Pathophysiology of pelvic organ prolapse:

Ruiz Zapata, A.M.

2015

CHAPTER 4

Changes in tissue composition of the vaginal wall of premenopausal women with prolapse

M.H. Kerkhof
A.M. Ruiz-Zapata
H. Brill
M.C.G. Bleeker
J.A.M. Belien
R. Stoop
M.N. Helder

American J Obstet Gynecol. 2014 Feb: 210(2):168.e1-9

ABSTRACT

Objective The objective of this study was to compare histological and biochemical features of the (normal) precervical anterior vaginal wall and the prolapsed anterior vaginal wall of women with pelvic organ prolapse (POP). These data were compared to tissue of the precervical anterior vaginal wall of age matched controls without POP to identify possible intrinsic and acquired effects.

Study design Biopsies were collected from the apex of the anterior vaginal cuff after hysterectomy from a control group of 13 premenopausal women undergoing hysterectomy for benign gynecological diseases, and a case group of 13 premenopausal women undergoing prolapse surgery (cystocele POP-Quantification stage ≥ 2). In women with POP an additional full-thickness vaginal wall sample was taken from the POP site during anterior colporrhaphy. Histomorphometric and biochemical analysis were performed for different components of the extracellular matrix.

Results There were no differences between case and control group in the precervical vaginal wall tissue with respect to the different components of the extracellular matrix and the biochemical parameters. However, there was a tendency toward a higher amount of collagen III and elastin, and a significant increase of smooth muscle cells and pyridinoline collagen cross-links in the POP site compared to the non-POP site of the same POP patient.

Conclusion Our findings suggest that the changes seen in connective tissue in the anterior vaginal wall of women with POP are the effect, rather than the cause, of POP.

INTRODUCTION

Pelvic organ prolapse (POP) is a global health problem, affecting adult women of all ages. It decreases their quality of life considerably[1,2]. POP is one of the most common reasons for gynecological surgery in women after the fertile period. The failure rate of surgery is relatively high: an estimated 17% of women require reoperation[2]. Despite this, little is known about the underlying pathophysiology of POP[3,4].

For decades, it has been speculated that POP occurs due to a structural defect in the vagina and its supportive tissues. Such defects could be a decrease in collagen content and quality, differences in collagen subtypes, changes in the amount and quality of elastin, and the density of smooth muscle cells (SMCs)[3,5]. Due to different biopsy sites, lack of clarity as to which layers of the vaginal wall are actually being analyzed, and very heterogeneous study populations, data are conflicting. Moreover, by comparing tissue that is prolapsed to one that is not prolapsed, it is impossible to distinguish between causes and effects of POP. In other words, it is impossible to distinguish whether the observed changes in the anterior vaginal wall are the result of an intrinsic (genetic) or an acquired (environmental) deffect.

The goal of this study was to identify the changes in the non-prolapsed anterior vaginal wall that occur in premenopausal women with POP, compared to age matched women without POP using histologically defined samples. The second goal was to determine whether the observed changes are caused by the prolapse, by comparing the histological and biochemical features of the anterior vaginal wall at the site of the POP with the same features at the non-POP site within the same patient. This approach helps us to identify possible changes in the connective tissue of the vaginal wall due to the increased pressure and stretch of the prolapsing pelvic organs on the vaginal wall. We hypothesize that the precervical anterior vaginal wall (non-POP site) of women with POP is comparable to the precervical anterior vaginal wall of controls, thus allowing us to consider this precervical tissue in POP patients as a proper control site. We expect to find differences in the histological and biochemical features at the POP site compared to the non-POP site within the same woman with prolapse. By testing the hypothesis that the changes seen in the connective tissue of the anterior vaginal wall are an acquired rather than an intrinsic effect causing POP, we hope to provide useful information that should help to develop new approaches in reconstructive pelvic surgery.

MATERIALS AND METHODS

Patient population

Upon medical ethical committee approval and acquired informed consent, biopsies were collected from 26 Caucasian premenopausal women at the Department of Obstetrics and Gynecology of the Kennemer Gasthuis, Haarlem, The Netherlands, from March 2009 through March 2011. Eligible women were divided into 2 groups: a control group of 13 women undergoing abdominal or laparoscopic hysterectomy for benign gynecological diseases with no sign of POP during gynecological examination, and a case group of 13 patients undergoing vaginal hysterectomy and reconstructive pelvic surgery of the anterior vaginal compartment because of a cystocele, POP-Quantification (POP-Q) stage ≥ 2. Signs of POP were recorded during the pelvic examination by the same urogynecologist (M.H.K.) and were described according to the International Continence Society POP-Q[6] Groups were matched for age, parity, smoking, and use of oral contraceptives. Premenopausal status was defined as having a regular period over the preceding 12 months. Women on oral contraception were asked to temporarily stop for 3 months to see whether a spontaneous regular menstrual cycle would occur. Endometrial biopsies were obtained from all women defining the stage of the menstrual cycle at time of operation. Exclusion criteria included the use of progestin-only hormone regimen, a history of pelvic surgery, pelvic malignancy or connective tissue disease affecting collagen or elastin remodeling, adhesions or scarring at the biopsy site, surgeons judgment that a biopsy may harm the patient, a history or presence of endometriosis, morbid obesity (body mass index > 35 kg/m^2), diabetes, chronic inflammatory disease, chronic infections, steroid use, and inability to provide informed consent. Standardized demographic and pertinent clinical information was recorded prospectively and stored in a dedicated database.

Tissue acquisition and preparation

The site of tissue collection was standardized because of potential differences in composition of the extracellular matrix (ECM) throughout the vagina[7]. After removal of the uterus in the controls, full-thickness samples of the anterior vaginal wall were obtained from the vaginal cuff at the anterior midline portion of the vaginal wall. In women with POP, an additional full-thickness anterior vaginal wall (midline) sample was taken from the POP site during anterior colporrhaphy (point Ba POP-Q). To minimize harm in the control subjects, only anterior vaginal wall tissue from the vaginal cuff was retrieved. The minimum size of the biopsy was 0.5 x 1.0 cm^2. All biopsies were large enough to perform (immuno-)histochemical as well as biochemical analysis. Biopsies were immediately passed off the surgical field and divided into 2 parts. For biochemical analysis, the biopsies were washed in phosphate-buffered saline solution and stored at –80^{o}C until further processing. For analysis by microscopy, the collected tissue was fixed in neutral-buffered formalin for 24 hours, dehydrated, and processed into paraffin blocks. Serial 3-mm sections

were cut from the paraffin blocks, mounted on slides, and stored until further processing. All measurements were performed without knowledge of sample identity.

(Immuno-)histochemistry

Hematoxylin-eosin staining was performed to verify that the collected samples represented the vaginal wall containing the epithelial layer, connective tissue, and muscularis. To detect the amount of elastin in the connective tissue, the Lawson elastic van Gieson stain kit, purchased from Klinipath (Duiven, The Netherlands), was used. As positive controls, tissues from lung, appendix, and liver were used. Monoclonal antibodies against desmin and CD31 were used to identify SMCs and blood vessels, respectively. Sections were also immunostained for the ECM proteins collagen I, III, and IV (Table 1).

Table 1. Antibodies used voor immuno histochemical analysis

Antibody	Company	Preparation	Titre	Positive control
Mouse anti human monoclonal antibody Desmin	DAKO, Copenhagen, Denmark	Peroxidase block	Ready to use anti-serum	Appendix
Mouse anti human monoclonal antibody CD 31	DAKO, Copenhagen, Denmark	Peroxidase block	Ready to use anti-serum	Tonsil
Rabbit anti human Collagen type I	ABNOVA, Heidelberg, Germany	Citrate pH 6.0	1:100	Kidney
Rabbit anti human Collagen type III	ACRIS, Herford, Germany	Pepsin 0.1%	1:1000	Skin
Mouse anti human IgG1 Collagen type IV	DAKO, Copenhagen, Denmark	Citrate pH 6.0	1:150	Skin

Morphometric analysis

To quantify SMCs, elastin, and microvessels we performed a morphometric analysis. Complete slides were scanned with a digital Mirax slide scanner system (3DHistech Ltd, Budapest, Hungary) equipped with a x20 objective with a numerical aperture of 0.75 and a Sony DFW-X710 Fire Wire 1/3, type progressive scan CCD camera pixel size 4.65 x 4.65 µm (3DHistech Ltd) (pixel size 4.65 x 4.65 µm). The actual scan resolution of all pictures at x20 was 0.23 µm. After scanning, representative areas of both the muscularis layer and the lamina propria layer between 0.55-0.64 mm^2 were randomly annotated by hand using Panoramic Viewer software (3DHistech Ltd). Resulting annotations were exported in the tagged image file format (tiff) image format. A computerized morphometric analysis of the desmin-, elastic van Giesone, and CD31-stained slides was executed, using Image J 1.44p software (http://rsbweb.nih.gov/ij/) with a modified macro from Hadi et al[8]. Analysis was performed for the lamina propria and muscularis layer separately. The amount of SMC was expressed as the total area of desmin-positive cells vs the total tissue area. The amount of elastin was expressed by total area of fibers vs total tissue area. The CD31 staining was used to quantify the amount of microvessels per area as well as the amount of nuclei per area.

Collagen staining was quantified in a blinded fashion using a 4-grade scoring system (absent, light, moderate, or strong) by 2 independent pathologists (Appendix,

Supplementary Figure). Slides were scored using a standard light microscopy technique with magnification of x100. An interrater reliability analysis using the Kappa statistic was performed to determine consistency between the 2 pathologists and was excellent (Kappa 0.97; P <.001).

Biochemical analysis

The epithelial layer of the vaginal wall biopsies was removed under a dissecting microscope. The remaining tissues were freeze-dried and weighed. Samples were hydrolyzed with 6 mol/L hydrogen chloride at 110°C for 20 hours. The amount of hydroxyproline and proline was determined by reverse-phase highperformance liquid chromatography (HPLC) of 9-fluorenylmethyl chloroformate-(Fluka, Buchs, Switzerland) derivatized amino acids, as described by Bank et al.[9] Collagen content was calculated assuming 300 residues hydroxyproline per triple helix and a molecular weight of 300,000 g/mol. The hydroxyproline/proline ratio was determined by dividing the hydroxyproline content per mmol/L by proline content per mmol/L, resulting in a dimensionless ratio. For the determination of the collagen cross-links lysylpyridinoline and hydroxylysylpyridinoline, acid-hydrolyzed samples were diluted to 50% acetic acid and injected on an HPLCsystem equipped with online sample purification on CC31 cellulose using a Prospekt solid-phase extractor (Separations, Jasco Benelux BV, IJsselstein, The Netherlands). The retained cross-links were eluted from the CC31 material and online chromatographed on a cation exchange column (Whatman Partisil SCX; Fisher Scientific, Waltham, MA). Eluting cross-links were detected by a Jasco fluorometer (model FP-920; Separations). The PYD/DPD HPLC calibrator (Metra, Palo Alto, CA) was used as standard. Values are expressed as total amount of residues per collagen molecule.

Statistics

The primary outcome of the study was to detect a difference in the amount of total collagen (hydroxyproline) or the amount of collagen type III in patients with and without POP. Secondary outcome measurements included a change in the amount of collagen I and IV, the amount of elastin and SMC, the concentration of non-collageneous proteins, and the maturity of the cross-links within the collagen fibrils in patients with and without POP. Based on previous studies[5,10], 10 women were required in the POP and in the control group to detect a difference of at least 10% in the group-specific amounts of total collagen and collagen type III for a power of 80% and a .05 significance level, using a 1-way analysis of variance. The final sample size was increased with at least 10% due to the use of nonparametric tests. All statistical calculations were performed using statistical software (SPSS 19.0; IBM Corp, Armonk, NY). Differences between continuous variables were identified using the Mann-Whitney *U* test and differences between categorical variables were identified using Pearson X^2 test, Fisher exact test, or likelihood ratio according to the expected cell size and number of degree of freedom. Comparison on paired samples was performed by using Wilcoxon signed ranks test. Results are expressed as mean ± SD for

continuous variables and as median and interquartile range for the ordinal variables. All statistical tests were 2-sided and differences were considered statistically significant when *P* value was <.05.

RESULTS

Demographics

In this study, patient selection and matching were very strict (Table 2). Furthermore, there was no difference in stage of menstrual cycle or in risk factors for POP such as heavy lifting, chronic obstructive pulmonary disease, or mean weight of first child and largest child between the groups. Stress urinary incontinence was more common in the POP group (P <.004) (Table 2). In the POP group the median stage of cystocele was 3 (range, 2-3). Six women had a cystocele stage II, defined as a mild POP. Seven women had severe POP with a cystocele POP-Q stage 3. The median stage of prolapse in the apical compartment and posterior compartment was 0 (range, 0-1) and 1 (range, 0-3), respectively. Within the control group 1 sample was lost before immunohistochemical analysis was performed, therefore the data of 12 controls were analyzed.

Table 2. Clinical characteristics

Characteristics	Control (n=12)	Case (n=13)	*P* value
Age, y[a]	44.5 ± 5.0	42.9 ± 6.0	.512[d]
BMI, kg/m^2[a]	24.5 ± 1.7	27.2 ± 6.0	.574[d]
Parity, births[b]	2.2 (1-3)	2.2 (1-4)	.852[d]
Vaginal delivery[b]	2.2 (1-3)	2.2 (1-4)	.820[d]
Assisted vaginal delivery[c]	4 (36)	4 (31)	1.000[e]
Smoking status[c]			
Never smoked	3 (25)	4 (31)	1.000[e]
Current	4 (33)	3 (23)	.673[e]
Previous	5 (42)	6 (46)	.821[f]
Oral contraception use[c]			
Until surgery	4 (33)	4 (31)	1.000[e]
Never used	1 (8)	1 (8)	1.000[e]
Stress urinary incontinence[c]	1(8)	9 (69)	.004[e]
POP-Q stage of prolapse[b]			
Anterior	0 (0)	3 (2-3)	
Central	0 (0)	0 (0-1)	
Posterior	0 (0)	I (0-3)	

BMI, body mass index; POP-Q, PO-Quantification. [a] Data presented as mean ± SD; [b] Data presented as median (interquartile range); [c] Data presented as number of patients (%); [d] Fisher exact test; [f] X^2 test.

(Immuno-)histochemical analysis

Examination of hematoxylin-eosine stained vaginal biopsies using standard light microscopy techniques confirmed that all layers were present in each biopsy (Figure 1). There was no evidence of inflammation in the subepithelial stroma.

Comparing the lamina propria and muscularis layer of the non-POP site of the POP group with the control group, no statistically significant differences were found with respect to the percentage of SMC, microvessel density, and cellularity (Table 3). Also no statistically significant differences were found in the ECM proteins evaluated: elastin and collagens I, III, and IV. Collagen IV was only detected in the basement membrane in all samples. Precervical tissue of women with POP and precervical tissue of controls seem to maintain a comparable amount and distribution of the different components of the ECM. A comparison between the POP site with the non-POP site within the same patient showed a significantly higher percentage of SMCs in the muscularis layer of the POP site (26.85% vs 19.47%, P <.05) (Table 3 and Figure 2). To determine whether the stage of POP influences the different components of the ECM, the case group was divided into a mild and severe POP group. Although no differences were found, the amount of desmin at the POP site in the severe POP group was higher compared to the mild group (mean 26.13% vs 23.44%, respectively). A similar pattern was seen for the amount of elastin with a mean 2.27% in the mild and 3.85% in the severe group. No differences were detected in the amount of collagen type I and III between the POP site and the non-POP site within the same patient. There was a tendency toward an increase of collagen III in the lamina propria and muscularis layer of the POP site compared to the non-POP site of the same patient. This pattern was more pronounced in severe POP compared to mild POP patients.

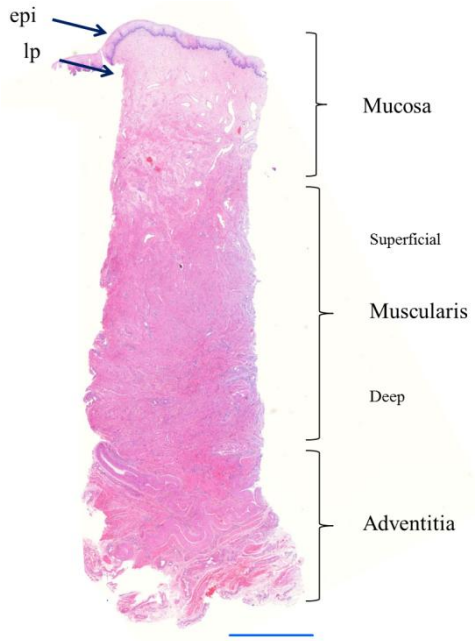


Figure 1. Histological features of anterior vaginal wall. Biopsies from anterior vaginal wall are full-thickness biopsy specimens represented by 4 layers. Vaginal mucosa consisted of squamous epithelium (epi) and underlying lamina propria (lp). Remaining vaginal wall, approximately 70-75% of total vaginal wall thickness consisted of muscularis and adventitia. Actual scan resolution (effective pixel size in sample plane) at x20 is 0.23µm. Blue bar represents 200µm.

Table 3. (Immuno-)histochemical analysis of anterior vaginal wall

Specimen muscularis layer	A Control	B Case non-POP site	C Case POP site	A-B *P* value[c]	B-C *P* value[d]
Desmin staining[a]					
Mean percent smooth muscle cells	20.99 (7.34)	19.47 (7.73)	26.85 (6.13)	.553	.016[e]
CD31 staining[a]					
N nuclei	922 (233)	955 (554)	925 (374)	.908	.583
N microvessels	51 (14)	54 (40)	47 (12)	.402	.844
Elastic van Gieson staining[a]					
Mean percent elastin	2.17 (1.12)	2.44 (1.51)	3.04 (2.97)	.458	.859
Collagen I[b]					
Connective tissue	4 (4 – 4)	4 (4 – 4)	4 (4 – 4)	1.000	1.000
Muscularis	2 (1.75 – 2)	2 (2 – 2)	2 (2 – 2)	.486	.157
Collagen III[b]					
Connective tissue	2 (2 – 2)	2 (2 – 2)	2 (2 – 2)	.254	.180
Muscularis	1 (1 – 1)	1 (1 – 1)	1 (1 – 1)	.204	.546

(Immuno-)histochemical analysis comparing different components of extracellular matrix in muscularis layer of precervical anterior vaginal wall (non-POP site) and prolapsed anterior vaginal wall (POP site) in women with POP and controls. A-B: precervical tissue of women with POP and precervical tissue of controls seem to maintain comparable amount and distribution of different components of the extracellular matrix. B-C: comparison between POP site with non-POP site within the same patient, showed a significantly higher percentage of smooth muscle cell in muscularis layer of the POP site. *POP*, pelvic organ prolapse. [a] Data presented as mean (SD); [b] Data presented as median (interquartile range); [c] Mann-Whitney test; [d] Wilcoxon signed rank test; [e] $P < .05$.

Biochemical analysis

Biochemical analysis showed that collagen content (expressed as collagen per dry weight) did not differ between the different harvesting sites within POP patients or between POP patients and healthy controls. This was confirmed by the observation that the hydroxyproline per proline ratio, which represents the amount of collagen per total protein, did not change either. However, a significant increase in collagen cross-linking (expressed as total amount of pyridinolines per collagen molecule) in the prolapsed tissue compared to nonprolapsed tissue within the same women was found ($P < .047$). Such differences were not seen between non-POP site and the age matched healthy controls (Table 4).

Table 4. Biochemical analysis of anterior vaginal wall

Variable	A Control	B Case non-POP site	C Case POP site	A-B *P* value[a]	B-C *P* value[b]
Percent of collagen compared to dry weight	58 (8)	46 (10)	46 (8)	.068	.972
Hydroxyproline/proline ratio	0.57 (0.28)	0.62 (0.19)	0.59 (0.18)	.136	.117
(HP + LP)/triple helix	0.29 (0.09)	0.33 (0.08)	0.38 (0.06)	.339	.047[c]

Biochemical analysis comparing controls and non-POP site with POP site within same women with POP. A-B: comparison control group vs case group non-POP site. B-C: comparison case group non-POP site vs case group POP site. All values are presented as mean (SD). *HP* hydroxylysinepyridinoline; *LP* lysylpyridinoline; *POP*, pelvic organ prolapse. [a] Mann-Whitney test; [b] Wilcoxon signed rank test; [c] $P < .05$.

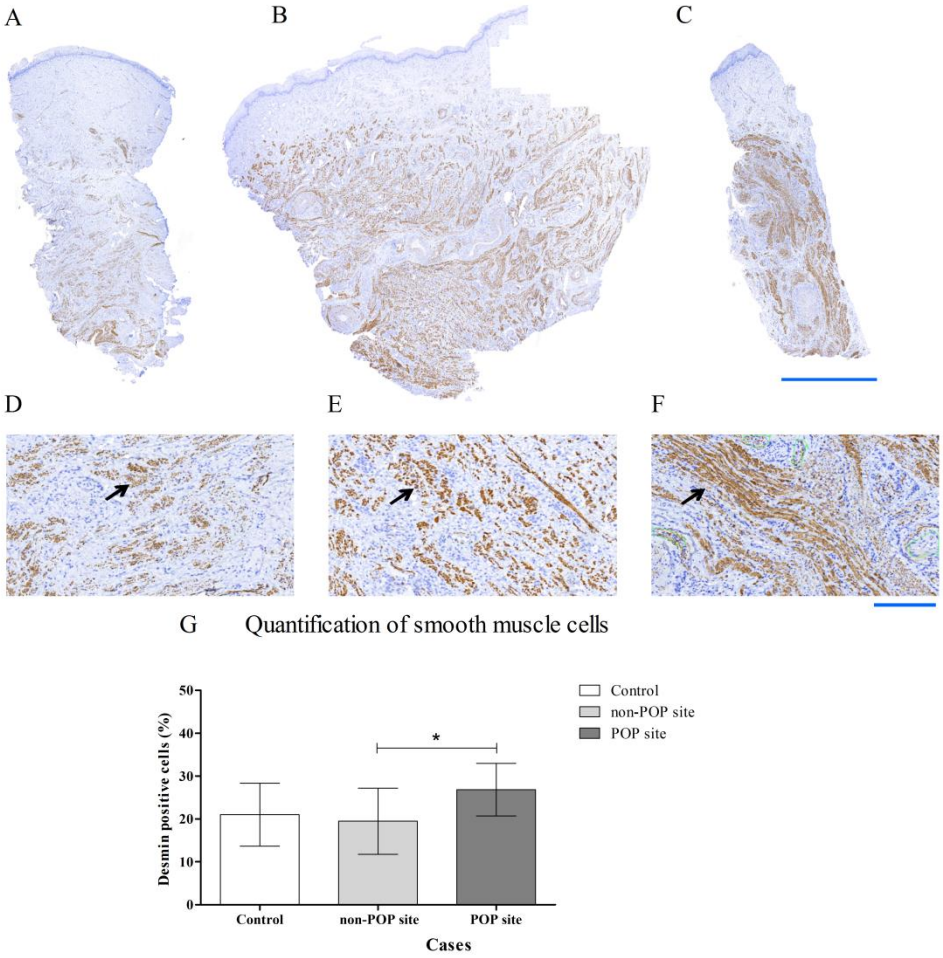


Figure 2. SMCs in anterior vaginal wall. Full-thickness anterior vaginal wall biopsy of **A**, control; **B**, non-pelvic organ prolapse (non-POP) site; and **C**, POP site of POP patient; with **D** to **F**, corresponding magnification of muscularis layer. Desmin-positive cells as marker for smooth muscle cells (SMCs) are brown (*arrows*). **C** and **F**, in women with POP SMCs are more tightly packed, better organized in fibers, and more oriented in longitudinal direction at prolapsed site compared to **B** and **E**, precervical (non-POP site) anterior vaginal wall of same patient. **A** and **D**, in controls, SMCs are fewer in number and poorly organized. **G**, morphometric analysis of amount of SMCs shows increased amount of desmin-positive cells at prolapse site (**P* = .016). Actual scan resolution (effective pixel size in sample plane) at x20 is 0.23µm. Blue bar represents 200µm.

COMMENT

Our results show that the non-POP tissue in the vaginal wall of POP patients does not differ in any of the parameters evaluated from the same tissue in healthy controls. However, there were marked differences observed between the prolapsed tissue of a POP patient, compared to nonprolapsed tissue of the same patient. There was a significant increase in mature pyridinoline cross-links in collagen molecules, and in the number of SMCs in the muscularis layer of the anterior vaginal wall. In addition, there was a tendency toward a higher amount of collagen III and elastin. No differences were found in the amount of collagens I and IV.

An increase in mature collagen cross-links has also been reported by Jackson *et al.*[11] Pyridinoline cross-links are the major type of mature cross-links in collagen in the fascia of the anterior vaginal wall[3,4]. An increase in cross-link density will result in slower metabolizing collagens, making them susceptible to nonenzymatic glycation, producing advanced glycation end products. Some of those advanced glycation end products, such as pentosidine, are found to be increased in women with POP and inhibit the turnover of collagen resulting in a more glycated collagen, which is brittle and susceptible for rupture[11]. Furthermore, an increased cross-linked collagen also makes tissues stiffer and the anterior vaginal wall from premenopausal women with POP has been reported to be stiffer than tissues from their healthy counterparts[12].

The tendency we found toward a higher amount of collagen III relative to the stronger type I collagen in the anterior vaginal wall of patients with POP is thought to result in thinner collagen fibers with correspondingly diminished biomechanical strength. The increase in collagen III expression in patients with POP, in combination with an increased activation of matrix metalloproteinase-9 (not analyzed in this study), is typical of tissues that are remodeling after injury or tissues that are remodeling to adapt to a progressively increasing mechanical load[5,13,14].

We demonstrate that the amount of SMCs is significantly increased in the prolapsed site and not in the unaffected site of the same patient. This increase in SMCs in the prolapsed tissue may indicate an accommodation of the connective tissue to the increased permanent mechanical load and stretch on the vaginal wall tissue caused by the prolapse. Mechanical forces induce a variety of responses in SMCs depending on the organ, the animal species, the nature of the mechanical forces, and the extent of the stretch[15]. In uterine smooth muscle, stretch is the primary stimulus for muscle hypertrophy and increase in force-generating capacity[16]. In bladder and intestinal smooth muscle, stretch induces an initial response that consists of hyperplasia and hypertrophy, but the force-generating capacity decreases[17]. Data on the response of the vaginal wall ECM to increased mechanical load or to factors that modify this response are lacking. Recent studies focus on the effect of decrease in mechanical load due to the use of synthetic meshes. Liang *et al.*[18] showed that vaginal wall tissue degenerates as a result of the decrease in loading, also known as stress shielding. Tissue degeneration that occurs in this context has been shown to

be the result of a deregulation of key structural proteins such as collagens, elastin, and glycosaminoglycans, as well as SMCs[19,20]. A decrease in vaginal SMCs following vaginal implantation with a polymeric mesh is associated with a loss of smooth muscle contractility[21].

It is clear that all tissues throughout the body depend on a certain amount of load to maintain their structure. Loss of load as well as increase of load leads to changes in the cellular and molecular responses. An increase of the number of SMCs at the site of the POP, together with an increase in pyridinolines, suggests that the tissue is trying to compensate for reduced tissue strength. After injury to levator ani muscles and/or the connective tissue attachments at childbirth, transient increases in intra-abdominal pressure are directly transmitted to the vaginal wall and its increased mechanical load to the vagina results in tissue stretch[22]. Mechanical loading has been shown to change the remodeling of tissues by inducing matrix metalloproteinases produced by the vaginal fibroblasts. Indeed vaginal connective tissue fibroblasts are mechano-sensitive with increased enzymatic activity[22,24].

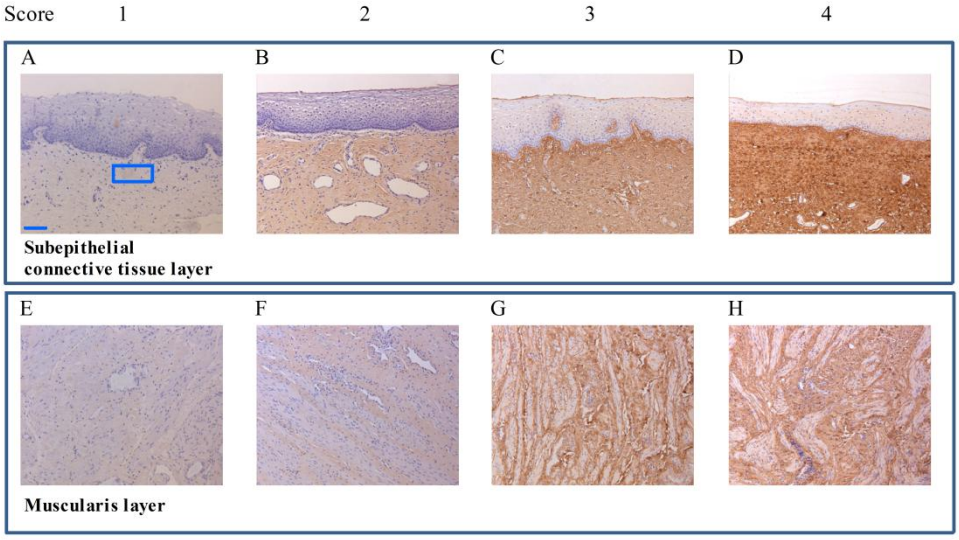
In most cases, the vaginal tissues are able to recover from those rather acute events by proper tissue remodeling. However, when repair mechanisms fail, these patients enter a chronic phase. In this phase, the vaginal wall tries to cope with the increasing biomechanical pressure of the prolapsed tissues and organs. This accommodation may be induced by the fibroblasts' reaction to increasing continuous mechanical stretch. The changes seen in the connective tissues in the anterior vaginal wall more likely reflect secondary effects of the prolapse on the tissue rather than a host predisposition to prolapse.

Due to our very strict patient selection with only Caucasian, premenopausal women and tightly matched controls, our study size was limited to the minimum according to the power calculation. The fact that we find significant differences underscores that our observations may pinpoint a general phenomenon occurring in POP. Also, one has to realize that this study was designed to examine the connective tissue of the anterior vaginal wall only. As the composition of the connective tissue of the vagina may vary throughout the vagina our data cannot be compared with connective tissue of the posterior vaginal wall nor with ligaments or other tissues responsible for the level-I support.

Our current findings cannot resolve the important questions on the etiology and or pathophysiology of POP.With our current approach with the women with POP being her own control we find that the changes in connective tissue of the prolapsed vaginal wall are an acquired effect, rather than an intrinsic defect in the connective tissue. Further studies investigating the effect of increased mechanical loading and stretch on vaginal tissue at different anatomic levels of support as well as the molecular mechanism resulting in the changes seen in the different components of the ECM are needed for further understanding of POP. This will be of utmost importance in the development of new treatment strategies.

ACKNOWLEDGMENTS: The authors thank Ron Glandorf, MSc (Kennemer Gasthuis, Haarlem, The Netherlands) for his expertise in the statistical analysis; Jessica Snabel, technician (TNO Health and Prevention, Leiden, The Netherlands) for conducting the biochemical analysis; Behrouz Zandieh Doulabi, PhD (Department of Oral Cell Biology, ACTA University of Amsterdam and VU University, Research Institute MOVE, Amsterdam, The Netherlands) for his assistance in interpretation of the data; and Hans A. M. Brölmann, MD, PhD (Department of Obstetrics and Gynecology, VU University Medical Center, Amsterdam, The Netherlands) for his critical review of the article.

SUPPLEMENTARY FIGURE



Scoring system used for immunihistochemical stainings for collagen. Scoring system with 4 grades was used to evaluate collagen type-1 and -3 stains (positive labeling brown). Increasing amount of collagen in **A** to **D**, subepithelial connective tissue layer and in **E** to **H**, muscularis layer ranging from 1 (absent) to 4 (strong). Magnification x10 objective. Blue bar represents 100µm.

REFERENCES

1. Jelovsek JE, Maher C, Barber MD. Pelvic organ prolapse. Lancet 2007;369:1027-38.
2. Denman MA, Gregory WT, Boyles SH, Smith V, Edwards SR, Clark AL. Reoperation 10 years after surgically managed pelvic organ prolapse and urinary stress incontinence. Am J Obstet Gynecol 2008;198:555.e1-5.
3. Kerkhof MH, Hendriks L, Brolmann HAM. Changes in connective tissue in patients with pelvic organ prolapseea review of the current literature. Int Urogynecol J Pelvic Floor Dysfunct 2009;20:461-74.
4. Chen B, Yeh J. Alterations in connective tissue metabolism in stress incontinence and prolapse. J Urol 2011;186:1768-72.
5. Moalli PA, Shand SH, Zyczynski HM, Gordy SC, Meyn LA. Remodeling of vaginal connective tissue in patients with prolapse. Obstet Gynecol 2005;106:953-63.
6. Bump RC, Mattiasson A, Bo K, et al. The standardization of terminology of female pelvic organ prolapse and pelvic floor dysfunction. Am J Obstet Gynecol 1996;175:10-7.
7. Jean-Charles C, Rubod C, Brieu M, Boukerrou M, Fasel J, Cosson M. Biomechanical properties of prolapsed or non-prolapsed vaginal tissue: impact on genital prolapse surgery. Int Urogynecol J 2010;21:1535-8.
8. Hadi AM, Mouchaers KTB, Schalij I, *et al.* Rapid quantification of myocardial fibrosis: a new macro-based automated analysis. Cell Oncol (Dordr) 2011;34:343-54.

9. Bank RA, Beekman B, Verzijl N, de Roos JA, Sakkee AN, TeKoppele JM. Sensitive fluorimetric quantitation of pyridinium and pentosidine crosslinks in biological samples in a single high-performance liquid chromatographic run. J Chromatogr B Biomed Sci Appl 1997;703:37-44.
10. Moalli PA, Talarico LC, Sung VW, et al. Impact of menopause on collagen subtypes in the arcus tendineous fasciae pelvis.Am J Obstet Gynecol 2004;190:620-7.
11. Jackson SR, Avery NC, Tarlton JF, Eckford SD, Abrams P, Bailey AJ. Changes in metabolism of collagen in genitourinary prolapse. Lancet 1996;347:1658-61.
12. Feola A, Duerr R, Moalli P, Abramowitch S. Changes in the rheological behavior of the vagina in women with pelvic organ prolapse. Int Urogynecol J 2013;24:1221-7.
13. Dviri M, Leron E, Dreiher J, Mazor M, Shaco-Levy R. Increasedmatrixmetalloproteinases-1,-9 in the uterosacral ligaments and vaginal tissue from women with pelvic organ prolapse. Eur J Obstet Gynecol Reprod Biol 2011; 156:113-7.
14. Goepel C. Differential elastin and tenascin immunolabeling in the uterosacral ligaments in postmenopausal women with and without pelvic organ prolapse. Acta Histochem 2008;110:204-9.
15. Boreham MK, Wai CY, Miller RT, Schaffer JI, Word RA. Morphometric analysis of smooth muscle in the anterior vaginal wall of women with pelvic organ prolapse. Am J Obstet Gynecol 2002;187:56-63.
16. Word RA, Stull JT, Casey ML, Kamm KE. Contractile elements and myosin light chain phosphorylation in myometrial tissue from nonpregnant and pregnant women. J Clin Invest 1993;92:29-37.
17. Gabella G. Hypertrophy of visceral smooth muscle. Anat Embryol (Berl) 1990;182:409-24.
18. Liang R, Abramowitch S, Knight K, et al. Vaginal degeneration following implantation of synthetic mesh with increased stiffness. BJOG 2013;120:233-43.
19. Gamble JG, Edwards CC, Max SR. Enzymatic adaptation in ligaments during immobilization. Am J Sports Med 1984;12:221-8.
20. Amiel D, Woo SL, Harwood FL, Akeson WH. The effect of immobilization on collagen turnover in connective tissue: a biochemicalbiomechanical correlation. Acta Orthop Scand 1982;53:325-32.
21. Feola A, Abromowitch S, Jallah Z, et al. Deterioration in biomechanical properties in the vagina following implantation of highstiffness prolapse mesh. BJOG 2013;120:224-32.
22. O'Dell KK, Morse AN, Crawford SL, Howard A. Vaginal pressure during lifting, floor exercises, jogging, and use of hydraulic exercise machines. Int Urogynecol J Pelvic Floor Dysfunct 2007;18:1481-9.
23. Zong W, Jallah ZC, Stein SE, Abramowitch SD, Moalli PA. Repetitive mechanical stretch increases extracellular collagenase activity in vaginal fibroblasts. Female Pelvic Med Reconstr Surg 2010;16:257-62.
24. Ruiz-Zapata AM, Kerkhof MH, Zandieh-Doulabi B, Brölmann HA, Smit TH, Helder MN. Fibroblasts from women with pelvic organ prolapse show differential mechanoresponses depending on surface substrates. Int Urogynecol J 2013;24:567-75.