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Time to redefine endometriosis including its pro-fibrotic nature

P. Vigano^{1*}, M. Candiani², A. Monno³, E. Giacomini¹, P. Vercellini⁴, E. Somigliana⁴

¹Reproductive Sciences Laboratory, Division of Genetics and Cell Biology, IRCCS Ospedale San Raffaele, Milan, 20132, Italy;

²Obstetrics and Gynaecology Department, IRCCS San Raffaele Scientific Institute, Milan, 20132, Italy;

³Innate immunity and Tissue Remodelling Unit, Regenerative Medicine Division, IRCCS San Raffaele Scientific Institute, Milan, 20132, Italy;

⁴Department of Clinical Sciences and Community Health, Università degli Studi, and Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via della Commenda 12, Milan 20122, Italy

28

29 **Abstract**

30 Endometriosis is currently defined as presence of endometrial epithelial and stromal cells at ectopic
31 sites. This simple and straightforward definition has served us well since its original introduction.
32 However, with advances in disease knowledge, endometrial stromal and glands have been shown to
33 represent only a minor component of endometriotic lesions and they are often absent within some
34 disease forms. In rectovaginal nodules, the glandular epithelium is often not surrounded by stroma
35 and frequently no epithelium can be identified in the wall of ovarian endometriomas. On the other
36 hand, a smooth muscle component and fibrosis represent consistent features of all disease forms.
37 Based on this facts, we believe that the definition of endometriosis should be reconsidered in terms
38 of ‘A fibrotic condition in which endometrial stroma and epithelium can be identified’. The main
39 reasons for this change are: 1. to foster the evaluation of fibrosis in studies on endometriosis
40 pathogenesis using animal models; 2. to potentially limit false negative diagnoses if pathologists
41 stringently stick to the current definition of endometriosis requiring the demonstration of
42 endometrial stromal and glands; 3. to consider fibrosis as a potential target for treatment in
43 endometriosis. This introductory discussion article is aimed at boosting up the attention to a largely
44 neglected aspect of the disease. Hopefully, targeting the fibrotic process might also reduce the high
45 attrition rate observed for new therapeutic approaches during the last decades.

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54 **Introduction**

55 With advances in knowledge, borders of diseases may change. Occasionally, these changes
56 are of such a magnitude that they require a redefinition of the disease. Also in endometriosis, since
57 the original description by John A. Sampson in 1927, there have been radical changes in our vision
58 of the disease, starting with a better description of the various manifestations and more specific
59 pathologic findings. Moreover, our understanding continues to ameliorate with increasing
60 knowledge of genetics and risk factors and progresses in biological mechanisms and animal models
61 of the disease. With these advances, clinicians and specialists in genetics, epidemiology, pathology
62 and basic science have developed their own conceptualizations of endometriosis which is much
63 more than the current simplistic definition that is based on the mere presence of endometrial
64 epithelial and stromal cells in ectopic sites.

65 Some main issues were indeed identified to challenge this obsolete definition. Even if the
66 presence of endometrial stromal and glands may be the starting point of the whole process leading
67 to endometriosis, it is unquestionable that endometrial stromal and glands represent only a minor
68 component of endometriotic lesions. Most importantly, this classical pathologic substrate may even
69 lack. In rectovaginal nodules, the glandular epithelium can often be observed deeply in the
70 fibromuscular tissue without any surrounding stroma (Donnez *et al.*, 1995), and in 40% of ovarian
71 endometriomas, no endometrial epithelium can be identified and the inner surface of the cyst is
72 covered only by fibrotic tissue (Muzii *et al.*, 2007). Finally, pelvic adhesions are typically free of
73 endometrial components despite being an essential pathologic characteristic of the disease
74 (Somigliana *et al.*, 2012). Noteworthy, pelvic adhesions may contribute to the determinism of some
75 classical endometriosis-related symptoms, such as deep dyspareunia, chronic pelvic pain and
76 infertility, and may play a role in the formation of endometriomas or deep nodules (Somigliana *et*
77 *al.*, 2012). This opinion paper is intended as an introductory discussion article, an opening of
78 dialogue in order to consider some changes in the general definition of endometriosis. The need for
79 a modification is also supported by previous attempts in this context (Holt and Weiss, 2000). We

80 will herein emphasize the consistent presence of fibrosis and myofibroblasts in endometriotic
81 lesions and their crucial role in the pathogenesis of the disease (Anaf *et al.*, 2000; Barcena de
82 Arellano *et al.*, 2011; Zhang *et al.*, 2016). Highlighting these features is aimed at boosting up the
83 attention of the scientific community to a largely neglected but essential disease aspect. Ultimately,
84 an enhanced sensitivity to fibrosis may orient the focus of researchers towards a more modern and
85 realistic vision of endometriosis, could the current animal models to the real nature of the disease,
86 may open new and more fruitful avenues of pharmacological research and may reduce the high
87 attrition rate observed for new therapeutic approaches of endometriosis during the last decades
88 (Vercellini *et al.*, 2011).

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90 **The biological basis of fibrosis development: the crucial role of myofibroblasts**

91 Myofibroblasts are contractile non-muscle cells that are usually activated in response to
92 injury with the intent to repair damaged extracellular matrix (ECM). These cells can differentiate
93 from different cellular lineages including tissue resident fibroblasts, endothelial cells undergoing
94 endothelial-to-mesenchymal transition, vascular smooth muscle cells and epithelial cells after
95 epithelial-to-mesenchymal transition. A myofibroblast is activated when the α -smooth muscle
96 isoform of actin (α -SMA) is neo-expressed and incorporated in stress fiber-like bundles which are
97 pivotal to promote the specific myofibroblast function of contracting the ECM. Two factors seem
98 critical to activate myofibroblasts from various precursor cells in the vast majority of organs
99 studied: Transforming Growth Factor (TGF)- β and the stiffness of the tissue. Indeed, TGF- β 1 is
100 able to induce neo-expression of α -SMA by fibroblasts *in vivo* and *in vitro* and cultures on stiff
101 substrates such as a fibrotic scar can activate a variety of different progenitors to become
102 myofibroblasts (Richter *et al.*, 2015; Hinz, 2016a).

103 When activated, myofibroblasts display increased proliferation, migratory ability,
104 production of cytokines and interstitial matrix with the consequence of disrupting the function of
105 intact residual tissues and altering the biochemical and biophysical microenvironment. A persistent

106 myofibroblast activity causes accumulation and contraction of collagenous ECM, a condition called
107 fibrosis. Macroscopically, due to accumulation of ECM, contraction of myofibroblasts and reduced
108 vasculature, fibrotic organs usually display an uneven surface, are pale and not elastic. This process
109 ultimately results in disruption of the normal anatomical structure (Bochaton-Piallat *et al.*, 2016).
110 Myofibroblasts are present in all fibrotic diseases, such as scleroderma, as well as liver, kidney, and
111 lung fibrosis and are prominent in heart failure and repair after myocardial infarction (Rockey *et al.*,
112 2015; Chistiakov *et al.*, 2016). Myofibroblast-produced tissue contractures can become life-
113 threatening when fibrosis affects vital organs (Rockey *et al.*, 2015).

114 Discriminating between myofibroblasts and smooth muscle cells may be demanding and
115 may be a matter of controversy (Hinz, 2016a). Neo-expression of α -SMA in stress fibers is the most
116 commonly used molecular marker for myofibroblasts that also express mesenchymal marker
117 proteins such as N-cadherin, vimentin and S1004A. However, these latter markers are also
118 expressed in smooth muscle cells, at least during tissue repair. Smooth muscle cells conversely
119 express a number of late differentiation markers, such as smooth muscle myosin heavy chain, h-
120 caldesmon, smoothelin and the muscle intermediate filament protein desmin, that are absent from
121 myofibroblasts in most organs. However, discriminating smooth muscle cells from myofibroblasts
122 is quite difficult in pathological conditions, so their distinction is usually a rather semantic issue
123 (Hinz, 2016a). Noteworthy, a metaplastic transformation from stromal cells to smooth muscle cells
124 via differentiation from fibroblasts to myofibroblasts has been also suggested (Zhang *et al.*, 2016).

125 Not surprisingly, the interest of researchers in various fields of medicine has recently
126 focused on anti-fibrotic therapeutic strategies aimed at blocking cytokines and factors that directly
127 control myofibroblast activation (Yang *et al.*, 2014). The complex presentation and activation
128 mechanisms of TGF- β 1 have led to develop various anti-TGF- β 1 approaches to prevent
129 myofibroblast formation and fibrosis development. Some initial findings were disappointing in
130 terms of both efficacy and safety. However, clinical trials using different anti-TGF- β 1 treatments
131 are ongoing in various diseases. Interestingly, since all the α v integrins have been shown to be able

132 to activate TGF- β 1 and are expressed in a tissue- and cell-distinctive manner, inhibiting their TGF-
133 β 1 activating function may be biologically more specific compared to the global inhibition of TGF-
134 β 1 itself. Some anti-integrin molecules are currently under investigation in clinical trials to treat
135 patients with lung fibrosis and initial findings seem promising (Hinz *et al.*, 2016b).

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137 **Fibrosis and myofibroblasts in endometriotic lesions**

138 *Peritoneal lesions*

139 The first study on peritoneal endometriosis with a monoclonal antibody against α -SMA was
140 published back in 1996 by Khare *et al.* (1996) who used immunoperoxidase and Masson's trichome
141 stains to determine respectively the presence of myofibroblasts and collagen in 10 pelvic wall
142 samples. Well-formed smooth muscle bundles and dense type I collagen were found in these
143 lesions. In 2000, Anaf and coworkers (2000) demonstrated by immunohistochemistry that all the 21
144 peritoneal lesions considered were variably but consistently positive for α -SMA staining, whereas
145 unaffected peritoneum and eutopic endometrial biopsies were negative. In 2002, Leyendecker *et al.*
146 analyzed 35 endometriotic lesions with specific α -SMA antibody by immunohistochemistry and all
147 of them stained positively for the marker. Although the authors did not formally discriminate
148 between various disease forms, they clearly showed representative sections of peritoneal
149 endometriotic lesions stained for α -SMA. The group of Sylvia Mechsner similarly evaluated
150 peritoneal endometriosis specimens in two different studies. In the first one, 76% of 120 lesions
151 showed α -SMA expression (2005) while in the second one, all 60 lesions showed positivity (2011).
152 Therefore, smooth muscle content seems to represent an important and consistent feature of
153 peritoneal endometriosis lesions.

154 Interestingly, TGF- β 1 levels were found to be significantly increased in the peritoneal fluid
155 of women with peritoneal lesions compared to women without the disease. Exposure of mesothelial
156 cells to TGF- β 1 increased the production of lactate, with reduction in the local pH. This increase in

157 the amount of lactate resulted in acid activation of the TGF- β ligand with secondary induction of
158 myofibroblast differentiation (Young *et al.*, 2014).

159

160 *Ovarian cysts*

161 The fact that fibrosis is present in the ovarian cyst wall is well known. The cysts
162 pseudocapsule is actually mostly constituted of fibrotic tissue. Noteworthy, the inner surface of the
163 cyst is usually not entirely covered by an endometrial lining and when the endometrial lining is
164 missing, only fibrotic tissue is identifiable. Positive immunostaining for α -SMA antibody was
165 demonstrated in all of 10 and 13 ovarian cysts by Khare *et al.* (1996) and Anaf *et al.* (2000),
166 respectively. According to Mechsner and coworkers (2005), who evaluated 40 ovarian lesions, the
167 smooth muscle content was present in 87% of the cases. Liu *et al.* (2017) investigated the histologic
168 features of deep and ovarian endometriotic lesions and observed a higher fibrotic content in the
169 former compared with the latter lesion type. Still, the 25 ovarian samples consistently showed
170 markers of fibroblast-to-myofibroblast transdifferentiation and stained positively for fibrosis at
171 Masson's trichrome technique.

172 Fibrosis was also identified in ovarian cortex surrounding the endometrioma. Indeed,
173 follicular density was found to be lower in the ovarian cortex adjacent to the endometriotic cyst and
174 this phenomenon is thought to be associated with tissue alterations, such as formation of fibrosis
175 and vascular deficiency, and does not seem to be related to mere mechanical stretching. Kitajima *et*
176 *al.* (2014) compared the histologic features in apparently normal ovarian cortical tissue from ovaries
177 with small endometriomas and from the contralateral healthy ovaries. Fibrosis, as determined by
178 Masson's trichrome staining with methyl green, was significantly more frequent in cortex from
179 ovaries with endometriomas (80%) than in those without (27%) and the presence of fibrosis with
180 concomitant loss of cortex-specific stroma was observed in 55% of cortical samples from ovaries
181 with endometriomas but in none of those from contralateral healthy ovaries.

182 Interestingly, the group of Sun-Wei Guo has recently shown that, in cells derived from
183 ovarian endometriosis, activated platelets promoted epithelial to mesenchymal transition, fibroblast-
184 to-myofibroblast transdifferentiation and differentiation to smooth muscle cells, resulting in
185 increased cell contractility, collagen production and ultimately to fibrosis, via the release of TGF- β 1
186 and the induction of TGF- β /Smad signaling pathway. TGF- β 1 blockade could reverse these
187 phenomena (Zhang *et al.*, 2016).

188

189 *Deep infiltrating endometriosis*

190 Donnez and coworkers (1995) demonstrated for the first time that deep endometriotic
191 nodules were histologically composed of scanty stroma and glandular epithelium disseminated in
192 extensive fibromuscular tissue. Gömöritrichrome stain was used to detect muscle tissue. They
193 speculated that this smooth muscle content pre-existed in the correspondent normal area and was
194 invaded by the ectopic endometrium. Subsequently, Anaf *et al.* (2000) based on the evaluation of 12
195 rectovaginal nodules and eight uterosacral lesions consistently positive for an anti- α -SMA antibody,
196 disputed the pre-existence of smooth muscle tissue in the rectovaginal nodules and conversely
197 supported a trans-differentiation of endometrial stromal cells. Itoga and coworkers (2003) examined
198 90 rectovaginal nodules for the presence of fibrosis by elastic-van Gieson staining of collagen and
199 for positivity to anti- α -SMA and anti-desmin antibodies. Fibrosis was observed in all but one of the
200 samples, and immunoreactivity for smooth muscle actin and desmin was observed in 89% of the
201 specimens. In deep nodules ($n=20$), staining levels for α -SMA, desmin, collagen I and extent of
202 fibrosis were shown to be higher than those of ovarian disease (Liu *et al.*, 2017). van Kaam *et al.*
203 (2008) not only showed that all the 20 deep infiltrating endometriotic lesions studied comprised
204 fibromuscular tissue containing α -SMA-, desmin- and myosin-positive myofibroblastic cells, but
205 again raised reasonable doubts on the origin of this muscle content. Indeed, they demonstrated that
206 the inoculation of human endometrium into a nude mouse could induce α -SMA expression in the
207 surrounding murine tissue. This would suggest that a reaction of the local environment to the

208 presence of ectopic endometrium, rather than the stromal differentiation toward smooth muscle
209 cells, could be at the basis of fibrosis development.

210 In spite of the identification of a fibrotic component in deep infiltrating disease, Matsuzaki
211 and coworkers (2017) showed that the TGF- β 1 signaling may be absent when culturing
212 endometriotic cells taken from this type of lesions. They suggested that endometrial stromal cells
213 from patients affected might differentiate into myofibroblasts without TGF- β 1 treatment and
214 produce collagen type I. Increased stiffness through increased myofibroblast collagen production
215 may then further increase matrix stiffness resulting in a fibrotic environment in deep disease over
216 time.

217

218 *Summary of the literature overview*

219 Regardless of the different hypotheses provided to explain the origin of myofibroblasts and
220 fibrosis in endometriotic lesions (summarized in Figure 1) (Young *et al.*, 2014; Zhang *et al.*, 2016;
221 Matsuzaki *et al.*, 2017 Albertsen and Ward, 2017), all investigators agree on the predominance of
222 this component. One may argue that fibrosis represents a secondary event triggered by an insult (the
223 presence of ectopic cells) in a suffering tissue (Walton *et al.*, 2017). However, fibrosis appears as
224 the phenomenon underpinning endometriosis-associated morbidity and some manifestations of the
225 disease (i.e. adhesions). Thus, in line with what is recognized for other conditions of unknown
226 etiology such as scleroderma (Tsou *et al.*, 2017), fibrosis seems to represent a self-amplifying
227 event of endometriosis.

228

229 **Why changing the definition**

230 There are essentially three reasons for including the term "fibrosis" in the definition of
231 endometriosis:

- 232 1. Myofibroblasts and fibrosis may receive more attention as potential targets of medical
233 treatments for endometriosis. Shifting the focus on fibrosis with a new definition may re-
234 orient current research efforts towards more effective therapies. When considering the
235 challenges of treating a fibrotic disease such as endometriosis, there is a pressing need to
236 identify effective pharmacological agents to block fibrosis, in addition to seek for agents
237 acting on ectopic endometrium (Somigliana *et al.*, 2012). When investigating the effects of
238 drugs interfering with endometrial tissue, researchers should concomitantly evaluate also the
239 impact on fibrosis. Moreover, the scientific community should pay utmost attention to the
240 progress on the management of fibrosis in other areas of medicine. If in the future some
241 effective and safe antifibrotic drugs will be developed for other disorders, endometriosis
242 might benefit as well.
- 243 2. Given the consistent presence of myofibroblasts and fibrosis in all disease forms, animal
244 models of endometriosis should also present this feature. The current definition could lead
245 researchers astray in this regard, as they tend to consider an animal model reliable merely
246 because endometrium is placed ~~in~~ at ectopic sites. However, endometriosis is much more
247 than that and fibrosis represents a crucial histological aspect. Mouse, hamster or rat models
248 have been developed so far by intraperitoneal or subcutaneous transplantation of autologous
249 endometrial tissue from the same or syngeneic donors, or from humans in nude mice.
250 “Endometriosis” is often induced surgically by suturing fragments of uterine tissue onto the
251 peritoneum or, in mice, an alternative procedure is to simply inject fragments of minced
252 uterine horns from donor mice into the peritoneum of recipient animals (Mariani *et al.*,
253 2012; Greaves *et al.*, 2017). A great variety of compounds with different functional
254 activities have been used in these models, and many of them have shown various degrees of
255 inhibition of lesion growth (Bedaiwy *et al.*, 2017). Unfortunately, to date, translation of
256 these findings to the clinic has been limited, with some paradoxical but enlightening results.
257 Raloxifene, for instance, was repeatedly demonstrated to be effective in rodent models

258 (Altintas *et al.*, 2010) but, when tested in women in a RCT, it even accelerated pelvic pain
259 recurrence after surgery when compared to placebo (Stratton *et al.*, 2008). A possible
260 explanation could be the poor alignment of the outcome measures evaluated in the current
261 animal models to the real nature of the disease. Disease features in an animal model should
262 also include the evaluation of fibrosis presence that can be done in several ways (Kushiyama
263 *et al.*, 2011; Dong *et al.*, 2017; Rittié *et al.*, 2017) (Figure 2).

264 3. A modification in the definition of endometriosis would not only aim at driving research
265 towards more successful therapies, but may also have some immediate clinical implications.
266 Indeed, from a diagnostic standpoint, based on histologic findings, endometriotic lesions can
267 sometimes be misjudged. Some cases of endometriosis-related extensive pelvic adhesions
268 may paradoxically remain without a definite diagnosis or erroneously considered long-term
269 consequence of pelvic inflammatory disease (PID). This may be particularly true in the
270 absence of endometriomas or deep peritoneal lesions and/or when surgical access to pelvic
271 organs at surgery is impeded by the severity of the adhesions. In fact, extending the
272 definition of endometriosis beyond the mere presence of ectopic endometrial tissue would
273 consent to classify women with extensive pelvic adhesions and without evidence of past
274 pelvic insults (such as for instance a damage of the tubal mucosa) as affected even in
275 absence of the two classic components of the histologic diagnosis, i.e., endometrial stroma
276 and glands. Noteworthy, even when available, surgical specimens are rarely serially
277 sectioned in standard practice, and lesions with no or only small areas with endometrial
278 lining can be missed by pathologists (Nisenblat *et al.*, 2016). False negative diagnoses can
279 occur if pathologists stringently stick to the current definition of endometriosis requiring the
280 concurrent demonstration of both endometrial stroma and glands. With a cautious approach
281 in order not to increase false positive cases, the definitive recognition of fibrosis as an
282 essential component of endometriosis may overcome these uncertainties. Noteworthy, the
283 debate on the reliability of non-invasive diagnosis of endometriosis may also be influenced

284 by a change in the definition of endometriosis. For instance, one cannot exclude that the
285 current high accuracy of transvaginal ultrasound for the diagnosis of endometriomas
286 (sensitivity of 93% and specificity of 94%) (Nisenblat *et al.*, 2016) may improve if the
287 definition of endometriosis will be modified. Sensitivity in particular may increase and
288 transvaginal ultrasound could reach the requirements to become a replacement test
289 (sensitivity $\geq 94\%$ and specificity $\geq 79\%$) and thus definitively substitute laparoscopy for the
290 diagnosis of these lesions. Noteworthy, for some fibrosis-based conditions such as
291 retroperitoneal fibrosis, the diagnosis relies more upon the typical imaging features on CT or
292 MRI, than on percutaneous biopsy (Cohan *et al.*, 2017).

293

294 **Conclusions**

295 The present definition of endometriosis based on the histologic feature of the concomitant presence
296 of endometrial stroma and epithelium in ectopic sites has been developed in order to guarantee a
297 uniform identification of the condition. However, with the increasing knowledge of the disease
298 mechanisms and the improvement of the diagnostic tools, this definition nowadays appears too
299 simplistic to represent the different histologic forms and clinical manifestations of this complex
300 disease. Therefore, if on the one hand the identification of the specific histopathologic
301 characteristics remains extremely important to diagnose endometriosis, on the other hand other
302 aspects need to be broadened from both a diagnostic and a therapeutic point of view. In our view,
303 the endometriosis definition should be reconsidered. ‘A fibrotic condition in which endometrial
304 stroma and epithelium can be identified’ could represent a realistic starting point.

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308 **Authors' roles**

309 All authors contributed to the development of the conceptions in this manuscript. A.M. performed
310 the staining in the mouse model. P.Vi. and E.S. drafted the manuscript, which was reviewed by all
311 co-authors.

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314 **Conflict of interest**

315 The authors have no conflicts of interest to declare.

316

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417 **Figure legends**

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419 **Figure 1.** Main pathogenetic models proposed to explain the presence of myofibroblasts and
420 the development of fibrosis in endometriosis. Epithelial to mesenchymal transition, fibroblast-to-
421 myofibroblast transdifferentiation, increased collagen production and ultimately fibrosis have been
422 suggested to be triggered in endometriotic cells in presence of stimulating factors (B and C,
423 platelets or a stiff tissue). Similar phenomena in other tissues (A, surrounding connective tissue or
424 C, mesothelial barrier) have been also proposed.

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426 **Figure 2.** Sirius red staining of an ectopic endometrial tissue in the mouse to visualize the area
427 occupied by fibrous collagen and usually used to assess a fibrotic phenomenon (Kushiyama *et al.*,
428 2011; Rittié *et al.*, 2017). Left panels, magnification 2.5X; right panels, magnification 16X.

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