potent stressor that can promote lesional development (14). In addition, these studies typically use the lesion size or coloration as the outcome measures (11-13). However, whether the coloration and/or the size of lesions can faithfully reflect the true progression is debatable at best.

Savaris et al found no correlation between revised American Society for Reproductive Medicine (rASRM) stage and patients' age and, as such, endometriosis does not seem to be a progressive disease (5), although this assertion rests upon an implicit, yet crucial, assumption that the rASRM stage is synonymous with stage of lesional progression. Despite its wide adoption, the rASRM staging system is known to be unrelated to the severity of symptoms or prognosis (15, 16), and it is certainly not synonymous with the lesional progression stage. Therefore, from the correlation of rASRM with age, or lack of it thereof, one simply cannot infer whether endometriosis is progressive or not.

There are also reports that seemingly defy easy explanation. In women with *asymptomatic* rectovaginal endometriosis, for example, progression has been reported to occur rarely (7). In contrast, in women with *symptomatic* deep endometriotic nodules infiltrating the rectosigmoid, disease progression, as measured by lesion size, does occur, especially in menstruating women (6). These conflicting reports demand answers from the lesional histology.

In the last few years, a growing body of evidence indicates that endometriotic lesions are fundamentally wounds undergoing repeated tissue injury and repair (ReTIAR) (17, 18) due to their cardinal hallmark of cyclic bleeding (19). Consequent to this ReTIAR, the endometriotic lesions, stimulated by various molecules released by activated platelets, immune cells, or sensory nerve fibers within the lesional microenvironment, undergo epithelial-mesenchymal transition (EMT) and fibroblast-to-myofibroblast transdifferentiation (FMT), resulting in increased collagen production and ultimately fibrosis (20, 21). In addition, endometriotic stromal cells are differentiated into smooth muscle cells (SMCs), leading to smooth muscle metaplasia (SMM) that is frequently seen in endometriotic lesions (22-25).

Viewing endometriosis through this looking glass, the true lesional natural history seems to be clear. Indeed, just by immunostaining E-cadherin (a marker of epithelial cells) in the epithelial component, stromal staining of α-smooth muscle actin (α-SMA, a marker of myofibroblasts), and evaluating the extent of lesional fibrosis—a representation of 3 landmarks in progression—the lesional age can be distinguished fairly accurately in mouse as well as baboon models of endometriosis (26, 27). Using a similar approach, the difference between ovarian endometriomas (OE) and deep endometriotic (DE) lesions has been characterized, providing explanation as why DE is more challenging to manage by pharmacological means (28).

In this study, we assembled 2 groups of patients with OE, one from adolescent patients, and the other from adult patients, and we secured their respective OE tissue samples. We hypothesized that, while endometriosis of the 2 groups might have different pathogenesis, their lesions, once established somewhat irreversibly and irrevocably so as to cause symptoms, should exhibit features consistent with different stages of progression. This is because, everything being equal, the adult lesions should have had much longer time to progress because of more episodes of ReTIAR, thus experiencing more thorough and complete EMT, FMT, and SMM and exhibiting more extensive fibrosis compared with the adolescent ones. Aside from this difference, the extent of lesional fibrosis also should positively correlate with the duration of symptoms such as dysmenorrhea, a strong risk factor for endometriosis, especially during adolescence (29, 30). In addition, we hypothesize that the dysmenorrhea, as a form of pain and thus a potent stressor, may activate the hypothalamic-pituitary-adrenal (HPA) axis, inducing lesional expression of adrenergic receptor β2 (ADRB2). Hence the severity of dysmenorrhea may correlate with the lesional expression levels of ADRB2. This study was designed to test these hypotheses.

Materials and Methods

Patients and specimens

This study was approved by the institutional ethics review board of Shanghai Obstetrics and Gynecology Hospital. Thirty adolescent patients, aged 15 to 19 years, and 32 adult patients, aged 35 to 39 years, all premenopausal and with laparoscopically and histologically diagnosed OE, who were admitted to the Obstetrics and Gynecology Hospital, Fudan University, from March, 2013, to July, 2018, were recruited into this study. For all recruited participants, their

demographic information, such as age, gravidity, parity, height, weight, body mass index (BMI), presence or absence of any genital anomaly, length of menstrual cycles, date of the last menstruation, date on which the surgery was performed, verbal rating scale (VRS, none, mild, moderate, or severe) and visual analogue scale (VAS) on the severity of dysmenorrhea and of pelvic pain, the duration of dysmenorrhea, the time elapsed since OE was detected by ultrasound, and the amount of menses (light, if no more than 1 sanitary pad was used in each menstruation, or heavy, if more than 3 pads were used; otherwise moderate), and rASRM scores were collected. We also reviewed the medical records to retrieve clinical data, such as laterality, lesion size (in cm), along with pathology reports from laparoscopic cystectomy. Their family history or previous history of deep venous thrombosis or coagulation disorders was queried but none was reported. None of the recruited participants smoked, or had taken any antiplatelet drug, steroid hormones, oral contraceptives, antidiabetic, or other medications 3 months prior to the surgery.

We collected OE tissue samples from all patients recruited into this study. All collected tissue samples were fixed with 10% formalin and paraffin-embedded for immunohistochemistry (IHC) analysis and for Masson trichrome staining, as described below. Written informed consent was sought before the patient was recruited into this study.

Immunohistochemistry analysis

Serial 4-mm sections were obtained from each block, with the first resultant slide stained with hematoxylin and eosin (H&E) to confirm the pathologic diagnosis, and IHC staining of E-cadherin, α-SMA, desmin, ADRB2, and neurokinin receptor 1 (NK1R) of OE tissue samples from all recruited patients. Routine deparaffinization and rehydration procedures were performed.

For antigen retrieval, the slides were heated at 98℃ in a citric acid buffer (pH 6.0) for a total of 30 minutes and cooled naturally to room temperature. Sections were then incubated with the primary antibody against E-cadherin (1:400; CST, MA), α-SMA (1:100; Abcam, Cambridge, UK), desmin (1:200; Abcam), ADRB2 (1:50; Abcam) or NK1R (1:100; Novus Biologicals, Centennial, CO) overnight at 4℃. After slides were rinsed, the horse radish peroxidase (HRP)-labeled secondary anti-rabbit/mouse antibody detection reagent (Shanghai Sun BioTech Company, Shanghai) was incubated at room temperature for 30 minutes. The bound antibody complexes were stained for 1 to 2 minutes or until appropriate for microscopic examination with diaminobenzidine and then counterstained with hematoxylin (30 seconds) and mounted. The positive staining was evaluated using a semiquantitative scoring system, as reported previously (31). Briefly, the number and intensity of positive cells were counted by Image-Pro Plus 6.0 (Media Cybernetics Inc, Bethesda, MD). A series of 3 to 5 randomly selected images on several sections were taken to obtain a mean value. The IHC parameters assessed in the area detected included (a) integrated optical density (IOD); (b) total stained area (S); and (c) mean optical density (MOD), which is defined as MOD = IOD/S, equivalent to the intensity of stain in all positive cells.

Human invasive breast cancer tissue samples were used as positive controls. For negative controls, human adenomyotic tissue samples were incubated with rabbit or mouse serum instead of primary antibodies (Supplementary Information Figure S1 (32)).

Masson trichrome staining

Masson trichrome staining was used for the detection of collagen fibers in tissues. Tissue sections were deparaffinized in xylene and rehydrated in a graded alcohol series, then were mordant in Bouin's solution at 37℃ for 2 hours. Bouin's solution was made with saturated picric acid 75 mL, 10% formalin solution 25 mL, and acetic acid 5 mL. The sections were stained using Masson's Trichrome Staining kit (Baso, Wuhan, China) following the manufacturer's instructions. The areas of the collagen fiber layer stained in blue were calculated by the Image Pro-Plus 6.0.

Statistical analysis

The comparison of distributions of continuous variables between or among 2 or more groups was made using the Wilcoxon and Kruskal tests, respectively, and the paired Wilcoxon test was used when the before-after comparison was made for the same group of subjects. A Pearson or Spearman rank correlation coefficient was used when evaluating correlations between 2 variables when both variables were continuous or when at least 1 variable was ordinal. Multiple linear regression analysis was used to identify which factor(s) were associated with the lesion weight or IHC measure (all squared-root or log-transformed to improve normality, where appropriate). Jonckheere's trend test was used to evaluate factors that may be associated with the severity of dysmenorrhea.

Here, we assumed that the severity of dysmenorrhea can progress through various stages, hence patients who start with "none" or "mild" can deteriorate to "moderate" or even "severe" and are unlikely to reverse this progression (33). If this is the case, then we can treat the severity data as if they were discrete "survival time" with no censoring. Note, however, that longer "survival time" actually means more severe condition of dysmenorrhea, hence the "risk factors" identified through this approach are actually "protective factors" for dysmenorrhea severity.

P values of less than 0.05 were considered statistically significant. All computations were made with R 3.6.1 (34) (www.r-project.org).

Results

Characteristics of the recruited patients

The characteristics of the recruited adolescent and adult patients are listed in Table 1. As expected, the 2 groups differed significantly in age, parity, and BMI, but not in age at menarche (Table 1). While the VRS and the VAS scores of dysmenorrhea severity were highly correlated (Spearman $r = 0.92$, $P < 2.2 \times 10^{-16}$), the adolescent patients had more severe dysmenorrhea ($P = 0.040$); however, even though the adolescents had a higher VAS scores, this did not differ significantly ($P = 0.17$; Table 1).

In contrast to the adult patients among whom there were no case of obstructive anomaly in the reproductive

Table 1. Characteristics of the Recruited Adolescent and Adult Patients with Ovarian Endometrioma

Variable	Adolescents (n = 30)	Adults (n = 32)	*P* value
Age (in years)			
Mean ± SD	17.7 ± 1.1	36.0 ± 1.1	5.4×10^{-12}
Median (range)	18 (15-19)	36 (35-39)	
Age at menarche (in years)			
Mean ± SD	13.0 ± 1.2	13.3 ± 1.1	0.29
Median (range)	13.0 (11-16) 2 missing	13.5 (11-15)	
Severity of dysmenorrhea			
None	3 (10.0%)	12 (37.3%)	
Mild	13 (43.3%)	7 (21.9%)	0.040
Moderate	5 (16.7%)	11 (34.4%)	
Severe	9 (30.0%)	2 (6.3%)	
Visual analogue scale on dysmenorrhea			
Mean ± SD	4.2 ± 2.8	3.2 ± 2.9	0.17
Median (range)	3 (0—8)	3 (0—9)	
Amount of menses			
Light	1 (3.3%)	0 (0.0%)	
Moderate	28 (96.6%)	29 (90.6%)	0.24
Heavy	0 (0.0%) 1 had no menses due to anomaly in reproductive tract (left unicornous uterus without cervix, right primordial uterus, vaginal atresia)	3 (9.4%)	
Duration of symptoms (in months)			
Mean ± SD	15.8 ± 15.1	24.4 ± 36.8	0.96
Median (range)	12 (0.0—60)	12 (0.0—168)	
Time interval between the first ultrasonic diagnosis and surgery (in months)			
Mean ± SD	6.4 ± 9.3	31.7 ± 33.1	7.1×10^{-7}
Median (range)	2.5 (0.5-36)	22 (1-168)	
rASRM score			
Mean ± SD	24.5 ± 21.6	37.4 ± 23.6	0.019
Median (range)	19 (8-96)	30 (8-96)	
rASRM stage			
II	13 (43.3%)	7 (21.9%)	
III	12 (40.0%)	14 (43.8%)	0.14
IV	5 (16.7%)	11 (34.4%)	
Laterality			
Left	10 (33.3%)	12 (37.3%)	
Right	10 (33.3%)	13 (40.6%)	0.66
Bilateral	10 (33.3%)	7 (21.9%)	
Anomaly in the reproductive tract			
No	24 (80.0%)	32 (100.0%)	0.0097
Yes	6 (20.0%) Vaginal atresia with atresia of the cervix: 1 Transverse vaginal septum: 1 Primordial uterus: 1 Didelphic uterus and cervix with oblique vaginal septum:1 Left unicornous uterus without cervix, right primordial uterus, absence of no vagina: 1 Infantile uterus: 1	0 (0.0%)	

tract, 6 (20.0%) of the adolescent cases had various anomalies ($P = 0.0097$; Table 1).

While there was no difference in the duration of symptoms between the 2 groups ($P = 0.96$; Table 1), the time from diagnosis to surgery differed significantly, with the adult group having a significantly longer duration ($P = 7.1 \times 10^{-7}$; Table 1). The duration of symptoms was not correlated with the time from the diagnosis ($r = 0.14$, $P = 0.29$). However, after removing patients who had no dysmenorrhea, the 2 factors became correlated ($r = 0.55$, $P = 6.7 \times 10^{-5}$). Compared with the adolescent patients, the adult patients appeared to have more rASRM stage IV patients, but the difference did not reach statistical significance ($P = 0.14$; Table 1). However, the 2 groups

had significantly different rASRM scores ($P = 0.019$; Table 1), due to possible obscuring of the difference by the staging system.

Differential lesional stages of progression between adolescent and adult patients

We first evaluated the immunostaining levels of E-cadherin, α-SMA, desmin (a marker for SMCs), NK1R (receptor for substance P), and ADRB2 (receptor for adrenaline and noradrenaline) in endometriotic lesions, as well as the extent of lesion fibrosis by Masson trichrome staining.

We found that E-cadherin staining was seen in glandular epithelial component of OE lesions, and was localized in the cell membrane. α-SMA and desmin staining was seen primarily in the stromal component. NK1R and ADRB2 staining was seen primarily in the epithelial component, and were localized in the cytoplasm (Fig. 1).

We found that, compared with the adolescents, the adults had significantly lower E-cadherin staining levels in the epithelial component ($P = 0.0004$; Fig. 2A) but significantly higher lesional staining levels of α-SMA, desmin and NK1R (all 3 P values $< 2.6 \times 10^{-6}$; Fig. 2B,C,E) but similar staining levels of ADRB2 ($P = 0.28$; Fig. 2F). However, multiple linear regression analyses incorporating bilaterality, lesion size, parity, and group identity consistently showed that the adult lesions had significantly lower staining levels of E-cadherin but higher staining levels of α-SMA, desmin, NK1R, and ADRB2 (all $P < 0.005$). Consistent with the more complete and more thorough EMT (lower E-cadherin staining), FMT (higher α-SMA staining), and SMM (higher desmin staining), the lesions in the adult group had significantly more fibrotic content than that of the adolescent group ($P = 1.2 \times 10^{-9}$; Fig. 2D).

Factors associated with the severity of dysmenorrhea

Our exploratory analyses showed that the duration of dysmenorrhea, rASRM scores, and lesional staining levels of ADRB2 were positively correlated with the severity of dysmenorrhea in both patient groups individually and combined (all Spearman $r > 0.35$, all $P < 0.004$; Fig. 3A-3F). Jonckheere's trend test also indicated that these 3 variables were positively associated with the severity of dysmenorrhea (all $P < 0.005$). While bilaterality appeared to be associated with more severe dysmenorrhea, this did not reach statistical significance for adolescent patients and was only marginally significant for adult patients ($P = 0.22$ and $P = 0.067$, respectively; Fig. 3G and 3H), even though the association was

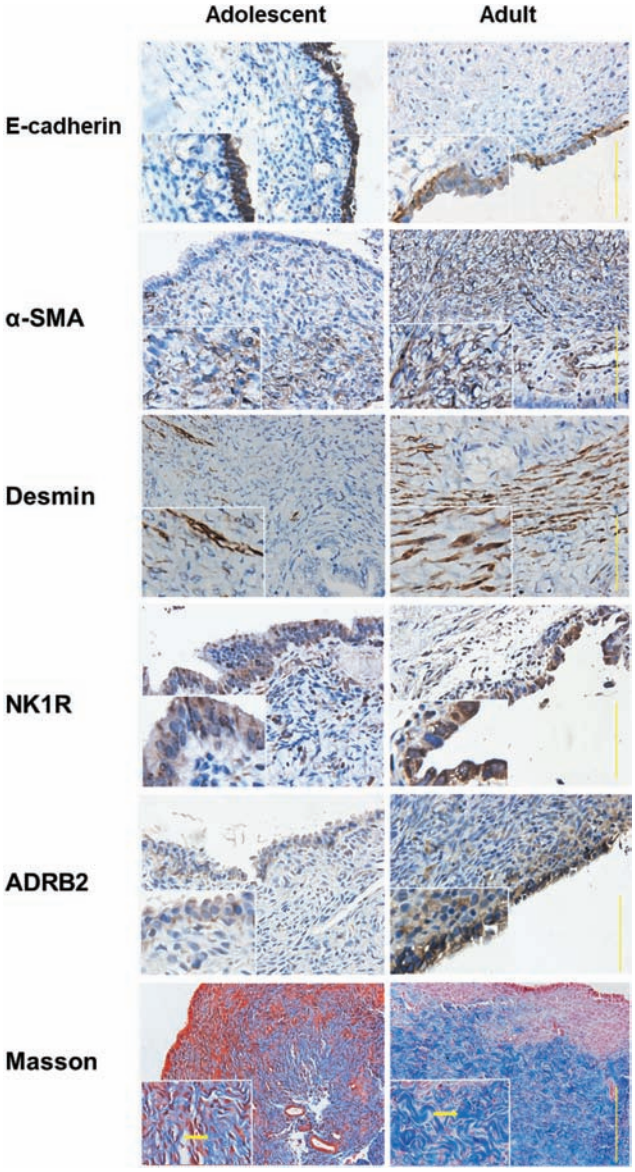

Figure 1. Representative immunoreactivity staining of E-cadherin, α-SMA, desmin, NK1R, and ADRB2, and Masson trichrome staining in ovarian endometrioma tissue samples from adolescent and adult patients. Left column: Adolescent group; Right column: Adult group. In Masson staining, yellow arrows point to collagen fibers stained in blue. Each yellow scale bar in immunoreactivity staining represents 125 μm, and the magnification is ×400; and yellow scale bar in Masson staining represents 251 μm, and the magnification is ×200. In each image, the inlet in white frame shows the further magnification of the original figure.

statistically significant when the 2 groups were combined ($P = 0.017$). Similarly, the maximal lesion size correlated *negatively* with the severity of dysmenorrhea for adolescent patients and when the 2 groups were combined ($r = -0.47$, $P = 0.009$, and $r = -0.28$, $P = 0.027$; Fig. 3I), but not for adult patients ($r = -0.10$, $P = 0.58$; Fig. 3J). Interestingly, the extent of lesional fibrosis correlated positively with the severity of dysmenorrhea in both adolescent and adult patients ($r = 0.43$, $P = 0.019$, and $r = 0.39$, $P = 0.026$; Fig. 3K and 3L). Hence, what

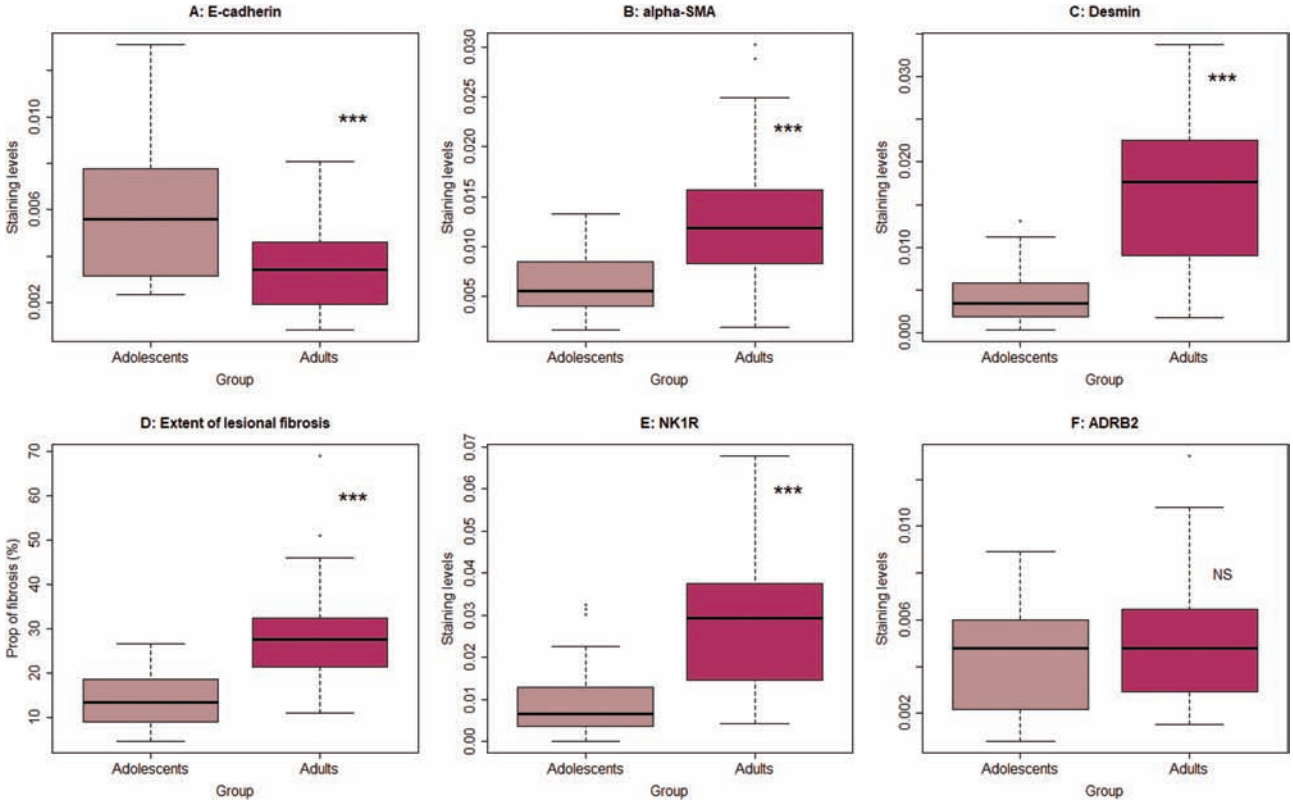

Figure 2. Summary results of immunohistochemistry and Masson trichrome staining. The boxplots showing the difference in E-cadherin (A), α-SMA (B), desmin (C), extent of lesional fibrosis (D), NK1R (E), and ADRB2 (F) between adolescent patients and adult patients with ovarian endometriomas. Symbols for statistical significance levels: NS, $P > 0.05$; ***$P < 0.001$; Wilcoxon's test.

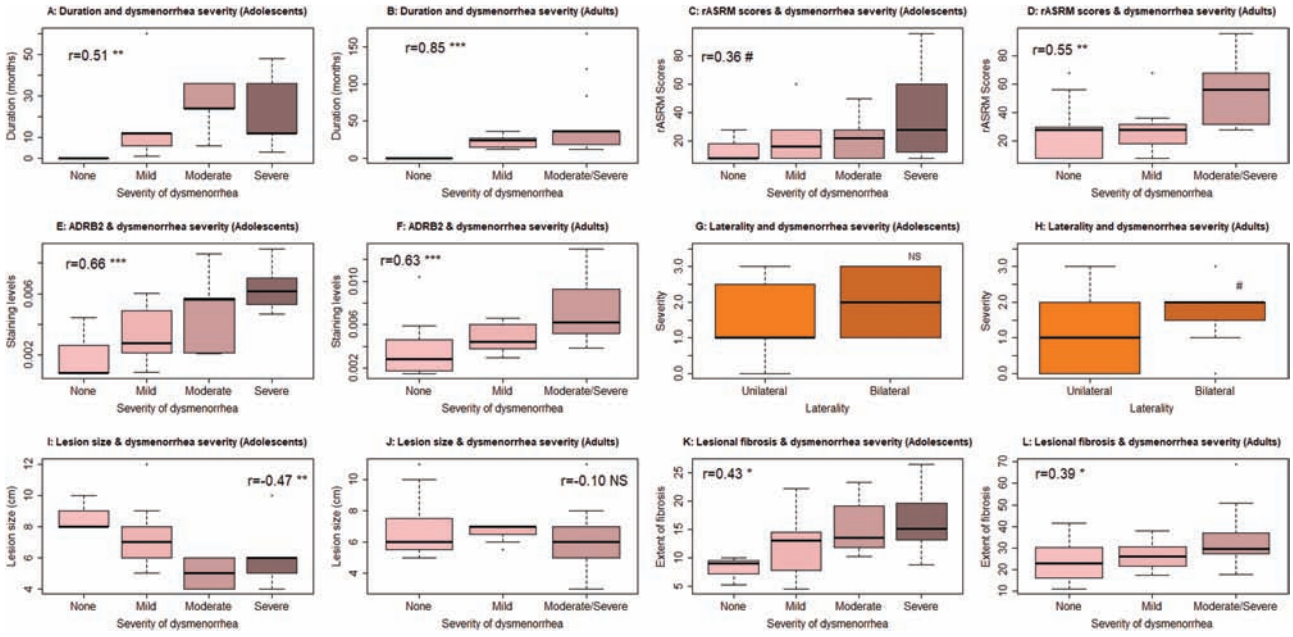

Figure 3. Summary results for potential factors that may be correlated with the severity of dysmenorrhea. The boxplots showing the difference in duration of dysmenorrhea in adolescents (A) and adults (B); rASRM scores in adolescents (C) and adults (D); lesional staining levels of ADRB2 in adolescents (E) and adults (F) with different severity of dysmenorrhea, the boxplot showing severity of dysmenorrhea in unilateral and bilateral cases (adolescents) (G) (adults) (H), and the difference in lesion size in adolescents (I), adults (J), and the difference in the extent of lesional fibrosis in adolescents (K) and adults (L) with different severity of dysmenorrhea. In panels (A-C) and (F-I), the Spearman correlation coefficient, along with its statistical significance level, is also shown. For panels (D) and (F), Wilcoxon's test was used. Symbols for statistical significance levels: NS: $P > 0.05$; #$P = 0.067$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

correlates with dysmenorrhea is the structure of the cyst wall, not the lesion size.

Using Cox regression model stratified by group (adolescent vs adult), the univariate analyses identified bilaterality, duration of dysmenorrhea, rASRM score, the extent of lesional fibrosis, and lesional ADRB2 staining levels as factors that are positively associated with severity of dysmenorrhea (Table 2). However, multivariate analysis incorporating age, parity, BMI, bilaterality, maximum lesion size, the extent of lesional fibrosis, and lesional staining of ADRB2, we found that the duration of dysmenorrhea and lesional staining levels of ADRB2 were the only 2 factors that were positively associated with the severity of dysmenorrhea ($P = 0.001$ and $P = 0.0007$, respectively). Since the Cox model was stratified by the patient group, these findings suggest that the 2 patient groups seem to have slightly different risk factors associated with the severity.

Factors associated with the extent of lesional fibrosis

Our current understanding on the natural history of endometriotic lesions is that fibrosis is the ultimate destiny (35, 36). As such, the extent of lesional fibrosis can arguably be viewed as the lesional "age" or the stage of lesional development. Therefore, we evaluated potential factors that were associated with fibrosis. First, we evaluated the relationship, if any, between the extent of lesional fibrosis and lesional staining levels of E-cadherin, α-SMA, desmin, NK1R, and ADRB2. We found that the extent of lesional fibrosis correlated negatively with the staining levels of E-cadherin ($r = -0.79$, $P = 1.8 \times 10^{-14}$; Fig. 4A) and positively with that of α-SMA desmin, NK1R, and ADRB2 (all $r \geq 0.62$, all $P < 7.6 \times 10^{-8}$; Fig. 4B-4E), suggesting that the extent of lesional fibrosis is indeed an end result of the EMT, FMT, and SMM.

While there was no difference in the extent of lesional fibrosis between unilateral and bilateral lesions ($P = 0.84$), the extent of lesional fibrosis correlated positively with the rASRM scores ($r = 0.54$, $P = 5.8 \times 10^{-6}$; Fig. 5A), stage ($r = 0.50$, $P = 3.2 \times 10^{-5}$; Fig. 5B), and the time since the imaging diagnosis of OE ($r = 0.58$, $P = 8.0 \times 10^{-7}$; Fig. 5C), correlated marginally with the duration of dysmenorrhea ($r = 0.24$, $P = 0.056$; Fig. 5D), and negatively but marginally with the (unilateral) lesional size ($r = -0.29$, $P = 0.055$; Fig. 5E) and the maximal size of lesions ($r = -0.24$, $P = 0.058$; Fig. 5F).

Multivariate linear regression analysis incorporating patient group identity (adolescent vs adult), parity, duration of dysmenorrhea, time since diagnosis, BMI, bilaterality, maximum lesion size, platelet count, prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (aPTT), fibrinogen, fibrin degradation products, and D-dimer indicated that the patient group was the only factor associated with the extent of lesional fibrosis ($P = 1.4 \times 10^{-9}$, R^2=0.46). If the group identity was replaced by age, then the multivariate linear regression analysis indicated that age was positively associated with the extent of lesional fibrosis ($P = 1.6 \times 10^{-9}$), while the lesional size was negatively associated with it ($P = 0.040$, R^2=0.49).

Multivariate linear regression analysis incorporating group identity, parity, duration of dysmenorrhea, time since diagnosis, BMI, bilaterality, maximum lesion size, and lesional immunostaining levels of E-cadherin, α-SMA, desmin, ADRB2, and NK1R indicated that the adult patient group ($P = 2.2 \times 10^{-5}$), and staining levels of α-SMA ($P = 0.0003$) and ADRB2 ($P = 0.042$) were positively associated with the extent of lesional fibrosis, while the lesional size ($P = 0.047$) and E-cadherin ($P = 5.6 \times 10^{-7}$) levels were negatively associated with it (R^2=0.85).

Discussion

Whether endometriosis is a progressive disease is a hotly debated issue, especially for early-onset endometriosis (1-5). Drawing on data from 7 clinical trials of adult endometriosis in which laparoscopy was performed twice without any treatment in the interim, Evers found that in 71% of the cases the disease did not progress, and 42% even regressed (3).

As pointed out by Benagiano et al (4), all the trials cited by Evers (3) were conducted in the 1990s when subtle endometriosis was diagnosed increasingly by laparoscopy but its relationship with symptomology

Table 2. Parameter Estimates of the Univariate Analysis Using the Cox Regression Model on factors Associated With the Visual Analogue Scale Rating of Dysmenorrhea Severity

Covariable	Estimate	Standard error	Hazard ratio	*P*-value
Bilaterality	-0.577	0.290	0.562	0.047
rASRM score	-0.0195	0.0064	0.981	0.0024
ADRB2 staining	-238.00	61.89	4.2×10^{-104}	0.0001
Extent of lesional fibrosis	-0.758	0.332	0.469	0.022
Duration of symptoms	-0.030	0.0086	0.970	0.0005

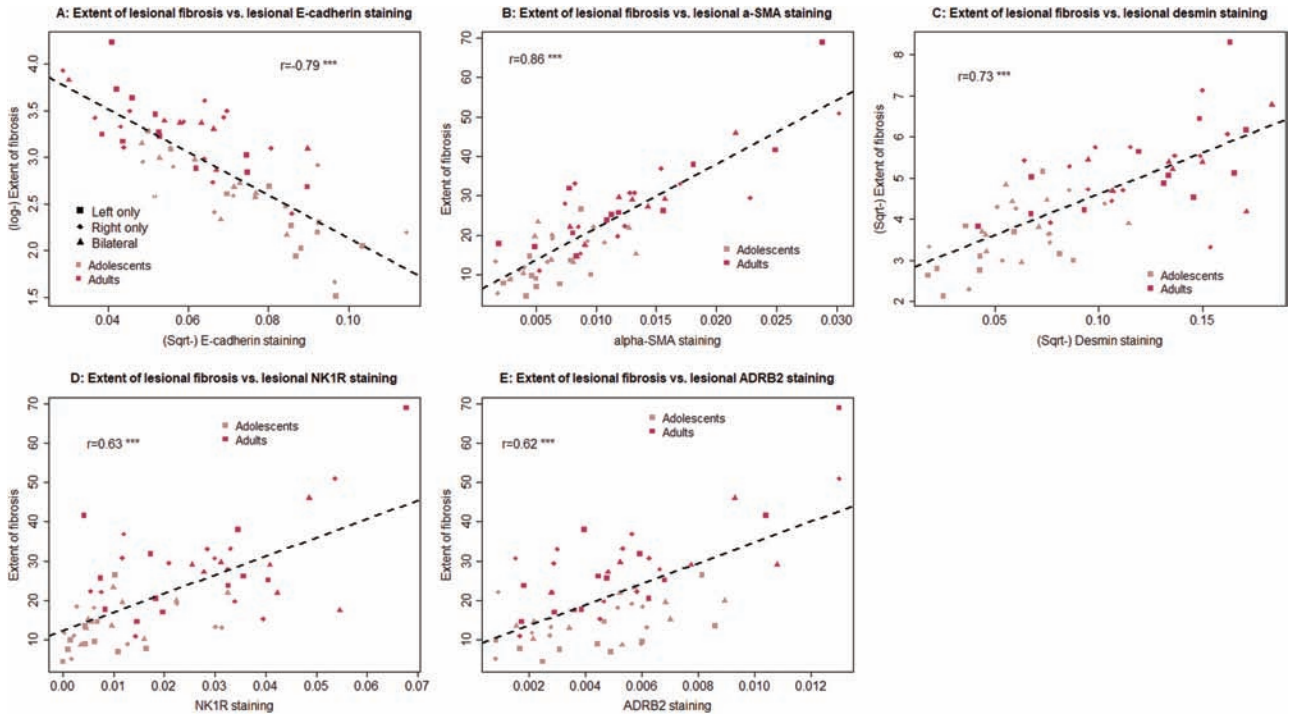

Figure 4. Correlation of the extent of lesional fibrosis with various lesional immunohistochemistry markers. Scatter plots showing the relationship between the extent of lesional fibrosis and lesional staining of E-cadherin (A), α-SMA (B), desmin (C), NK1R (D), and ADRB2 (E). For all panels, the Pearson correlation coefficient, along with its statistical significance level, is also shown. In addition, data from the adolescent and adult patient groups are represented in different colors, and the lesion laterality is also depicted with different shapes (Panel A). The dashed line represents the regression line. Symbols for statistical significance levels: $***P < 0.001$.

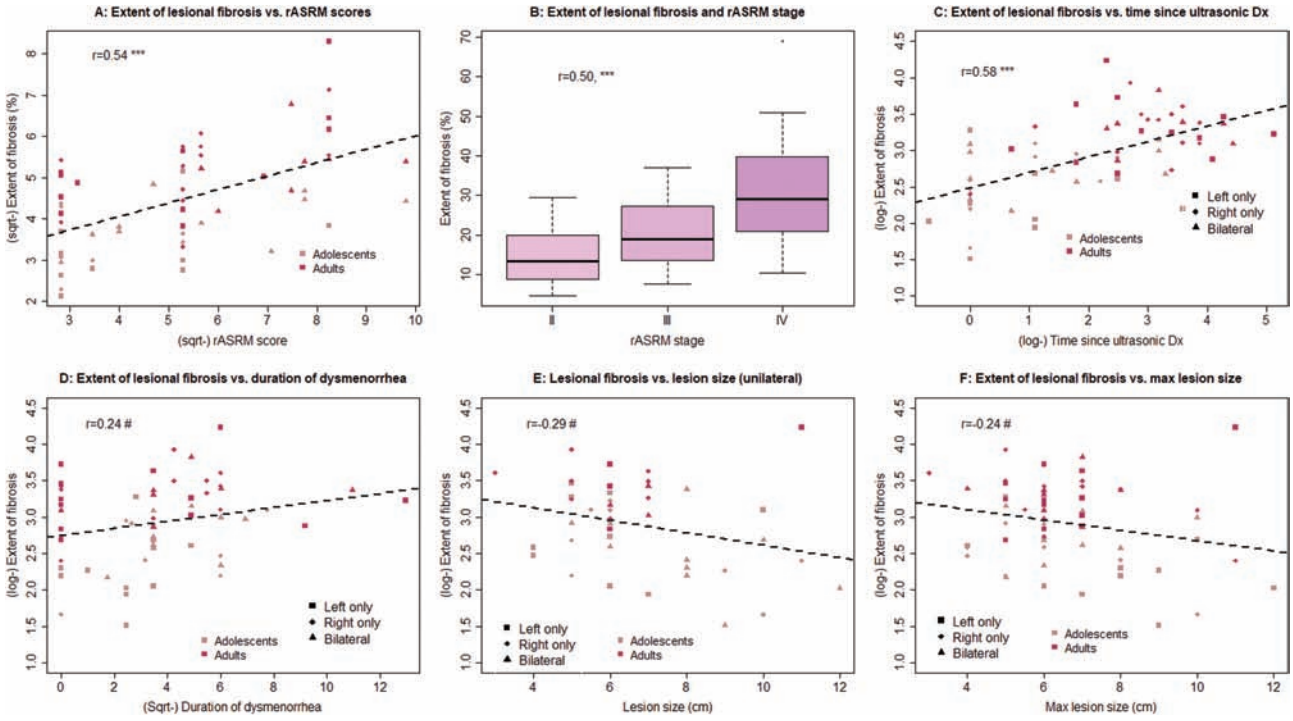

Figure 5. Correlation of the extent of lesional fibrosis with different variables. Scatter plots showing the relationship between the extent of lesional fibrosis and rASRM scores (A), the time since the imaging diagnosis of ovarian endometriomas (C), duration of dysmenorrhea (D), (unilateral) lesion size (E) and the maximum lesion size (if bilateral) or the lesion size (if unilateral). (B) The boxplots showing the difference in lesional fibrotic content in girls/women with ovarian endometriomas complaining different severity of dysmenorrhea. For all panels (except B), the Pearson correlation coefficient, along with its statistical significance level, is also shown. In addition, data from the adolescent and adult patient groups are represented in different colors, and the lesion laterality is also depicted with different shapes. The dashed line represents the regression line. For panel (B), the Spearman correlation coefficient and its statistical significance level are shown. Symbols for statistical significance levels: $\#P < 0.06$; $***P < 0.001$.

was obscure. It was assumed at that time that subtle lesions can appear and regress spontaneously, but may also progress to peritoneal lesions, OE, or DE (37, 38). The finding of microscopic lesions in apparently normal peritoneum (39), and the seemingly active remodeling of lesions (40, 41), prompted Koninckx (42) and Evers et al (43) to propose that superficial lesions may be a transient or even a physiological condition. However, others suggested that superficial lesions may still progress when located on ovarian cortex, uterine ligaments or peritoneal defects (44).

Capitalizing on the recently outlined natural history of endometriotic lesions characterized by several key and landmark molecular processes (26, 35, 36), we employed markers of EMT, FMT, SMM, and the extent of lesional fibrosis to discern the difference, if any, between adolescent and adult OE. We found that, compared with the adolescents, the adult lesions demonstrated more complete and more thorough EMT, FMT, and SMM, and, as a result, more extensive fibrosis. In addition, a longer duration of dysmenorrhea was found to be positively associated with the extent of lesional fibrosis (Figs. 1 and 2). This indicates that, on average, lesions in adult women progressed in a more advanced manner than in adolescents. In addition, we found that the duration of dysmenorrhea and the lesional staining levels of ADRB2 were 2 factors significantly associated with the severity of dysmenorrhea. Moreover, adult lesions and higher staining levels of α-SMA and ADRB2 were positively associated with more fibrotic content, while the lesion size and the E-cadherin staining were negatively associated with the extent of lesional fibrosis. Since lesions in the adult have had longer to progress, our data provide a more definitive piece of evidence suggesting that OE is a progressive disease.

One alternative explanation is that OE in adolescents may be more aggressive than the adult form, originating from, say, neonatal uterine bleeding (2, 45). This is possible, and our study cannot rule out this possibility due to the study design. However, this explanation would entail, by necessity, the assumption that some early adolescent lesions, growing out of stem/progenitor cells seeded at birth and lying dormant until thelarche (45), are intrinsically different from the adult form, possibly endowed with higher survival, anti-apoptotic, inflammatory, and invasive propensity. Unfortunately, data in support for this propensity are at present completely lacking. Since about 20% of the adolescent patients had obstructive anomalies, while there were none in adult patients, this difference in fact suggests that the adolescent form may be no different from the adult form—these adolescents had OE simply because of unfortunate massive retrograde menstruation starting right after menarche. The other 80% of these adolescents may have experienced equally adverse events that predisposed them to OE. In other words, both adolescent and adult OE are likely to have identical pathogenesis, and, as such, the 2 forms of OE are very similar except the age at onset.

In fact, due to the general tendency to normalize dysmenorrhea and the reluctance to adopt aggressive treatment modalities (such as laparoscopy or even transvaginal ultrasonography) for adolescent girls with or without endometriosis, it is likely that the adolescent endometriosis, as compared with the adult form, may have more time to develop, leading to seemingly more severe symptomology when eventually diagnosed in adulthood. This surely would leave the impression that adolescent form is more aggressive than the adult one.

The initial discovery of lesional expression of ADRB2 was made in the context of chronic stress that accelerates the development of endometriosis (46, 47). Surgery, as a stressor, can also activate the HPA axis and promote the lesional development through inducing the lesional expression of ADRB2 (14). Pain and infertility are 2 major complaints that prompt women with endometriosis to seek medical attention, especially incapacitating cyclic and noncyclic pain in adolescents (48). Consequently, women with endometriosis are reported to have higher levels of psychological stress, depression, and anxiety (49-54). In particular, endometriosis-associated pain (EAP) is frequently intense and debilitating and typically chronic and uncontrollable (55, 56), the resultant psychological stress appears to contain all the ingredients for exerting a potent negative effect on women with endometriosis (57). As a result, it is likely to induce systemic activation of the HPA and the sympathoadrenal-medullary (SAM) axes, resulting in increased release of glucocorticoids and catecholamines. The catecholamines, especially adrenaline and noradrenaline, would activate the ADRB2/CREB signaling pathway in lesions, inducing angiogenesis and proliferation and leading to accelerated progression of endometriosis (47). The accelerated progression may further exacerbate EAP, effectively forming a vicious cycle and resulting in a situation of being "stuck between a rock and a hard place" (58). This may explain as why the lesional ADRB2 staining, along with the duration of dysmenorrhea, are two most prominent factors associated with the severity of dysmenorrhea. It also explains as why lesional ADRB2 staining is one of factors that are associated with the extent of lesional fibrosis, a surrogacy for lesional progression stage. Of note, our results also explain as why the progression of *asymptomatic* rectal

nodules is unlikely to occur (7) simply because no such a vicious cycle existed. Fig. 6 depicts various cells in the lesional microenvironment and several factors that may impact on lesional progression, either positively or negatively.

In fact, our data can also explain why there is a risk of progression of *symptomatic* deep endometriotic nodules infiltrating the rectosigmoid, especially in menstruating women (6). First, being symptomatic, particularly in DE patients, means a great deal of persistent and chronic stress and the subsequent (and likely persistent) activation of the HPA and SAM axes, hence ADRB2 may be overexpressed in lesions, resulting in accelerated lesional progression. Second, amenorrhea, induced by drugs, pregnancy, or lactation, effectively shuts down ReTIAR, thus effectively stalling EMT, FMT, SMM, and fibrogenesis. However, amenorrhea may not completely suppress EMT, FMT, SMM, and fibrogenesis, since endometriotic cells may still secrete molecules that activate platelets (63), other immune cells (64), and even sensory nerve fibers (65), that collectively maintain a microenvironment that is still conducive to the maintenance or even growth, albeit at much slower pace, of endometriotic lesions. Stress arising unavoidably from everyday life may also facilitate lesional progression. This may explain the data in (6) that, in a span of about 3 years, about 11.6% of *symptomatic* DE patients regressed, 60.5% were stable, and 27.9% progressed, and that the majority of those regressed (94.1%) were amenorrhoic, while only 19.2% of stabilized and 15.1%

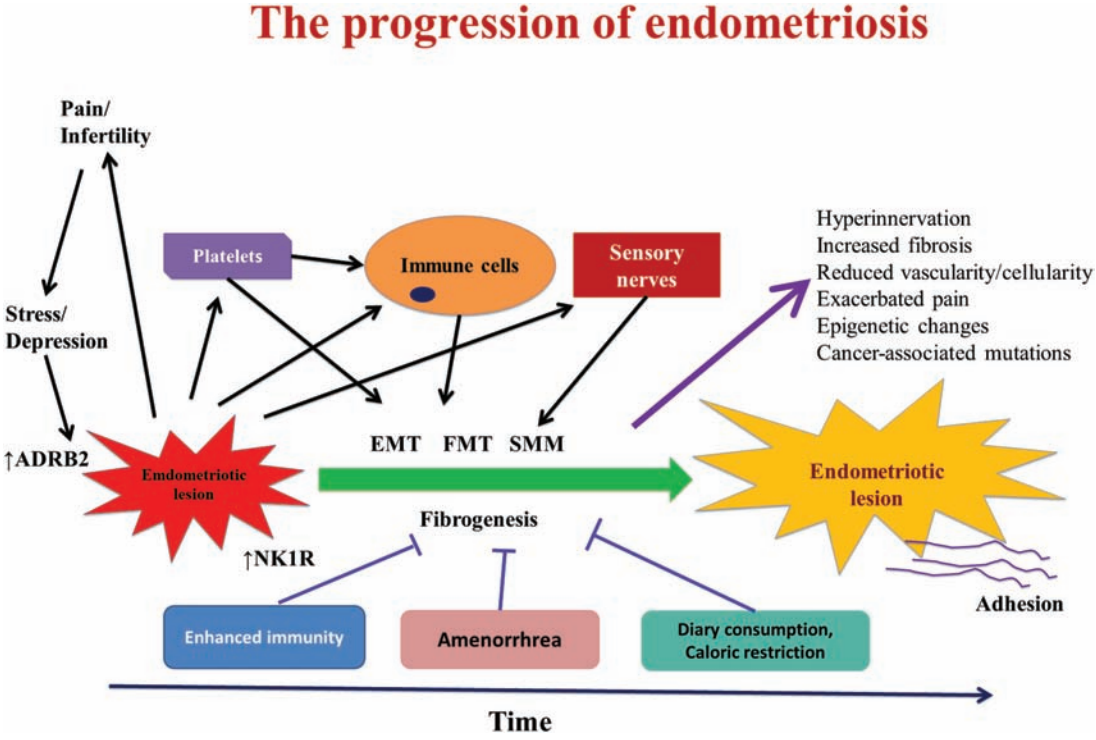

Figure 6. Diagram that depicts various cells in the lesional microenvironment and various factors that may impact on the lesional development. Endometriotic lesions are fundamentally wounds undergoing repeated tissue injury and repair (ReTIAR) (17, 18) due to their cardinal hallmark of cyclic bleeding (19). Consequent to this ReTIAR, the endometriotic lesions, stimulated by various molecules released by activated platelets, immune cells, or sensory nerve fibers within the lesional microenvironment, undergo epithelial-mesenchymal transition (EMT) and fibroblast-to-myofibroblast transdifferentiation (FMT), resulting in increased collagen production and ultimately fibrosis (20, 21). In addition, endometriotic stromal cells are differentiated into smooth muscle cells (SMCs), leading to smooth muscle metaplasia (SMM) that is frequently seen in endometriotic lesions (22-25). Endometriotic cells engage active crosstalk with platelets, immune cells and sensory nerve fibers by secreting various molecules, and these cells in turn also release various factors that, through receptors expressed in lesions such as NK1R, collectively promote the lesional progression. Moreover, endometriosis-associated pain and infertility and the resultant psychological stress, depression, and anxiety may induce systemic activation of the hypothalamic-pituitary-adrenal (HPA) and the sympatho-adrenal-medullary (SAM) axes, resulting in increased release of glucocorticoids and catecholamines.The catecholamines, especially adrenaline and noradrenaline, would activate the ADRB2/CREB signaling pathway in lesions, inducing angiogenesis and proliferation and leading to accelerated progression of endometriosis (47). The accelerated progression may further exacerbate symptoms, forming a vicious cycle that promotes lesional progression. Lesion development also induces hyperinnervation, increased lesional fibrosis, adhesion, reduced vascularity and cellularity, and promotes epigenetic changes and cancer-associated mutations, rendering medical treatment more difficult. On the other hand, enhanced host immunity due to changing to a healthier diet or lifestyle may help to stall the lesion progression (59) and may even help clear the minor lesions. Amenorrhea effectively shuts down the ReTIAR, interfering molecular processes involved in lesional development, such as EMT, FMT, SMM, and fibrogenesis, leading to lesion arrest. Caloric restriction (60), enriched environment with more social interactions and physical activity (61), and more dairy consumption (62) also may retard lesional progression. Abbreviations: ADRB2, adrenergic receptor β2; NK1R, neurokinin receptor 1; EMT, epithelial-mesenchymal transition; FMT, fibroblast-to-myofibroblast transdifferentiation; SMM, smooth muscle metaplasia.

of progressed patients were amenorrhagic. This may also explain as why progestin-induced amenorrhea, while effective in alleviating EAP, somehow permits the progression of deep endometriosis (66, 67), as the lesions may still progress, albeit at a much slower pace. In other words, menstruation is a prerequisite for ReTIAR, and EAP and stress perpetuate the progression.

Our data are consistent with the observation that adolescent lesions are typically characterized by red hemorrhagic, clear/polypoid, or vesicular lesions (8, 68, 69) and frequently lack SMCs (70), which are signs of early lesions. Due in part to the relatively shorter time span for progression in the adolescents as compared with the adults, the processes of EMT, FMT, and SMM are typically not thorough or complete, yielding higher expression of E-cadherin in the epithelium and lower expression of α-SMA and desmin, as well as less fibrosis in lesions, as observed precisely in this study. In contrast, lesions in adults have much longer time to develop, yielding more thorough and complete EMT, FMT, and SMM and more extensive lesional fibrosis. Indeed, the typical lesions in the adult tend to be black with white scar (the classic "powder-burn" lesion) (8, 68, 71), and white-colored OE lesions have been shown to be more fibrotic than blackish/brownish ones (17).

Granted, our observations are based exclusively on OE and, as such, our conclusions may not be applicable to other subtypes of endometriosis such as peritoneal endometriosis. However, as we have shown previously, irrespective of endometriosis subtypes or even adenomyosis, all lesions appear to undergo identical cellular processes such as EMT, FMT, SMM, and fibrogenesis (72-75). The difference in phenotype among these subtypes lies in their microenvironment (36, 73, 75).

Given the lack of longitudinal data, one could argue that endometriosis in the adolescent represents a transient condition that may regress later, especially given its morphology that is different from that of adult lesions (70). Unfortunately, there has been no data to demonstrate such a transient nature. Barring a dramatic change in diet (60) or lifestyle (61), or intervention that would engender enhanced immunity, endometriotic lesions are unlikely to regress or even disappear spontaneously. This is because endometriotic cells constantly engage in active crosstalk with other cells in their microenvironment (63-65, 76, 77); hence, in effect these cells are partners-in-crime that jointly promote the lesional progression (21), practically a maladaptation of the evolutionarily conserved tissue repair mechanism. Indeed, the morphological appearance (8, 68, 69) and the absence of SMCs (70) in adolescent lesions are signs of early stages of lesional development. In contrast, the data presented in this study further demonstrate that adult lesions exhibit features that are consistent with a more advanced stage of progression (28).

One seemingly puzzling observation in our study is that the lesion size is negatively associated with the extent of lesional fibrosis. One possible explanation for this is that the size of OE may initially increase but later on decrease due to reduced vascularity as lesions progress and become more fibrotic (28), while at the same time the chocolate fluid may be gradually absorbed, resulting in decreasing lesion size. Hence the OE size is not a determinant for its progression. Rather, it is the structure of the wall and its microenvironment that determine the lesional progression. This seems to be different from deep lesions (6).

But how does this notion of progression reconcile with the reported lesional regression, that is, the transient nature of mild endometriosis?

As now becomes evident, the lesional microenvironment is crucial in determining the destiny of the lesions (73, 75). While retrograde menstruation can deposit viable endometrial fragments in the peritoneal cavity and may form occult or microscopic lesions (39, 78) or even mild lesions, these lesions may also be removed by immune cells. Whether the lesion would regress, stay unchanged, or further progress may depend on the immune function of the host, whose lifestyle, diet, and exposure to other modifiable factors may play a positive or negative role in immunity. For example, a high-fat diet (79, 80) and more meat consumption (81) can promote lesional progression, but more dairy consumption (62), caloric restriction (60), or living in an enriched environment (61, 82) may stall the progression. Of note, whether the patient experiences pain or under chronic psychological stress would also be crucial to determine the lesional fate. Pregnancy, lactation, or drug-induced amenorrhea may also substantially slow down lesional progression, and may facilitate the clearance of mild lesions by the immune cells.

Given the progressive nature of endometriosis, it seems logical to conclude that to intervene as early as possible when endometriosis is diagnosed or even suspected should stall or even terminate the lesional development. This conclusion is based not only on the progressive nature of endometriosis but also on the observation that older, and thus more fibrotic, lesions have less vascularity and cellularity as well as more extensive epigenetic (26, 28) and genomic changes (83,

84), which, conceivably, would be more refractory to hormonal treatment. Given the documented promotional role of chronic psychogenic stress and the apparent activation of the HPA system and lesional expression of ADRB2 (47, 85), it seems that the intervention procedures should not be confined to surgery or hormonal treatment but rather should include an effective measure in relieving EAP at the very minimum. Alternatively, drug-induced amenorrhea should also dramatically slow down the tempo and pace of progression. Teaching patients effective coping strategies (86, 87) may also help to attenuate or suppress the activation of the HPA axis, breaking the vicious cycle of lesion–pain–lesion development–more pain. Unfortunately, research in this area is very scanty and the topic warrants more investigation.

The finding that 20% of adolescent patients had obstructive Müllerian anomalies is generally consistent with the documented role of the anomalies in adolescent (and in adult as well) endometriosis (88-90) and in particular with the report that 15 out of 63 (23.8%) adolescents (≤ 20 years) with endometriosis in China had congenital anomalies in the reproductive tract (91). This suggests that the adolescent endometriosis, or early-onset endometriosis, has a risk factor profile that is somewhat different from that of adult endometriosis, in that obstructive anomalies can lead to early onset of endometriosis. Aside from this, it highlights the pathogenic role of retrograde menstruation since the anomalies facilitate the retrograde flow (90).

In summary, we found that, compared with adolescent OE lesions, the adult lesions exhibit more thorough and complete EMT, EMT, SMM, and more extensive lesional fibrosis due to more time for lesional development in the adults. In particular, the duration of dysmenorrhea also correlates with the advanced lesional progression. Moreover, the lesional ADRB2 staining levels, in conjunction with the duration of dysmenorrhea, are associated with the severity of dysmenorrhea. These findings, taken as a whole, provide more definitive evidence in support for progressive nature of OE lesions. This may call for early intervention for endometriosis, especially in adolescent girls.

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Additional Information

Data Availability: Data are available to those who send written request to the corresponding authors, detailing their purpose of intended use.

Correspondence: Xishi Liu, M.D., Ph.D., E-mail: lxsdoc@hotmail.com; or Sun-Wei Guo, Ph.D., Shanghai Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200090, China. E-mail: hoxa10@outlook.com.

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