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ORIGINAL ARTICLE

Elastin density: Link between histological and biomechanical properties of vaginal tissue in women with pelvic organ prolapse?

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Abstract
Introduction and hypothesis The aim of the study was to correlate histological and biomechanical characteristics of the vaginal wall in women with pelvic organ prolapse (POP).
Methods Tissue samples were collected from the anterior [point Ba; POP Questionnaire (POP-Q)] and/or posterior (point Bp; POP-Q) vaginal wall of 15 women who underwent vaginal surgery for POP. Both histological and biomechanical assessments were performed from the same tissue samples in 14 of 15 patients. For histological assessment, the density of collagen and elastin fibers was determined by combining high-resolution virtual imaging and computer-assisted digital image analysis. For biomechanical testing, uniaxial tension tests were performed to evaluate vaginal tissue stiffness at low (C_0) and high (C_1) deformation rates.
Results Biomechanical testing highlights the hyperelastic behavior of the vaginal wall. At low strains (C_0), vaginal tissue appeared stiffer when elastin density was low. We found a statistically significant inverse relationship between C_0 and the elastin/collagen ratio (p=0.048) in the lamina propria. However, at large strain levels (C_1), no clear relationship was observed between elastin density or elastin/collagen ratio and stiffness, likely reflecting the large dispersion of the mechanical behavior of the tissue samples.
Conclusion Histological and biomechanical properties of the vaginal wall vary from patient to patient. This study suggests that elastin density deserves consideration as a relevant factor of vaginal stiffness in women with POP.

Keywords Vagina · Pelvic organ prolapse · Collagen · Elastin · Connective tissue · Biomechanics

Abbreviations

POP	Pelvic organ prolapse
ECM	Extracellular matrix
αSMA	Alpha smooth-muscle actin
RGB	Red–green–blue
SHM	Scanning haptic microscope
LOX	Lysyl oxydases

Introduction

Pelvic organ prolapse (POP) is a global health problem that can seriously impact on a woman's quality of life (QoL). The lifetime risk of undergoing surgery for prolapse or incontinence in women is 20 % by the age of 80 years [1]. Vaginal childbirth, obesity, and advancing age are important risk factors for developing POP [2, 3]. The high recurrence rate of POP (almost 30 %) after surgical repair using weak native

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tissue [4] suggests that a genetic predisposition can also play a role in POP development [5, 6]. Investigations into vaginal tissue composition in women with POP remain limited and have led to controversial results [7]. To date, the contribution of specific connective tissue components to the support of pelvic organs and to mechanical properties of the vaginal wall is still difficult to determine.

The vaginal wall is composed of four layers: The epithelial layer is a superficial nonkeratinized squamous epithelium. The subepithelial layer, or lamina propria, is a dense connective tissue layer mainly composed of fibrillar collagens and elastin populated by fibroblasts. The muscularis is mainly composed of smooth muscle cells embedded in connective tissue. The adventitia is a loose connective tissue layer that separates the vaginal muscularis and paravaginal tissue. Connective tissue of the vaginal wall is composed of cellular elements, fibroblasts, and smooth muscle cells surrounded by an extracellular matrix (ECM). The main fibrillar components of the ECM, collagen and elastin, are thought to contribute the most to its biomechanical properties. Their alterations are most probably involved in the physiopathology of POP [8]. Collagen fibers are very rigid and do not easily distort, while elastin fibers provide elasticity and recoil to the tissue. Elastic fibers are important for mainting vaginal structural integrity against mechanical strain [9].

Histology alone does not explain changes in connective tissues or their involvement in the pathogenesis of POP. We previously described the histological changes observed between two different locations of the vaginal wall in women with POP by using immunohistochemistry combined with computer-assisted digital image analysis [10]. A significant decrease in elastin density was observed in the most distal portion of the vaginal wall compared with that of the precervical area of the same women. According to these previous findings, we hypothesized that elastin density can be involved in POP pathophysiology, but the mechanism underlying this disorder is still unclear. Therefore, we attempted to establish whether there is a link between histological and mechanical properties of vaginal tissue by analyzing both parameters in the same group of women previously described [10].

Materials and methods

Patients

Tissue samples for both histological and biomechanical testing were obtained from 14 of the 15 women who underwent vaginal surgical procedures for POP at the Department of Gynecological Surgery of the University Hospital Jeanne de Flandre, Lille, France. Institutional Review Board approval was obtained prior to the start of the study (CPP 09/62), which was conducted between February and May 2012. All patients underwent an assessment of their POP stage according to the International Pelvic Organ Prolapse Quantification system [11]. Demographic characteristics (age, parity, BMI, menopausal status, POP-Q stage and type of surgery) were collected prospectively and stored in a dedicated database.

Full-thickness vaginal tissue samples were taken from each woman in the longitudinal axis of the anterior and/or posterior vaginal wall for histological and biomechanical assessment. Biopsies were collected only in the compartment involved during POP repair. Samples were obtained with Metzenbaum scissors after carrying out deep infiltration with diluted lidocaine solution, followed by a sagittal midline incision on either side of the tissue at the Ba and/or Bp POP-Q points. The left side was frozen in a saline solution at −18 °C for biomechanical testing. The right side was collected and fixed in 4 % formalin for histological analysis.

Histological material

Histological analysis was performed by using the same protocol as described previously [10]. Vaginal samples were embedded in paraffin and serially sectioned at a thickness of 5 μm. Tissue sections were stained with routine dyes [hematoxylin and eosin H&E)] for conventional histopathological evaluation or with specific stains, such as Masson's trichrome or orcein, to evaluate collagen and elastin fibers, respectively [10, 12]. Masson's trichrome coloration identified vaginal wall collagen fibers, which appeared green. The green component of the red–green–blue (RGB) color image was extracted, and a median filter was applied to eliminate noise [13]. Collagen fibers were then extracted using automatic entropy thresholding [14]. Orcein appears to be an appropriate specific coloration, providing optimal contrast between elastin fibers and tissue background (Fig. 1). After splitting the original color image into its RGB components, elastin fibers could be directly extracted from the red component using automatic entropy thresholding.

Image analysis

Virtual images were acquired with the fully automated digital microscopy system dotSlide (Olympus, BX51TF, Aartselaar, Belgium) coupled with a Peltier-cooled high-resolution digital color camera (1376×1032 pixels; XC10, Olympus). Digital images of the entire tissue sections were digitized at high magnification (100×), producing virtual images in which pixel size was 0.65 μm. Image analysis was performed, as previously described [10]. This methodology combines high-resolution virtual imaging of full-thickness biopsies and computer-assisted image analysis [15, 16]. On the processed images, we measured elastin density (area occupied by elastin fibers per unit surface) in the lamina propria and muscularis, total collagen density (area occupied by collagen fibers per

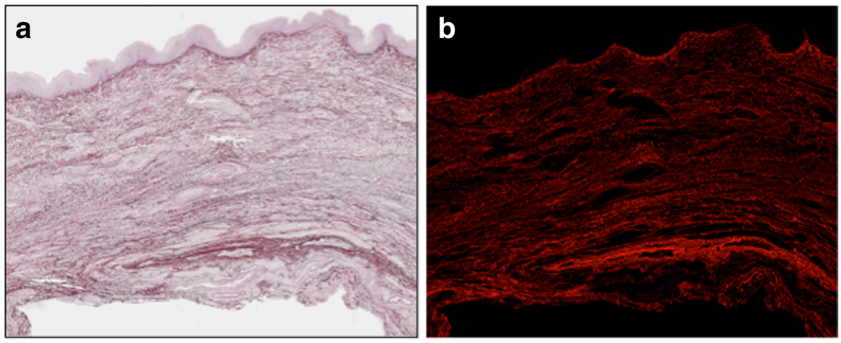

Fig. 1 **a** Orcein-stained, full-thickness histological section of the anterior vaginal wall, and **b** binary image corresponding to elastin fibers in the lamina propria and muscularis

unit surface) in the lamina propria, and muscularis and the ratio of elastin/collagen in the muscularis and lamina propria.

Biomechanical test conditions

The experimental method for biomechanical characterization of vaginal tissue was conducted following the protocol of Rubod et al. [17, 18], which allows biomechanical testing in controlled and reproducible conditions (temperature, hygrometry, deformation rate). The vaginal biopsies were unfrozen before the tests, and samples of 25×4 mm were excised from the tissue using a punch. Due to sample geometry, only the uniaxial tension test could be performed. The thickness of each sample was measured with a caliper rule to further determine nominal stress (F/S_0, where F is the load and S_0 is the size of the initial cross section). Each sample was clamped in a tightening grip designed to prevent tissue slippage during the test [17] and were directly loaded without a preloading phase. Results included the complete response from zero force to rupture. Rupture tests were performed using a conventional tension machine (Instrom 5882). A low-capacity load cell (1 kN) was used to measure nominal stress (force per unit of surface) during the test, knowing the force and initial cross section of samples. Load-cell sensitivity was 0.01 N (0.001 class), which is in agreement with the quantity measured. Strain (l-l_0/l_0, where l is the length and l_0 the initial length) was measured with a contactless video extensometer. Subsequently, stress–strain curves were obtained and analyzed to characterize biomechanical behavior of the considered tissues. Mechanical response before rupture was then studied. For a comparative statistical analysis of all experimental data, a behavior model was incorporated taking into account nonlinear elasticity phenomena during major deformation (Mooney-Rivlin model) [19, 20]. This model requires at least the application of two parameters—C_0 and C_1—characterizing the biomechanical behavior under low and high deformation, respectively, using the least-squared roots method: $\sigma=2\ (\lambda - 1/\lambda^2)\ [C_0+C_1\ (\lambda^2+2/\lambda - 3)]$, where σ is nominal stress and λ is the stretch. Each sample was then tested, obtaining two parameters per sample, C_0 and C_1.

Statistic analysis

Statistical analysis was performed with the statistic toolbox of Matlab (9.2) software (Mathworks, Inc.). The coefficient of multiple correlation (R^2) was calculated to determine goodness of a linear fit between biomechanical and density parameters. Significance of the linear fit, i.e., if the slope differs from zero (no correlation), was assessed with Fisher's F test (considered significant at $p<0.05$).

Results

Demographic characteristics of women in the study are presented in Table 1. Tissue samples were taken only from the compartment involved in POP repair. Among the 15 procedures initially conducted [10], vaginal tissues from 14 patients were analyzed for both histological and biomechanical assessments, with nine samples from the anterior vaginal wall and seven from the posterior vaginal wall (Table 2). In one case, the sample collected during surgery was not adequate to perform biomechanical testing. Most patients were postmenopausal (86.7 %), and none had received hormonal treatment in the 3 months prior to surgery.

For biomechanical analysis, uniaxial tension tests were preformed. We observed a nonlinear relationship between stress and strain (Fig. 2), confirming the hyperelastic behavior of vaginal tissue in large deformation [18]. C_0 and C_1 parameters, characterizing the mechanical behavior of vaginal tissue

Table 1 Patient characteristics

Characteristic	No.=14
Age [year (mean ± SD)]	62±11.2
BMI [kg/m^2 (mean ± SD)]	29.3±4.2
Vaginal parity [median (range)]	3 (1–8)
Menopause [*n*, (%)]	12 (85.7)
POP stage [median (range)]	3 (2–3)

SD standard deviation, *BMI* body mass index *POP* pelvic organ prolapse

Table 2 Biomechanical and biochemical measurements of vaginal tissue (n=16) derived from 14 patients undergoing POP repair. C_0 (rigidity under low deformation) and C_1 (rigidity under large deformation) values are expressed in megapascal (MPa). Collagen and elastin density values are presented in percent

Patient no.	Vaginal rigidity		Lamina propria layer			Muscularis layer		
	C_0 (MPa)	C_1 (MPa)	Collagen (%)	Elastin (%)	Elastin/collagen ($\times 10^{-2}$)	Collagen (%)	Elastin (%)	Elastin/collagen ($\times 10^{-2}$)
1 (anterior)	0.00	0.15	72.89	5.9	8.1	56.44	4.1	7.3
2 (anterior)	0.07	0.12	84.12	9	10.7	45.83	5.3	11.6
3 (anterior)	0.14	0.26	85.24	3.6	4.2	59.32	7.5	12.6
4 (anterior)	0.07	0.09	87.7	11	12.5	62.54	4.9	7.8
5 (posterior)	0.03	0.07	70.71	7.7	10.9	44.83	7.3	16.4
6 (posterior)	0.18	1.16	85.44	6.6	7.7	66.64	12.6	18.9
7 (anterior)	0.01	0.42	87.29	3.7	4.2	64.57	17.7	27.4
7 (posterior)	0.01	0.09	86.19	8.3	9.6	40.75	10	24.5
8 (posterior)	0.04	0.25	77.96	10.5	13.5	42.53	8.9	20.9
9 (anterior)	0.10	0.46	74.83	7.1	9.5	52.7	10.6	20.1
10 (posterior)	0.25	0.33	87.58	6.2	7.1	63.19	4.9	7.7
11 (anterior)	0.10	0.80	85.41	10.5	12.3	47.22	5.9	12.5
12 (posterior)	0.02	0.22	89.21	15.3	17.1	67.05	6.7	10
13 (anterior)	0.00	0.37	88.37	14.5	16.4	67.44	9.1	13.5
13 (posterior)	0.00	0.22	73.58	14.2	19.3	58.49	15.5	26.5
14 (anterior)	0.00	0.20	92.02	14.2	15.4	68.97	4.1	5.9

at low and high strains, were calculated and are given in Table 2. We also observed a great variability of response, especially in case of large deformation (C_1), which can be explained by the interindividual differences already observed in previous work [21, 22].

In parallel, we also measured the density of collagen and elastin and the ratio of elastin/collagen in the lamina propria and muscularis. In a majority of cases, we observed that a low C_0 was correlated with high elastin density in the lamina propria, and a high C_0 was always correlated with low elastin density. Using linear regression, we attempted to determine a link between histological and biomechanical properties of vaginal tissue in genital prolapse (Fig. 3). We found an inverse relationship between elastin density in the lamina propria and vaginal tissue stiffness at low deformation (C_0) (p=0.068). We also observed a statistically significant inverse relationship between C_0 and elastin/collagen ratio (p=0.048) (Fig. 3).

Nevertheless, we observed no relationship between collagen density and he C_0 or C_1 values. C_1 parameter is more difficult to correlate with histomorphometric parameters of the different layers and does not allow identifying a connective tissue fibrillar component, which could be responsible for the observed biomechanical properties at high strains. In addition, the larger dispersion of results at greater strains precludes the demonstration of a correlation between tissue composition and its mechanical features under large strains.

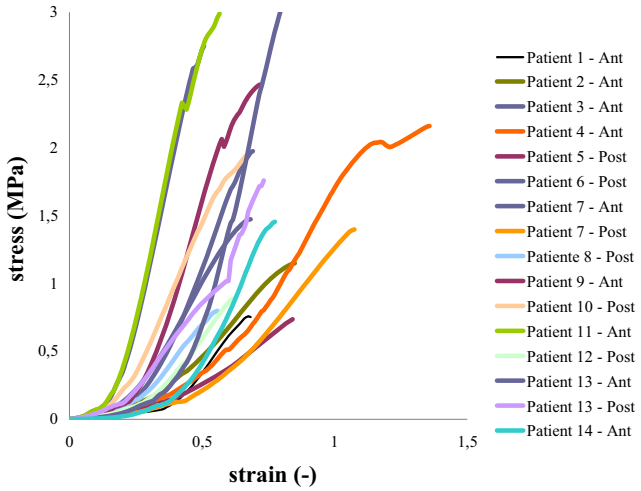

Fig. 2 Mechanical response of vaginal wall to tension loading. *Stress–strain curves* showing hyperelastic behavior of tissue and the great interindividual variability of mechanical response, especially in large deformation

Discussion

We simultaneously analyzed fibrillar tissue composition and biomechanical properties of the vaginal wall in women with POP, with the aim to assess whether a correlation exists between these properties. First, we confirmed the nonlinear

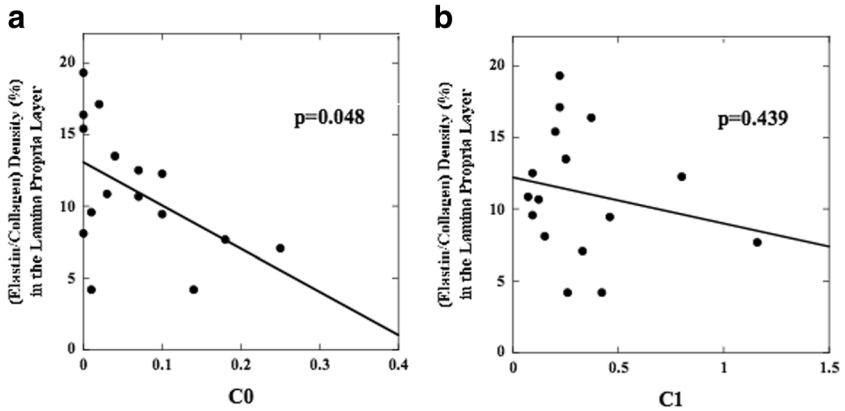

Fig. 3 Correlation between elastin/collagen density ratio in the lamina propria and vaginal rigidity under **a** low deformation (C_0) and **b** high deformation (C1) in 16 samples derived from 14 patients. In the first case, there is significant inverse relationship between elastin/collagen ratio and C_0 (p=0.048) with a coefficient of determination of R^2=0.25, whereas in the second case, the relationship is not significant (p=0.439, R^2=0.04)

relationship between stress and strain and hyperelastic behavior, finding great interindividual variability of vaginal tissue under large deformation (C_1). Using linear regression, we found an inverse relationship between the density of elastin in the lamina propria and rigidity of vaginal tissue at low deformation (C_0). We also observed a statistically significant inverse relationship between C_0 and elastin/collagen ratio in the lamina propria. Our results suggest that vaginal tissue is probably less rigid during the first part of the mechanical test, when the amount of elastin is high, corresponding to a preliminary alignment phase of elastin fibers. In case of high rigidity during the first part of the tests, elastin density of vaginal tissue is decreased, and the alignment phase of elastin fibers is shorter. Under large strains, the relationship between rigidity and tissue composition is less obvious, probably due to high variability of mechanical behavior in the second part of mechanical tests.

The relationship between elastin density and rigidity was most evident in the lamina propria compared with the muscularis. In our previous report, we also found that differences in elastin density were more pronounced in this layer [10]. Histologically, the lamina propria is a dense connective tissue predominantly composed of collagen and elastin. This fibroelastic layer may thus play an important role in the mechanical behavior of vaginal tissue.

To date, few studies have investigated the relationship between biochemical changes in vaginal tissue composition and the potential impact of these alterations on the biomechanical properties in women with POP. Previously, Zhou et al. found that vaginal tissue of women with POP was significantly stiffer than that of controls and was associated with lower content in collagen III [23]. However, it is important to note that biomechanical assessment was performed using scanning haptic microscope (SHM) instead of uniaxial tension tests. This technique relies on detecting resonance shifts through physical contact with tissue to determine local tissue stiffness. The advantage of this method is that it can be performed on small tissue samples, but the reliability of results has not been demonstrated by a comparative study of these two techniques. For biochemical assessment, collagen content was assessed using Western blot. Those authors found that collagen III expression was significantly decreased in menopausal women with POP and that collagen I expression was not significantly different between controls and cases [23]. A recent study conducted on animal model analyzed the influence of reproductive status on biochemical tissue composition and biomechanical properties of ovine vagina [24]. This method allows obtaining larger biopsies than in humans. Samples were collected from virgin, parous, and pregnant parous sheep, showing that parous sheep had the lowest elastin-tissue-associated protein (ETAP) content and highest collagen content and that pregnant sheep had the highest ETAP content. Biomechanically, pregnant ovine vagina was the most extensible but weakest tissue, whereas parous and virgin tissues were strong and elastic [24]. These observations are consistent with our findings, showing the possible relationship between changes in collagen and elastin contents and POP. In that study, total collagen content was measured by hydroxyproline assay and ratio of collagen III to collagen I was determined by sodium dodecyl sulfate polyacrylamide gel electrophores (SDS-PAGE) electrophoresis. There was no significant difference in collagen III relative levels between pregnant, virgin, and parous sheep. It is well documented that collagen I endows tissue with strength, while collagen III contributes to its elasticity [8]. Published data on collagen composition of vaginal tissue and POP are inconclusive; some authors report a reduction of collagen III in the vaginal wall of patients with POP [25–27], while others observed that it is the major fibrillar collagen of the vagina in patients with POP [28]. These conflicting results are mainly due to study heterogeneity in terms of patients, histological definition of tissues, location of tissue sampling, and methods of quantification [7]. Moreover, the

potential role of elastin in tissue stiffness was not evaluated in that study.

Other works have investigated the role of elastin fibers for maintaining integrity of the female pelvic floor [29, 30]. The lysyl oxidase (LOX) family of proteins are involved in the crosslinking of collagen and elastin monomers to form insoluble polymers. In animal studies, a large proportion of *LOXL1*-deficient mice developed weakening vaginal tissue and POP after parturition [29]. In the study of Alarab et al., expression of LOX enzymes was reduced in the anterior vaginal wall of premenopausal women with advanced POP [30]. These findings support the importance of collagen- and elastin-fiber homeostasis in pelvic floor tissue integrity.

This study has some limitations: We used histological staining to evaluate collagen content, and we did not use specific biochemical assessment of collagen subtypes. Therefore, we cannot determine the potential role of different collagen subtype variable density in biomechanical properties of vaginal tissue. We also cannot provide information on the histoarchitecture of the vagina, such as orientation of collagen or elastin fibers, diameter of fibers, and tissue quality. Another potential limitation is the relatively small number of patients and their heterogeneity in terms of age, menopausal status, and parity, which does not allow us to draw definite conclusions on the correlation between rigidity and elastin density. This underlines the difficulty, mainly for ethical reasons, of obtaining good-quality samples for both biochemical and biomechanical assessment on the same patient. For the same reasons, it is also impossible to obtain such biopsies on control patients undergoing surgery for indications other than POP. We analyzed tissue derived from both anterior and posterior vaginal tissue together. This is a potential limit because we observed previously different biomechanical properties between anterior and posterior vaginal walls in nonprolapsed patients [18]. However, the aim of this study was not to draw important conclusions about biomechanical behavior of the vaginal wall in POP patients but, rather, to establish whether or not there is a correlation between stiffness and tissue composition. Moreover, patient heterogeneity and tissue localization can explain the high interindividual variability and the wide range of biomechanical behavior observed in this study.

In conclusion, our study reveals a potential link between elastin density and vaginal wall stiffness. It underlines the interest of considering elastin density as an important factor of tissue stiffness. Therefore, this work provides new insights into the relationship between histological and biomechanical properties of the vaginal wall in women with POP. We recommend that future research protocols, designed for better understandings of the biomechanical behavior of vaginal tissue, should include quantification of collagen and elastin fibers. These novel observations are also worth considering in the development of prostheses used for POP surgery.

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Compliance with ethical standards

Conflicts of interests The authors have no conflicts of interests except Michel Cosson, who is consultant for Ethicon, AMS, Boston, Allergan, and Olympus. He is owner of patents in the field of urogynecology in development, and has one Research project funded by Ethicon.

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