Dating Endometriotic Ovarian Cysts Based on the Content of Cyst Fluid and its Potential Clinical Implications

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Abstract

This study was undertaken to test the hypotheses that, due to gradual accumulation of dead erythrocytes and their ingested products resulting from repeated hemorrhage, older endometriomas (whitish in color) contain chocolate fluid with higher iron content than younger (brownish/blackish in color) ones with concomitant higher collagen content and more adhesions. We recruited 30 premenopausal women with histologically confirmed ovarian endometriomas and collected samples of their endometriotic lesions and chocolate fluid and measured the viscosity, density, and the concentration of total bilirubin, ferritin, and free iron of the chocolate fluid. We also evaluated the lesion color and adhesion scores. In addition, we performed Masson trichrome and Picro-Sirius red staining on all endometriotic cysts and evaluated the extent of fibrosis in the lesions. We found that fluids taken from white-colored endometriomas had significantly higher concentration of total bilirubin, ferritin, and free iron, respectively, than black/brown-colored ones. In addition, older cysts had fluids that had significantly higher density and viscosity. Fluid density correlated positively with the concentrations of total bilirubin, ferritin, and free iron. Older lesions had significantly more collagen content and higher adhesion scores. Taken together, these data supports the notion that older cysts, having experienced more bleeding episodes, contain chocolate fluid that is higher in viscosity, density, and iron content and higher fibrotic content than younger ones. This provides another piece of evidence that endometriotic lesions are wounds that undergo repeated injury and repair, resulting ultimately fibrotic lesions that are resistant to hormonal treatment.

Keywords

chocolate fluid, collagen I, iron content, lesion age, ovarian endometriomas

Introduction

Ovarian endometriomas, also called endometriotic ovarian cyst, is one of the three major subtypes of endometriosis¹ and are very common.² Since Sampson's 1921 description of chocolate cysts of the ovary,³ numerous studies have been published to characterize the disease and to elucidate its pathogenesis^{1,4-8} yet its pathogenesis still is an enigma and its treatment is inadequate.

As with any quest for the genesis of a complex phenomenon, to pinpoint exactly what the initial and pivotal events are that lead to the genesis of endometriomas can be very challenging. We can argue that, for interventional purposes, it suffices to understand just how endometriomas *progresses*. Unfortunately, the natural history of any ovarian cyst is poorly understood, although there is a general consensus that the color of the peritoneal endometriotic lesions changes as the lesions age, starting as a red lesion, then progressing to black, and finally to white.^{1,4,9} The endometrioma lining is frequently surrounded by fibrous tissues,^{4,5} especially in white-colored lesions.¹ Therefore, the color of endometrioma

internal cyst wall can be used as a rough proxy for the age of the lesion. However, the coloration as a way of dating may be too crude to be clinically useful.

Dating the endometriomas can be clinically important since the age of the cyst would unveil its current state of progression and thus provide a basis for the choice of the best treatment modality. For example, a cyst that is comprised of mostly fibrotic tissues would signal the terminal stage of the disease, which is unlikely to be responsive to hormonal treatment. In this regard, the existing revised American Society for Reproductive Medicine (rASRM) staging system¹⁰ is not very

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helpful, since it merely provides a summary measure about the extensiveness of endometriosis, giving no information on the developmental stage of the lesions. It is known to be uncorrelated with the pain severity¹¹ and related only tangentially with the recurrence risk.^{11,12} It provides little guidance for the choice of a right treatment modality. Therefore, a better dating method is sorely needed.

Ovarian endometriosis contains chocolate-like fluid as the result of accumulation of menstruation-like hemorrhagic blood entrapped in the cyst.^{8,13} Yet bleeding is a cardinal sign of tissue damage. Repeated bleeding signals repeated tissue injury, which is known to cause tissue fibrosis,^{14,15} as, in fact, seen frequently in endometriomas. We found recently that platelets are significantly aggregated in ovarian cysts and that activated platelets induce epithelial-mesenchymal transition (EMT) and subsequent myofibroblast activation, resulting in the acquisition of the smooth muscle cell marker, increased contractility, and increased production of collagen I (D. Ding, MSc, unpublished data, January 2015).¹⁶ As myofibroblasts are known to be the primary extracellular matrix (ECM) secreting cells during wound healing and fibrosis,¹⁷ our data provide evidence that endometriosis are wounds that undergo repeated tissue injury (bleeding) and repair, ultimately leading to fibrosis.

Erythrocytes entrapped within the cyst contain iron that is bound to hemoglobin (Hb). Macrophages acquire most of their iron by phagocytosis of senescent erythrocytes. Digestion of Hb through erythrophagocytosis results in liberated iron, which is either released from macrophages or stored in a protein complex either as ferritin or hemosiderin.¹⁸ The presence of iron itself is a major trigger for the production of ferritin,¹⁹ which is a primary intracellular protein that stores and releases iron. The amount of ferritin stored reflects the amount of iron stored. Erythrocytes within the cyst are disposed of when they are senescent or damaged, from which the Hb is released, which, in turn, is metabolized into bilirubin.

High iron concentration within endometriotic cysts has been reported previously,^{20,21} which is thought to be of diagnostic value.^{22,23} In the peritoneal fluid of patients with endometriosis, higher levels of iron²⁴⁻²⁷ and higher ferritin levels²⁶⁻²⁸ also have been reported. The elevated concentration of iron, ferritin, and total bilirubin, along with debris therein, would conceivably result in increased thickness or viscosity and the density of the cyst fluid. In other words, the physical properties of the cyst fluid may also contain information on the age of the cyst.

Aside from repeated bleeding episodes and the gradual accumulation of iron in the cyst fluid, we have recently found that activated platelets are critically involved in the development of endometriosis (D. Ding, MSc, unpublished data, January 2015).¹⁶ In addition, coculture of endometriotic epithelial cells with platelets results in EMT and also results in myofibroblast activation in endometriotic stromal cells, leading to excessive deposition of collagens and thus fibrosis in endometriosis.¹⁶ In view of the above, we hypothesized that (1) older endometriomas contain fluid with higher free iron, soluble ferritin, and total bilirubin content than younger ones due to gradual accumulation of dead erythrocytes and their ingested

products resulting from repeated hemorrhage and (2) since repeated injury leads to tissue fibrosis, older cysts have higher collagen content and more adhesions than younger ones. This study was undertaken to test these hypotheses.

Materials and Methods

Patients and Sample Collection

Thirty premenopausal women with histologically confirmed ovarian endometriomas who underwent laparoscopy between December 2012 and March 2013 in our hospital were recruited consecutively for this study after informed consent. For each patient, the demographic and clinical information, such as age, gravidity, parity, body mass index (BMI), severity of dysmenorrhea as measured by the Verbal Descriptor Scale (none, mild, moderate, or severe), complaint of infertility or not, the use of any hormonal medication ever and, name of the medication and duration, was recorded. After the artificial pneumoperitoneum was set up at the beginning of the surgery, the entire pelvic cavity was thoroughly explored and evaluated. The laterality, size (the diameter of the ovarian cyst), and presence of adhesion to neighboring organs or peritoneum were carefully evaluated and recorded. The rASRM scores were determined by the presence and the size of ovarian endometriomas, status of the posterior *cul-de-sac*, and presence and nature of tuboovarian adhesions, among other things. For each cyst, the adhesion score, as part of the rASRM scoring system, was also recorded.

Once the evaluation was done, the ovarian cyst was punctured by a Transfix needle (Bile duct needle, $\phi 5 \times 330$ mm, 302.513, Optcla, Hangzhou, China) connected with a 20-mL sterile syringe and the fluid content (5-15 mL) was sucked into the syringe. If the patient had bilateral ovarian cysts (4 cases), the cyst fluid was obtained from both cysts in an identical manner and processed separately. The procured fluid was divided into 2 aliquots, and then stored on ice and underwent measurement immediately.

In all cases, the endometriomas were removed by stripping the cyst wall from the ovaries. After the cyst walls were taken out, they were rinsed with sterile saline to remove the residual chocolate-like fluid, and the color of the endometriotic lesion was recorded. For all 30 patients, the ovarian endometriomas were grouped by coloration as black or white, as the following. If over two thirds of the internal surface of the cyst wall surface area was blackish or brownish, the lesion was described as black, and the portion of the blackish/brownish specimen was collected. Black lesions were formed likely because of old hemorrhage. Lesions considered to be blackish/brownish but partially yellowish (no more than one third of the entire surface area) were classified as black lesions. Similarly, if over two thirds of the interior surface area of the cyst was whitish, the lesion was described as white in color, and the portion of the whitish specimen was collected. Whitish lesions were likely to be fibrotic tissues. The fresh, reddish hemorrhagic spots could also be seen in some brownish/blackish specimens, and their number ranged from a few to as many as ~20 per

Figure 1. Representative coloration of ovarian endometriomas. The blackish/brownish lesions and whitish lesions were marked with different type of black arrows. The fresh red hemorrhaging spot was marked with blue arrow. The ruler shown in the picture is in unit of centimeter (cm). (The color version of this figure is available in the online version at <http://rs.sagepub.com/>.)

specimen. In contrast, in whitish specimens, these spots were very rare. The representative pictures of ovarian cysts with different coloration are shown in Figure 1. All cyst tissue samples were fixed for 24 hours with 10% formalin and paraffin embedded. The histologic diagnosis of endometrial glands, stroma, fibrous tissues, and hemosiderin-carrying macrophages was made by pathologists after hematoxylin and eosin (H&E) staining. This study was approved by the Ethics Review Committee of Shanghai OB/GYN Hospital.

Measurement of the Density, Shear Viscosity of Cyst Fluid and its Total Bilirubin, Soluble Ferritin, and Free Iron

Depending on the availability, 2 or 4 mL of cyst fluid was weighted at room temperature by the Electronic Balance scale (FA2004, FangRui, Shanghai) to calculate the density of the fluid (in g/mL), and the shear (dynamic) viscosity of the fluid was measured at room temperature by Automatic Blood Rheology Test Instrument, (ZS9200, Zonci, Beijing, China) at the different shear stress of 1 mPa•s (low-shear force), 50 mPa•s (medium-shear force), and 200 mPa•s (high-shear force) in the hospital clinical laboratory. The use of different shear stress was necessary since the cyst fluid, like blood, is essentially a non-Newtonian liquid, in which viscosity is not a constant but rather depends on the magnitude of the shear stress.²⁹

Another aliquot of the cyst fluid was diluted 5 times to measure the concentration of total bilirubin (in $\mu\text{mol/L}$) and free iron (in $\mu\text{mol/L}$) by the Automatic Blood Biochemical Analyzer (7600-120, Hitachi, Tokyo, Japan) and of soluble ferritin (in mg/L) by ChemiLuminescence ImmunoAssay System (Roche, Basel, Switzerland).

Tissue Staining

Serial 4- μm sections obtained from each block, with the first resultant slide being stained for H&E to confirm pathologic diagnosis, and the subsequent slides were used for Masson trichrome staining and Picro-Sirius red 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°C for 2 hours. Bouin's solution was made with saturated picric acid 75 mL, 10% formalin solution 25 mL, and acetic acid 5 mL. Tissue sections were stained using Masson's Trichrome Staining kit (Baso, Wuhan) following the manufacturer's instructions. The areas of the collagen fiber layer stained in blue in proportion to the entire field of the ectopic implants were calculated by the Image Pro-Plus 6.0 (Media Cybernetics, Inc, Bethesda, Maryland).

Picro-Sirius red staining was used for the detection of collagen fibers in tissues.³⁰ Tissue sections were heated in an oven at 60°C for 45 minutes, and then deparaffinized in xylene and hydrated using graded ethanol solutions. The tissue slides were placed in a Picro-Sirius red stain solution kit (Goodbio, Wuhan) for 20 minutes following the manufacturer's instructions and washed in running tap water for 1 minute. Under the optical microscope, the areas of the collagen fiber layer stained in red. To quantify the extent of fibrosis, the thickest staining area of each section was chosen to calculate the collagen concentration. Following Street et al,³¹ the percentage of fibrotic tissues within lesions in Picro-Sirius red stained sections was quantified in pixels. Briefly, a background intensity threshold was set by Image Pro-Plus 6.0 (and then kept constant for all slides), showing the appearance of collagen fibers, and then the percentage of unmasked pixels above the threshold relative to the total pixels within the whole area of the lesion was calculated, yielding the average percentage of the collagen content for 2 to 3 nonoverlapping 20 $\times$ fields. Under the dark zone of the microscope equipped with polarizing illumination (Nikon Eclipse 55i, Tokyo), the collagen fibers appeared bright while the interstitial space and noncollagen elements appeared darker. Collagen I appeared thick and in red color and yellow, while collagen III appeared thin and in green.

Statistical Analysis

The comparison of distributions of continuous variables between or among 2 or more groups was made using the

Table 1. Characteristics of Recruited 30 Patients With Ovarian Endometriomas.

Variable Name	Statistics/Distribution
Age, year	Mean = 32.0 (SD = 7.1) Median = 31.0 Range = 18-44
Gravidity	
0	16 (53.3%)
I	6 (20.0%)
≥2	8 (26.7%)
Parity	
0	18 (60.0%)
I	11 (36.7%)
≥2	1 (3.3%)
rASRM score	Mean = 22.7 (SD = 20.5) Median = 13.5 Range = 4-64
Severity of dysmenorrhea	
None	13 (43.3%)
Mild	6 (20.0%)
Moderate	5 (16.7%)
Severe	6 (20.0%)
Complaint of infertility	
No	28 (93.3%)
Yes	2 (6.7%)
Cyst size (diameter, in cm; all 34 cysts included)	Mean = 6.3 (SD = 1.9) Median = 6.5 Range = 2-10
rASRM adhesion scores (all 34 cysts)	Mean = 5.4 (SD = 4.6) Median = 4.0 Range = 0-16
Cyst color	
Brown/black	15 (44.1%)
White	19 (55.9%)

Abbreviations: rASRM, revised American Society for Reproductive Medicine; SD, standard deviation.

Wilcoxon's and Kruskal's test, respectively, and the paired Wilcoxon test was used when the before-after comparison was made for the same group of patients. Pearson's or Spearman's rank correlation coefficient was used when evaluating correlations between 2 variables when both variables were continuous or when at least 1 variable was ordinal. To evaluate the potential factors associated with the cyst color, a multivariate logistic regression analysis using backward elimination procedure was employed. Multidimensional scaling was used to see the relationship of cyst color with cyst fluid density and viscosity (medium-shear stress).

The *P* values of less than .05 were considered statistically significant. All computations were made with R 3.1.0.³²

Results

All cyst samples were confirmed to be ovarian endometriomas with presence of both endometrial glands and stroma by histopathological evaluation. The characteristics of the 30 patients with ovarian cysts are listed in Table 1. Among the 30 patients,

28 (93.3%) had cysts of ≥ 4 cm in diameter. Only 2 had cysts of 2 cm in diameter: 1 patient underwent an exploratory laparoscopy because of unexplained infertility, and the other due to severe adenomyosis-associated dysmenorrhea.

Both Masson trichrome staining and Picro-Sirius red staining indicated that all cysts had various amount of collagen fibers, especially in white-colored ones (Figures 2A and B). In addition, Masson staining showed that in the stromal component of ectopic endometrium, many cells were stained in red, suggestive of muscle fibers or myofibroblasts (Figure 2A). Picro-Sirius red staining shows that white-colored lesions had mostly type I collagen fibers while black-colored lesions also had type I collagen fibers and some type III fibers (Figure 2B). Normal endometrium (data not shown), endometrial epithelium, and vascular epithelium of ectopic endometrium all appeared in dark black. Overall, white-colored lesions had more collagen fibers than that of black-colored ones (Figures 2A and 2B; *P* = .043 and *P* = .004 by Masson staining and Sirius staining methods, respectively; Figure 3A and B). By Masson staining, the average percentage of areas containing collagen fibers was 54.4% and 68.2% for black- and white-colored lesions, respectively, with an overall percentage of 62.1% (standard deviation [SD] = 20.3%). We calculated the proportion of collagen-fibers in the entire section fields and found that the 2 staining methods yielded correlated measures (*r* = 0.71, *P* = 5.7×10^{-6}). By Sirius staining, the average percentage of areas containing collagen fibers was 40.7% and 52.2% for black- and white-colored lesions, respectively, with an overall percentage of 47.1% (SD = 12.6%).

As expected, the viscosity measured under different shear stress was different for the same cyst fluid sample, with decreasing value for higher shear stress characteristic of a non-Newtonian liquid (Figure 4A). We found that the severity of dysmenorrhea did not correlate with the rASRM score (*r* = -0.14, *P* = .44), nor with the adhesion score (*r* = -0.17, *P* = .34). The cyst color was not associated with either cyst size (*P* = .97) or the rASRM score (*P* = .20). Although women with black-colored ovarian cysts appeared to have more severe dysmenorrhea than those with whitish ones, the difference did not reach statistical significance (*P* = .072). However, white-colored cysts were associated with higher density of the cyst fluid (*P* = 8.2×10^{-6}) and with higher shear viscosity (all *P* values <.004; Figure 5). White-colored cysts were also associated with higher concentration of total bilirubin (*P* = .016), soluble ferritin (*P* = .0035), and iron (*P* = .0045) and higher adhesion scores (*P* = .0046; Figure 5), but not BMI (*P* = .39).

We found that, compared with black-colored cysts, white-colored ones had significantly higher content of collagen fibers (Figure 3A and B). In fact, the extent of fibrosis, as measured by the proportion of collagen fibers, was correlated most closely with the iron concentration in the cyst fluid (Figure 3C and D), less so with the total bilirubin or soluble ferritin (data not shown). The adhesion score, the viscosity of the cyst fluid, and the soluble ferritin, iron, and total bilirubin levels in the cyst fluid were also correlated with the

Figure 2. Panel A, representative Masson trichrome staining results of normal human endometrium (top panel) and ectopic human endometrium (ovarian endometrioma: black lesion and white lesion, in middle and lower panels). Magnification in all left, middle, and right column of figures are as indicated: 100 $\times$, 200 $\times$, and 400 $\times$. The scale bar represents 502, 251, and 125 μ m, accordingly. Yellow arrows point to collagen fibers stained in blue, while black arrows indicate muscle fibers stained in red. Panel B, representative Picro-Sirius red staining results of black- and white-colored endometriotic ovarian cysts viewed under a polarized microscope. Magnification in all left, middle, and right column of figures are as indicated: 100 $\times$, 200 $\times$, and 400 $\times$. The scale bar represents 502, 251, and 125 μ m accordingly. Collagen fibers stained in red are marked with white long arrows. Collagen fibers stained in red or yellow are type I, and those stained in green are type III. Col: collagen; I: type I; III: type III. (The color version of this figure is available in the online version at <http://rs.sagepub.com/>.)

Figure 3. Boxplot of the proportion of areas containing collagen fibers between black- and white-colored cysts, as measured by Masson trichrome staining (A) or Sirius red staining (B). The statistical significance of between-group difference is shown by the P value. C and D, Scatter plot of the proportion of areas containing collagen fibers, as measured by Masson trichrome staining (C) or Sirius red staining (D), versus the iron content in the cyst fluid. The squared dots represent black-colored cysts while the crosses represent white-colored cysts. The Pearson correlation coefficient, along with its statistical significance, is shown in the figure.

density of cyst fluid (Figure 6; P values are shown therein). In addition, the fluid density correlated with the proportion of collagen fibers as measured by the Picro-Sirius red staining ($P = .37$, $P = .035$), but not by the Masson staining ($r = 0.22$, $P = .21$).

The iron concentration in the cyst fluid correlated with both the soluble ferritin level ($r = 0.38$, $P = .028$, both log-transformed) and the total bilirubin level ($r = 0.36$, $P = .0498$, iron level square-root transformed). It also correlated with the adhesion score ($r = 0.51$, $P = .0019$). However, it was not correlated with either the cyst size ($r = -0.03$, $P = .85$) or the severity of dysmenorrhea ($P = -.22$, $P = .21$).

We performed a logistic regression analysis to evaluate the potential factors that may be associated with the cyst color and found that the cyst fluid density, viscosity (low-, medium- or high-shear force), ferritin level, iron level, total bilirubin level, and adhesion score, but not cyst size, BMI, or rASRM score, were all associated with the cyst coloration (all P values $<.047$). The proportion of collagen fibers of the cyst as determined by Sirius red staining was also associated with the cyst color ($P = .019$) but marginally associated with that as determined by Masson trichrome staining ($P = .056$). In

particular, 2 physical properties of the cyst fluid, that is, density ($P = .04$) and medium-shear force viscosity ($P = .04$) were both found to be associated with the cyst color. The multidimensional scaling using just the cyst fluid density and viscosity (medium-shear) suggested that the 2 variables could be used to distinguish the black-colored and white-colored cysts reasonably well (Figure 4B).

Discussion

This study shows that older cysts, ostensibly seen as white-colored cysts that presumably had experienced more bleeding episodes than younger ones, have higher iron, soluble ferritin, and total bilirubin concentrations in the cyst fluid than younger ones. It also shows that older cysts have higher fibrotic content most likely resulting from repeated hemorrhage signaling repeated tissue injury. This study provides one piece of evidence that ovarian endometriomas are wounds that undergo repeated injury and healing, resulting ultimately in fibrotic lesions with extensive adhesions. This may explain as why ovarian endometrioma is tough to treat since in general fibrosis is difficult to treat, let alone cure.³³ In other

Figure 4. A, Measured viscosity of cyst fluid as a function of shear stress. Different lines represent data from different samples. The thick black line represents the mean values, with their standard deviations. B, Classification of ovarian cyst tissue samples by multidimensional scaling using the density and the viscosity (medium-shear force) of the cyst fluid. B: black-colored cysts; W: white-colored cysts.

words, older cysts, which have elevated iron, ferritin, and total bilirubin concentration in the cyst fluid concordant with higher density and viscosity, may be resistant to hormonal treatment simply because fibrotic tissues in general do not respond well to hormonal drugs.³⁴ Of course, other explanations, yet to be elaborated, may also be possible.

Our findings are in broad agreement with many published studies. Aside from the contents of the cyst fluid, the proportion of collagen fibers as seen in this study can explain the finding reported in Muzii et al³⁵ that an endometrial lining covered the internal surface of the cyst wall in 60% of the entire area, with a range of 10% to 98%. When the endometriotic cells are transdifferentiated and become part of the ECM, they inevitably lose the morphology characteristic of endometrial stroma and/or glandular epithelium. As such, altered angiogenesis, changes in hormone biosynthesis and receptivity, and changes in expression profile in the lesion (which are expertly reviewed in³⁶) are sure to occur.

In endometriosis, iron overload has been demonstrated in different components of the peritoneal cavity (peritoneal fluid, endometriotic lesions, peritoneum, and macrophages).^{27,37,38} Increased oxidative stress resulting from iron overload also would result in nuclear factor κ B activation, creating an environment favorable for increased proliferation, survival,

angiogenesis, proinflammatory cytokine/chemokine production, and invasiveness.^{18,39-41} However, iron overload is known to contribute to fibrosis in the liver,⁴² lung,⁴³ and the heart.⁴⁴

Although the natural history of endometriosis is still unclear, the consensus view is that the color of endometriotic lesions, possibly including ovarian endometriomas, is a rough proxy for the lesion age, with red-colored lesion being the younger ones that progressing to black color and finally to white.^{1,45-48} Beside the conspicuous difference in coloration, red-colored, and thus younger, lesions have higher expression of vascular endothelial growth factor (VEGF) and consequently higher angiogenic and proliferative propensity than black ones.^{49,50} Interestingly, lower VEGF expression has been reported in idiopathic pulmonary fibrosis.⁵¹ It is possible that higher VEGF expression may facilitate scarless or nonfibrotic healing of wounds⁵² and inhibit EMT during the progression to fibrosis,^{53,54} but lower expression would lead to the opposite direction. Myofibroblasts generated from epithelial cells through EMT could be the primary sources of ECM-producing myofibroblasts in injured tissues.¹⁷

In pelvic endometriotic lesions, molecular aberrations consistent with EMT have been reported.⁵⁵ It is recently reported that the Wnt/ β -Catenin signaling induced myofibroblast activation may be a possible molecular mechanism underlying fibrosis in endometriosis.⁵⁶ Our own data show that activated platelets induced not only EMT in endometriotic epithelial cells but also subsequent myofibroblast activation, leading to increased ECM deposition¹⁶ (D. Ding, MSc, unpublished data, January 2015). As seen in our Masson staining of endometrioma tissues, there is increased portion of muscle fibers in both stromal and epithelial components of the lesions (Figure 2), suggesting that some fibroblasts may have already been differentiated into myofibroblasts, very likely mediated through tumor growth factor β 1 (TGF- β 1) signaling since activated platelets release a great deal of TGF- β 1.

Our findings pieces together several seemingly unrelated findings regarding the contents of endometrioma fluid (see³⁶). The increased reactive oxygen species in the cyst fluid (summarized in³⁶) may facilitate EMT.^{57,58} The reported metaplastic changes into smooth muscle cells in endometrioma⁵⁹ and the ovarian smooth muscle cell metaplasia^{60,61} may result from the activation of myofibroblasts in the lesions, which produce large amount of collagens in the process. In addition, the activation of the coagulation system due to cyclic hemorrhage of the ectopic endometrium results in the activation of the extrinsic coagulation pathway with resultant elevation of plasminogen activator inhibitor 1 (PAI-1) levels in cyst fluid, as reported.⁶² Elevated PAI-1 would inhibit urokinase-type plasminogen activator/tissue-type plasminogen activator/plasmin and plasmin-dependent matrix metalloproteinase activities, tipping the fibrolysis and fibrogenesis balance further toward fibrogenesis.⁶³

Ovarian endometriomas contain chocolate-like fluid as the result of accumulation of menstruation-like hemorrhagic blood in the cyst.^{8,13} Since the blood, presumably shed cyclically by the ectopic implants, is entrapped in the cyst, they soon become senescent and ingested by macrophages,

Figure 5. Boxplots of the density (A), viscosity of cyst fluid (B-D), total bilirubin (E), soluble ferritin (F), iron in cyst fluid (G), and adhesion scores (H) between black- and white-colored cysts. The statistical significance of between-group difference is shown by the *P* value.

releasing Hb, which in turn, is turned into bilirubin, iron, and ferritin. Barring a substantial leakage outside of the cyst and assuming a regular and cyclic bleeding of the functional implant, the concentration of iron, soluble ferritin, or total bilirubin in the cyst fluid should be proportional to the number of hemorrhage episodes experienced, or the age of the ovarian cyst since existence. At the same time, the elevated concentration of iron, soluble ferritin, and total bilirubin, along with accumulating cell debris, would thicken the fluid, resulting in increased density as well as viscosity of the cyst fluid, as we have shown. However, it is unclear as why these parameters do not correlate with the size of the cyst. Conceivably, the cyst size may be determined by factors that are different from the number of bleeding episodes.

Our study suggests a possibility that the age of an ovarian cyst could be estimated more accurately than the cyst color by the content of the cyst fluid, assuming a more or less regular menstrual cycle. Other physical measurements, such as density and viscosity of the cyst fluid, may also be useful for this purpose. In the absence of any better method tracking the natural history of endometriotic lesions, this dating method

should be more valuable than the coloration of the implants. If we accept this notion, then it could explain as why there exist conflicting data regarding the same type of endometriotic lesions simply because of different ages or stages of the tissue sample used. Clearly, more research is needed to further illuminate this issue.

Although rASRM, the most widely used staging system for endometriosis, captures the extensiveness of endometriosis, it fails to provide any information on the developmental stage of the disease and is of limited value in prognosis or the aid to decide the best therapeutic modality, among other deficiencies.⁶⁴ For infertility patients diagnosed with endometriosis, the Endometriosis Fertility Index that has recently been emerged as a staging system superior to the rASRM in predicting pregnancy rates following surgical diagnosis and treatment of endometriosis.⁶⁵ For endometriosis-related pain, unfortunately, there is no better staging system yet.

We note that most, if not all, staging systems for endometriosis have emulated those for malignant disease.⁶⁴ These systems may capture the extensiveness (amount, spread, or depth of infiltration) of the disease, or anatomic locations, but none of

Figure 6. Scatter plots showing the relationship between density of cyst fluid and (A) adhesion score of the ovarian cyst, (B-D) viscosity of the cyst fluid, (E) soluble ferritin, (F) total bilirubin, (G) iron concentration in the cyst fluid, and (H) the proportion of areas containing collagen fibers per Sirius red staining. The squared dots represent black-colored cysts while the crosses represent white-colored cysts. The Pearson's correlation coefficient, along with its statistical significance, is shown in the figure.

them make an attempt to outline the time dimension on which the disease progresses. For endometriosis, the extensiveness is not synonymous with the developmental stage of the disease. Conceivably, different stages of endometriosis would require different treatment modalities. In this sense, the dating of ovarian cysts based on the measurement of cyst fluid content may be of great clinical potentials. As we can see from Figure 4B, the use of just 2 physical measurements, that is, density and viscosity of the cyst fluid, can classify the cysts fairly well.

This study also outlines, in broad strokes, a root cause for fibrosis and adhesion in ovarian cysts and possibly in other types of endometriosis. It is well-documented that a major component of the nodular lesion frequently is fibromuscular tissue with sparse, fingerlike extension of glandular and stroma tissues,¹ which are responsible for an elevated risk of bowel obstruction, chronic abdominal pain, and infertility.⁶⁶

Although the general mechanism underlying adhesion formation is thought to be the activation of the extrinsic pathway of the coagulation cascade leading to a fibrogenesis state,⁶⁷ our study also adds the time dimension to the adhesion formation, that is, repeated injury leads to the formation of adhesion or

tissue fibrosis.^{14,15} Viewed through this lens, it may be easy to understand as why the Douglas pouch and volume are reduced in women with deep endometriosis, as repeated injury and thus increased fibrosis would generate more fibrotic tissues that forms a false bottom.⁶⁸

In summary, this study shows that the cyst fluid content contains information on the age of the cyst. This “age” information, gleaned from the “fossil record” inherent in the cyst fluid, may aid in the choice of treatment modality, as in the case of cervical cancer and other malignancies.

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Authors' Note

SWG conceived and designed the study, performed data analysis and data interpretation, and drafted the article. DD carried out most of the study, XSL recruited patients and secured their biological samples, and MHS carried out some histological studies. All participated in writing the article.

Declaration of Conflicting Interests

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References

- Nisolle M, Donnez J. Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities. *Fertil Steril*. 1997;68(4):585-596.
- Vercellini P. Endometriosis: what a pain it is. *Semin Reprod Endocrinol*. 1997;15(3):251-261.
- Sampson JA. Perforating hemorrhagic (chocolate) cysts of the ovary. *Tr Am Gynec Soc*. 1921;46(1):162-236.
- Martin DC, Berry JD. Histology of chocolate cysts. *J Gynecol Surg*. 1990;6(1):43-46.
- Brosens IA, Puttemans PJ, Deprest J. The endoscopic localization of endometrial implants in the ovarian chocolate cyst. *Fertil Steril*. 1994;61(6):1034-1038.
- Nezhat C, Nezhat F, Seidman DS. Classification of endometriosis. Improving the classification of endometriotic ovarian cysts. *Hum Reprod*. 1994;9(12):2212-2213.
- Brosens IA, Puttemans P, Deprest J, Rombauts L. The endometriosis cycle and its derailments. *Hum Reprod*. 1994;9(5):770-1.
- Busacca M, Vignali M. Ovarian endometriosis: from pathogenesis to surgical treatment. *Curr Opin Obstet Gynecol*. 2003;15(4):321-326.
- Redwine DB. Age-related evolution in color appearance of endometriosis. *Fertil Steril*. 1987;48(8):1062-1063.
- Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril*. 1997;67(5):817-821.
- Vercellini P, Fedele L, Aimi G, Pietropaolo G, Consonni D, Crognani PG. Association between endometriosis stage, lesion type, patient characteristics and severity of pelvic pain symptoms: a multivariate analysis of over 1000 patients. *Hum Reprod*. 2007;22(1):266-71.
- Liu X, Yuan L, Shen F, Zhu Z, Jiang H, Guo SW. Patterns of and risk factors for recurrence in women with ovarian endometriomas. *Obstet Gynecol*. 2007;109(6):1411-1420.
- Nisolle M. Ovarian endometriosis and peritoneal endometriosis: are they different entities from a fertility perspective? *Curr Opin Obstet Gynecol*. 2002;14(3):283-288.
- Friedman SL. Liver fibrosis—from bench to bedside. *J Hepatol*. 2003;38 Suppl 1:S38-S53.
- Liu Y. Renal fibrosis: new insights into the pathogenesis and therapeutics. *Kidney Int*. 2006;69(2):213-217.
- Ding D, Liu X, Duan J, Guo SW. Platelets are a unindicted culprit in the development of endometriosis. *Hum Reprod*. In press.
- Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol*. 2008;214(2):199-210.
- Defrere S, Lousse JC, Gonzalez-Ramos R, Colette S, Donnez J, Van Langendonck A. Potential involvement of iron in the pathogenesis of peritoneal endometriosis. *Mol Hum Reprod*. 2008;14(7):377-385.
- Theil EC. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu Rev Biochem*. 1987;56(1):289-315.
- Iizuka M, Igarashi M, Abe Y, Ibuki Y, Koyasu Y, Ikuma K. Chemical assay of iron in ovarian cysts: a new diagnostic method to evaluate endometriotic cysts. *Gynecol Obstet Invest*. 1998;46(1):58-60.
- Yamaguchi K, Mandai M, Toyokuni S, et al. Contents of endometriotic cysts, especially the high concentration of free iron, are a possible cause of carcinogenesis in the cysts through the iron-induced persistent oxidative stress. *Clin Cancer Res*. 2008;14(1):32-40.
- Sugimura K, Takemori M, Sugiura M, Okizuka H, Kono M, Ishida T. The value of magnetic resonance relaxation time in staging ovarian endometrial cysts. *Br J Radiol*. 1992;65(774):502-506.
- Takahashi K, Okada S, Okada M, Kitao M, Kaji Y, Sugimura K. Magnetic resonance relaxation time in evaluating the cyst fluid characteristics of endometrioma. *Hum Reprod*. 1996;11(4):857-860.
- Arumugam K. Endometriosis and infertility: raised iron concentration in the peritoneal fluid and its effect on the acrosome reaction. *Hum Reprod*. 1994;9(6):1153-1157.
- Arumugam K, Yip YC. De novo formation of adhesions in endometriosis: the role of iron and free radical reactions. *Fertil Steril*. 1995;64(1):62-64.
- Van Langendonck A, Casanas-Roux F, Dolmans MM, Donnez J. Potential involvement of hemoglobin and heme in the pathogenesis of peritoneal endometriosis. *Fertil Steril*. 2002;77(3):561-570.
- Lousse JC, Defrere S, Van Langendonck A, et al. Iron storage is significantly increased in peritoneal macrophages of endometriosis patients and correlates with iron overload in peritoneal fluid. *Fertil Steril*. 2009;91(5):1668-1675.
- Polak G, Wertel I, Tarkowski R, Morawska D, Nowakowski A, Kotarski J. Ferritin levels in the peritoneal fluid—a new endometriosis marker? [in Polish]. *Ginek Pol*. 2006;77(5):389-393.
- Baskurt OK, Meiselman HJ. Blood rheology and hemodynamics. *Semin Thromb Hemost*. 2003;29(5):435-450.
- Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J*. 1979;11(4):447-455.
- Street JM, Souza AC, Alvarez-Prats A, et al. Automated quantification of renal fibrosis with Sirius Red and polarization contrast microscopy. *Physiol Rep*. 2014;2(7): pii: e12088.
- Team R. *A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing; 2013.

33. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med*. 2012;18(7):1028-1040.
34. Rivlin ME, Krueger RP, Wiser WL. Danazol in the management of ureteral obstruction secondary to endometriosis. *Fertil Steril*. 1985;44(2):274-276.
35. Muzii L, Bianchi A, Bellati F, et al. Histologic analysis of endometriomas: what the surgeon needs to know. *Fertil Steril*. 2007;87(2):362-366.
36. Sanchez AM, Vigano P, Somigliana E, Panina-Bordignon P, Vercellini P, Candiani M. The distinguishing cellular and molecular features of the endometriotic ovarian cyst: from pathophysiology to the potential endometrioma-mediated damage to the ovary. *Hum Reprod Update*. 2014;20(2):217-230.
37. Van Langendonck A, Casanas-Roux F, Donnez J. Iron overload in the peritoneal cavity of women with pelvic endometriosis. *Fertil Steril*. 2002;78(4):712-718.
38. Defrere S, Van Langendonck A, Vaesen S, et al. Iron overload enhances epithelial cell proliferation in endometriotic lesions induced in a murine model. *Hum Reprod*. 2006;21(11):2810-2816.
39. Guo SW. Nuclear factor-kappaB (NF-kappaB): an unsuspected major culprit in the pathogenesis of endometriosis that is still at large? *Gynecol Obstet Invest*. 2007;63(2):71-97.
40. Gonzalez-Ramos R, Donnez J, Defrere S, et al. Nuclear factor-kappa B is constitutively activated in peritoneal endometriosis. *Mol Hum Reprod*. 2007;13(7):503-509.
41. Gonzalez-Ramos R, Van Langendonck A, Defrere S, et al. Involvement of the nuclear factor-kappaB pathway in the pathogenesis of endometriosis. *Fertil Steril*. 2010;94(6):1985-1994.
42. Delima RD, Chua AC, Tirmitz-Parker JE, et al. Disruption of hemochromatosis protein and transferrin receptor 2 causes iron-induced liver injury in mice. *Hepatology*. 2012;56(2):585-593.
43. Zakyntinos E, Vassilakopoulos T, Kaltsas P, et al. Pulmonary hypertension, interstitial lung fibrosis, and lung iron deposition in thalassaemia major. *Thorax*. 2001;56(9):737-739.
44. Sampaio AF, Silva M, Dornas WC, et al. Iron toxicity mediated by oxidative stress enhances tissue damage in an animal model of diabetes. *Biomaterials*. 2014;27(2):349-361.
45. Walter AJ, Hentz JG, Magtibay PM, Cornella JL, Magrina JF. Endometriosis: correlation between histologic and visual findings at laparoscopy. *Am J Obstet Gynecol*. 2001;184(7):1407-1411.
46. Stripling MC, Martin DC, Chatman DL, Zwaag RV, Poston WM. Subtle appearance of pelvic endometriosis. *Fertil Steril*. 1988;49(3):427-431.
47. Wykes CB, Clark TJ, Khan KS. Accuracy of laparoscopy in the diagnosis of endometriosis: a systematic quantitative review. *BJOG*. 2004;111(11):1204-1212.
48. Stratton P, Winkel CA, Sinai N, Merino MJ, Zimmer C, Nieman LK. Location, color, size, depth, and volume may predict endometriosis in lesions resected at surgery. *Fertil Steril*. 2002;78(4):743-749.
49. Donnez J, Smoes P, Gillerot S, Casanas-Roux F, Nisolle M. Vascular endothelial growth factor (VEGF) in endometriosis. *Hum Reprod*. 1998;13(6):1686-1690.
50. Van Langendonck A, Eggermont J, Casanas-Roux F, Scholtes HE, Donnez J. Expression of platelet endothelial cell adhesion molecule-1 in red and black endometriotic lesions. *Fertil Steril*. 2004;82(4):984-985.
51. Willems S, Verleden SE, Vanaudenaerde BM, et al. Multiplex protein profiling of bronchoalveolar lavage in idiopathic pulmonary fibrosis and hypersensitivity pneumonitis. *Ann Thorac Med*. 2013;8(1):38-45.
52. Colwell AS, Beanes SR, Soo C, et al. Increased angiogenesis and expression of vascular endothelial growth factor during scarless repair. *Plast Reconstr Surg*. 2005;115(1):204-212.
53. Lian YG, Zhou QG, Zhang YJ, Zheng FL. VEGF ameliorates tubulointerstitial fibrosis in unilateral ureteral obstruction mice via inhibition of epithelial-mesenchymal transition. *Acta Pharmacol Sin*. 2011;32(12):1513-1521.
54. Hong JP, Li XM, Li MX, Zheng FL. VEGF suppresses epithelial-mesenchymal transition by inhibiting the expression of Smad3 and miR192, a Smad3-dependent microRNA. *Int J Mol Med*. 2013;31(6):1436-1442.
55. Matsuzaki S, Darcha C. Epithelial to mesenchymal transition-like and mesenchymal to epithelial transition-like processes might be involved in the pathogenesis of pelvic endometriosis. *Hum Reprod*. 2012;27(3):712-721.
56. Matsuzaki S, Darcha C. Involvement of the wnt/beta-catenin signaling pathway in the cellular and molecular mechanisms of fibrosis in endometriosis. *PLoS One*. 2013;8(10):e76808.
57. Rhyu DY, Yang Y, Ha H, et al. Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol*. 2005;16(3):667-675.
58. Radisky DC, Levy DD, Littlepage LE, et al. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature*. 2005;436(7047):123-127.
59. Fukunaga M. Smooth muscle metaplasia in ovarian endometriosis. *Histopathology*. 2000;36(4):348-352.
60. Mechsner S, Bartley J, Loddenkemper C, Salomon DS, Starzinski-Powitz A, Ebert AD. Oxytocin receptor expression in smooth muscle cells of peritoneal endometriotic lesions and ovarian endometriotic cysts. *Fertil Steril*. 2005;83 Suppl 1:1220-1231.
61. Odagiri K, Konno R, Fujiwara H, Netsu S, Yang C, Suzuki M. Smooth muscle metaplasia and innervation in interstitium of endometriotic lesions related to pain. *Fertil Steril*. 2009;92(5):1525-1531.
62. Boss EA, Massuger LF, Thomas CM, et al. Clinical value of components of the plasminogen activation system in ovarian cyst fluid. *Anticancer Res*. 2002;22(1A):275-282.
63. Ghosh AK, Vaughan DE. PAI-1 in tissue fibrosis. *J Cell Physiol*. 2012;227(2):493-507.
64. Roberts CP, Rock JA. The current staging system for endometriosis: does it help? *Obstet Gynecol Clin North Am*. 2003;30(1):115-132.
65. Adamson GD. Endometriosis classification: an update. *Curr Opin Obstet Gynecol*. 2011;23(4):213-220.
66. Gonzalez-Quintero VH, Cruz-Pachano FE. Preventing adhesions in obstetric and gynecologic surgical procedures. *Rev Obstet Gynecol*. 2009;2(1):38-45.
67. Alpay Z, Saed GM, Diamond MP. Postoperative adhesions: from formation to prevention. *Semin Reprod Med*. 2008;26(4):313-321.
68. Vercellini P, Aimi G, Panazza S, Vicentini S, Pisacreta A, Crosignani PG. Deep endometriosis conundrum: evidence in favor of a peritoneal origin. *Fertil Steril*. 2000;73(5):1043-1046.