**Identifying distinct nanoscopic features of native collagen fibrils towards early diagnosis
of pelvic organ prolapse**

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Abstract

Pelvic organ prolapse (POP) is characterized by weakening of the connective tissues and loss of support for the pelvic organs. Collagen is the predominant, load-bearing protein within pelvic floor connective tissues. In this study, we examined the nanoscopic structures and biomechanics of native collagen fibrils in surgical, vaginal wall connective tissues from healthy women and POP patients. Compared to controls, collagen fibrils in POP samples were bulkier, more uneven in width and stiffer with aberrant D-period. Additionally, the ratio of collagen I (COLI) and collagen III (COLIII) is approximately doubled in POP with a concomitant reduction of the amount of total collagen. Thus, POP is characterized by abnormal biochemical composition and biophysical characteristics of collagen fibrils that form a loose and fragile fiber network accountable for the weak load-bearing capability. The study identifies nanoscale alterations in collagen as diagnostic markers that could enable pre-symptomatic or early diagnosis of POP.

Keywords:

Collagen fibril, pelvic organ prolapse, biomechanics, D-period.

Introduction

Pelvic organ prolapse (POP) is a debilitating condition that affects millions of women and compromises their quality of life. In this disorder, pelvic organs become unsupported and protrude through the vaginal inlet because of inherent abnormalities of the supporting endopelvic connective tissues, with a concomitant decrease in their biomechanical strength. The etiology of POP remains obscure due to its multifactorial nature. However, one purported cause is defects in supportive structures of the pelvic organs. The connective tissue at the vaginal wall contains fibrous collagen, elastin and non-fibrous extracellular components, such as smooth muscle, fibroblasts and proteoglycans. Collagen is the main supportive protein within the connective tissue. Structural changes of collagen fibers, such as altered dimension and alignment, are frequently accompanied by mechanical alterations resulting in many diseases and disorders, such as osteogenesis imperfecta [1], breast cancer [2-4], stress urinary incontinence and POP [5, 6]. The biomechanical strength of collagen fibers is attributed to the hierarchical organization of collagen. Microfibrils consisting of collagen triple helix molecules pack in a staggered fashion to form fibrils with a periodicity of ~67 nm, called D-period. Individual fibrils further form interdigitated networks of collagen fibers. The pathological changes in POP may start at the level of molecular collagen, and elevate to higher organizational levels of the architecture of supportive structures to cause progressive and irreversible structural and functional defects.

Many researchers linked abnormal collagen biosynthesis, deposition, assembly and degradation to weakened pelvic floor supportive tissue structure leading to prolapse [6, 7-12]. Collagen I (COLI) and collagen III (COLIII) are the predominant types of collagen in the connective tissue. Typically, fibrillar COLI offers high tensile strength to tissues. COLIII is commonly found alongside COLI in tissues that require increased flexibility and distension and

that are subject to periodic stress, for instance, in vagina, skin and intestines [13-16]. Aberrant COLI/COLIII ratios were found in connective tissue-related disorders such as dilated cardiomyopathy, inguinal hernia, POP, dermal fibrotic disease, and keratoconus cornea [13-15]. However, contradictory evidences associated with mechanically weak vs. strong tissues are frequently presented in the literature. Such a controversy may be due to the tendency to correlate unjustifiably the biochemical analysis of molecular collagen with the mechanical properties of collagen network derived at the bulk tissue level.

Methods such as ultrasound or magnetic resonance elasticity imaging [17-20] and methods using miniature puller or indentation systems [21-23] evaluate the macroscopic biomechanics of bulk tissues. In these cases, any alterations in the biomechanical properties are only evident at advanced stages of the disorder/disease. Additionally, variability in tissue biopsy results in specimens with heterogeneous structural composition. Thus, the characterization of tissue elasticity in bulk specimens is uncoupled from the biochemical identity and integrity of tissue constituents - the intrinsic determinants of tissue biomechanics. Furthermore, the sensitivity of bulk measurements is too low to detect subtle changes at the cellular and molecular levels - the scale at which biological processes associated with the onset of POP may initiate. Atomic force microscopy (AFM) is a powerful technique that allows simultaneous nano- to micro-scale imaging and evaluation of nanoscale elasticity of a material. Thus, an AFM study can potentially signal initial changes of the connective tissue that cannot be assessed by the current diagnostic methods. AFM studies reporting ultrastructural analysis of collagen in relatively stable, dense connective tissues, such as bone, tendon and skin, are common; yet similar studies on loose connective tissues, e.g. the vaginal wall tissues, are rare owing to the challenges in handling and examining such tissues. Our previous work established a method for *in situ* AFM examination of

the connection between collagen ultrastructure and mechanical property in fresh connective tissues [24]. The method was sensitive enough to discern the subtle differences in the morphology and elasticity profiles of collagen in vaginal wall connective tissues from pre- and post-menopausal women. A significant merit of the approach is the ability to directly visualize and quantify the nanoscopic to microscopic variations in the collagen fibrils in its natural context. In this study, we applied the method to unravel the interplay between identified nanostructural defects and varied biochemical composition in collagen fibrils and fiber network that provokes the biomechanical failure in tissues under the pathological condition of POP. The on-site nanoscopic analysis of collagen in its native tissue environment sheds light on how COLI and COLIII impact the biomechanics of collagen in multi-scale of its hierarchical organization. Thus, it clarifies the controversial results obtained from bulk measurements of POP and non-POP tissues.

Methods

Handling of Clinical Tissue Samples

Vaginal wall connective tissues from four healthy pre-menopausal (pre-M) women (ages 25-41), five healthy post-menopausal (post-M) women (ages 58-73), and five POP women (post-M, ages 51-73) were obtained with their consent following the approved IRB protocols at Rush University Medical Center (ORA # 08081108) and University of Illinois at Chicago (IRB protocol #2011-1025). After the blood was removed from the biopsy sample, the bulk tissue piece was cooled to 0 °C, and sequentially sectioned by a CM1850 Cryostat Ultratome (Leica Microsystems Inc., Buffalo Grove, IL) at a thickness of 5 μ m or 10 μ m. The tissue slices were naturally spread and settled on cover slides in chilled RPMI medium. The sequential thin

sectioning of the tissue specimen allowed for adjacent slices to be nearly identical in all aspects. As shown in Figure 1, among three slices of tissues from sequential thin sectioning, the first and third slices of the tissue were fixed and stained by Gomori trichrome to identify collagen (blue) from other constituents in the tissue and to verify the region of interest, whereas the fresh tissue slice in between was used in AFM studies at the identified collagen-rich region.

Gomori staining and imaging

Gomori trichrome staining was carried out to distinguish collagen from other components of a tissue sample according to manufacturer's protocol. In brief, the tissue samples on cover slides were briefly rinsed with 1× PBS at pH 7.4; they were fixed for 15 min. with 4% paraformaldehyde in PBS at pH 7.2 at room temperature, followed by immersing in Bouin's solution at 56 °C for 1 h. The slides were thoroughly rinsed with DI water prior to applying the trichrome stain for 10 min., then rinsed in acid-alcohol solution for 5 s and air-dried. The slides were then preserved by progressive rinsing with 95% alcohol (5 min., twice) and xylene (10 min.) and mounted on a coverslip using permount medium. Optical images were collected by using a Nikon TE-U 2000 microscope (Nikon Instruments Inc., Melville, NY).

Immunofluorescent staining

Antibodies against COLI and COLIII were purchased from Abcam (Cambridge, MA). The tissue samples were fixed with ice cold 4% paraformaldehyde in PBS, pH 7.4 for 15 min. at room temperature, followed by rinsing and blocking with freshly prepared 1% BSA-PBS for 30 min. The primary antibodies were diluted as recommended by the manufacturer (1:100 for COLI and 1:400 for COLIII), and were incubated for 1 h in a humidified chamber. The secondary

antibodies, purchased from Invitrogen (Carlsbad, CA), were used at a dilution of 1:200 and incubated for 1 h in a dark, humidified chamber. The slides were then subject to fluorescent imaging using a Nikon TE-U 2000 microscope. The relative amounts of COLI and COLIII in the connective tissues were analyzed by ImageJ software (National Institute of Health, Bethesda, Maryland).

AFM imaging and elasticity measurement

AFM studies of collagen structure and elasticity in fresh, unfixed tissue samples were carried out in PBS (pH 7.4) medium using a multimode Nanoscope IIIa AFM (Veeco Metrology, Santa Barbara, CA), equipped with a J-scanner. The measurements were conducted at the collagen-rich sub-mucosa region. The triangular Si₃N₄ probes (OTR4 probes from Veeco Metrology) at a thermal resonance frequency of 8-10 kHz were used. The spring constant of the cantilevers was 0.06 ± 0.01 N/m, as calibrated using reference cantilevers with known spring constants [25-27]. Guided by Gomori staining on the adjacent tissue slices (Figure. 1), an AFM tip was positioned at a desired region for high-resolution imaging and force mapping at the same area. Individual collagen fibrils, with characteristic D-period, were identified on the high-resolution image (512 pixels per line), whereas the elasticity was derived from the corresponding force map (32 pixels per line), in which a force-distance curve was collected at each pixel, and the Young's modulus, E, was derived from the force-indentation (F- δ) curve taking into account the geometry of the probe and the fibril dimension [24, 25,28-30]. Note that the E-value is a measure of the level of fibril deformation against a substrate in response to a vertical load, thus is characteristic for fibril elasticity. While it does not fully reflect the tensile elasticity of fibrils, it can reliably quantify the relative elasticity or stiffness among spatially distributed fibrils and among comparable samples.

To make reasonable comparison, the indentation depth of all measurements was fixed at $\delta=10$ nm. The comparison of the topographic image and the force map allows the nanoscopic quantification of the elasticity of an identified collagen fibril. Histograms and scatter plots were generated based on the E values derived from at least 200 force curves for collagen fibrils on each tissue sample.

To understand the effect of collagen composition on material stiffness, purified bovine COLI and COLIII (Millipore Corp., Temecula, CA) were purchased. The collagen was dissolved in 0.02 N acetic acid and mixed at ratios of 1:1, 1:2 and 1:4 between COLI and COLIII at a total collagen concentration of 1.5 mg/ml. The solutions were then used to make hydrogels for AFM imaging and elasticity measurements.

Results

Nanoscopic structures of native collagen fibrils in fresh tissues

Figure 2 shows the AFM images revealing the apparent morphological differences in collagen fibrils from tissues of both post-M and POP women with respect to those of pre-M women. At the nanoscopic scale, pre-M collagen fibrils are densely packed and uniformly aligned with respect to each other (Figure 2A). These fibrils bundle into collagen fibers that form the fiber network visible in Gomori staining and immunofluorescent images. It is apparent from the micrometer scale images that these fibrils are part of distinctive ropes or braids that are 2-3 μm wide (Figure. 2B). The post-M fibrils are aligned but appear loosely packed at the nanoscopic scale (Figure. 2C). At the microscopic scale, braided bundles are largely absent. It is also evident that collagen fibrils in pre-M and post-M samples are uniform in width along their lengths. In POP samples, however, the fibrils are uneven and are flatter with some stunted ends,

which indicate deterred fibril growth (Figure. 2E, white arrows). Like in post-M samples, braided bundles of collagen fibrils are absent at the micrometer scale in POP samples (Figure. 2B vs. D, F), which likely cause the decreased mechanical strength of the collagen, as delineated in the Discussion section.

Fibril width was measured from the AFM images and analyzed statistically as shown in Figure. 3A. The median of fibril width is 55 nm in pre-M samples and 57 nm in post-M samples, whereas it is 130 nm in tissues from POP patients. The width of most fibrils in non-POP samples falls within a narrow range of 40-80 nm. In contrast, the fibril width of POP samples varies broadly between 60 - 210 nm. The characteristic D-period of the fibrils is apparent in the AFM images and permits the quantitative and statistical analysis. In pre-M and post-M samples, the medians of the D-period are 68.2 nm and 68.0 nm, respectively, and their difference is statistically insignificant ($p = 0.2$). The value is consistent with the reported value in healthy soft connective tissues [31]. However, in the POP samples, the median of D-period is reduced to 65.0 nm and the values have a broader distribution. The difference with respect to the D-period of non-POP samples is statistically significant ($p < 0.0001$) according to the t-Test analysis (Table 1). The fibril-specific correlation between D-period and fibril width is demonstrated in the scatter plot in Figure 3C. While the D-period of pre-M and post-M samples is largely independent of fibril width, the D-period of POP samples decreases with the increase of fibril width from 50 nm to 150 nm. However, the D-period fluctuates drastically as widths increase beyond 150 nm. The results suggest that abnormal collagen fibril assembly occurs in POP tissues.

Nanoscopic characterization of the elasticity of native collagen fibrils

We applied the nano-indentation method to probe fibril elasticity in response to a vertical load. The elasticity values indicate that collagen fibrils in POP samples are stiffer than those in non-POP samples. The fibril elasticity of pre-M and post-M samples falls within a narrow range of 0.2-1.0 MPa, whereas in POP samples, the fibril elasticity ranges 0.6-4.0 MPa (Figure. 3D). The elasticity was measured on those fibrils identified by topographic imaging in order to correlate fibril elasticity with fibril width. Compared with the fibril width (Figure 3A), the probe size (~15 nm in radius) is smaller but at a similar scale. Pixels in the force map were carefully chosen by overlaying the topographic image and the force map to ensure only E values measured on fibrils were included in the analysis. As illustrated in Figure 3E, collagen fibrils in pre-M and post-M samples are thinner and softer, and the E value is largely width-independent. Fibrils in POP samples vary dramatically in both fibril dimension and E value; there is a tendency that thicker fibrils are stiffer. Additionally, non-POP fibrils of a particular width (e.g., ~90 nm) are softer (~ 0.4 MPa) than POP fibrils (~0.7 MPa) of the same width, whereas the D-periods of non-POP and POP fibrils of the same width are essentially the same. The observation that POP fibrils are stiffer than controls for any given fibril width suggests that the POP fibrils are likely biochemically different from non-POP fibrils.

We further examined the elasticity profile of individual collagen fibrils in POP samples. As illustrated in Figure 4A and 4B, the topographic image and the force map were collected at the same region. E-values measured at the labeled pixels are shown in the Table. It is apparent that E-values are largely invariant along the fibril in uniform fibrils (e.g., Pixels 1, 2, 9 and 10); fibrils exhibiting uneven width along a fibril show marked variations in E-value (e.g., Pixels 3, 4, 6 and 7). The abnormal, bulged regions of a fibril are significantly stiffer. These results reinforce our observation from Figure. 3E: the frequently observed, aberrant fibrils with stunted ends in

POP samples lead to a drastic variation in E-value whereas the uniform fibrils in non-POP samples result in a narrow range of E-values. The abnormality is deliberated in the Discussion section.

Effect of collagen composition on collagen fibril elasticity and D-period

COLI and COLIII are predominant fibrous collagens of connective tissues. They combine to form hybrid fibrils and have distinctive roles in tissue biomechanics. We studied the relative amount of COLI and COLIII in the connective tissue samples. Figure 5 shows the immunofluorescent images of collagen in pre-M, post-M and POP tissue samples at the mucosa region stained against COLI (green) and COLIII (red). Apparently, both collagen types are present, largely co-exist and are evenly distributed, as seen in the overlap images. It is also evident that they are the major components in the vaginal wall connective tissues. The collagen fibrils shown in the high-resolution AFM images (Figure 2) are presumably composed of both COLI and COLIII, consistent with the reports from the literatures [13, 16, 32, 33].

The immunofluorescent images reveal that the COLI and COLIII are less brightly stained in POP samples in comparison to pre-M and post-M samples. Unlike the whorled pattern of fibers in non-POP samples as reported by others as well [6], collagen fibers in POP samples appear stressed and loose. We estimated the relative amounts of COLI and COLIII in the samples by ImageJ analysis of their fluorescent intensities (Figure 5). The data are summarized in Table 1. When compared with the pre-M and post-M samples, we observed a significant drop in the amount of both COLI and COLIII in POP samples. Also, the intensity ratio of COLI to COLIII in pre-M, post-M and POP samples was 1.4, 1.7 and 2.6, respectively. In this quantification, COLI was stained in green and COLIII was in red. To rule out the influence of dye color in the

fluorescent intensity measurement, the same experiment was repeated by staining both COLI and COLIII with the same green fluorescent dye on adjacent tissue slices containing nearly identical composition and morphology. According to these images, the ratio of COLI/COLIII in pre-M and POP samples was 1.2 and 2.9, respectively, confirming that the trend is true.

We speculated that the change in the relative amounts of COLI and COLIII in POP contributes to the increased fibril width and stiffness as well as the decreased D-period. To unravel the effect of COLI/COLIII ratio, we prepared hybrid fibrils of COLI and COLIII by mixing the proteins from commercial sources at ratios of 1:1, 2:1 and 4:1 before they were gelled to form fibrils. The relative amount of COLI and COLIII in the matrix is verified by ImageJ analysis of the fluorescence intensity in the immuno-fluorescent images. E-values of the fibrils were measured and the correlation with COLI/COLIII ratio is illustrated in Figure. 6. Data collected from pre-M, post-M and POP tissue samples are also presented for comparison. The E-value of the reconstituted fibrils is lower than that in tissues since the total collagen concentration in the artificial hybrid fibrils is lower [34]. In the tissue samples, doubling of COLI/III ratio (from 1.4:1 in pre-M samples to 2.6:1 in POP samples) increases the fibril stiffness by 2.4 times, accompanied by a decrease in collagen D-period from 68 nm to 65 nm. Doubling of COLI/III ratio (from 1:1 to 2:1) in the artificial hybrid fibrils increased the fibril stiffness by 1.9 times but without any change in D-period. It implies that while fibril stiffness in POP is associated with collagen composition, the D-period is not. Note that the post-M collagen fibrils are slightly softer than the pre-M fibrils even though the COLI/COLIII ratio seems slightly higher in the post-M samples. This may be relevant to estrogen deficiency in post-M individuals (see Discussion section). Nevertheless, significant differences between POP and non-POP samples were consistently observed in multiple measurements.

Discussion

POP is often correlated to changes in the amount, assembly, degradation and aging of collagen in the extracellular matrix of pelvic floor connective tissues [13,35]. In the current study, we identify such changes *in situ* at the microscopic and nanoscopic scales to derive their direct correlations underlying the biomechanical failure in POP. We have discovered that the POP collagen fibrils are thicker, stiffer, more uneven, and characterized by a small but significant decrease in D-period. Changes in D-period are usually ascribed to the combination of molecular elongation, an increase in the gap region between longitudinally adjoining molecules along the fibril axis, and the relative slippage of laterally adjoining molecules along the fibril axis [36]. These changes are likely associated with elastic energy storage, transmission and dissipation [37-39]. Though mechanical strength of tissues emerges from multiple factors, a change in collagen D-period denotes deviations in the building block of tissue architecture, thus contributes at least in part to the tissue biomechanics. This is especially true in connective tissues, where collagen is the predominant constituent. Changes in D-period in connective tissues due to strain [40, 39] and dehydration [41] have been reported. A noticeable change of 2.5 nm in D-period was also found in osteoporosis [42, 43], and the change was ascribed to COL1 mutation [44] which causes conformational changes in the triple helix and consequently in collagen fibril assembly [45]. Here we report a 3 nm decrease of collagen D-period in pelvic floor connective tissues from POP patients with respect to the control counterparts. While it is unclear if such changes are arisen from mutation and/or defect of cross-links in fiber assembly, the flat, bulky and uneven collagen fibrils in POP samples resemble fibrils of mutated collagen observed by others [42, 43, 44, 46]. On the other hand, our preliminary data showed that when collagen was

extracted from POP tissues and reconstituted *in vitro*, the fibrils exhibited a longer D-period, suggesting that the reduced D-period in POP tissues likely emerges from defective fibril cross-linking. Further study is needed to clarify the cause of the change in D-period. Nevertheless, the 3 nm change in D-period is expressive enough to be used as a potential physical marker for POP diagnosis.

Kotova et al [47] also reported an ultrastructural AFM analysis of collagen fibrils in patients' skin tissue of the perianal area in relation to POP. Their observation of relatively disorganized collagen fibrils in POP samples agrees with our results. However, their claims of conserved collagen D-period and thinner fibrils in the skin connective tissues of POP patients contradict our results of reduced D-period and thicker fibrils in the vaginal wall connective tissues. The discrepancies are likely caused by the different sources of tissues. Their samples were also exposed to a series of chemical treatments prior to AFM imaging. The use of native, fresh tissues in our study eliminates the effects of potential artifacts. Our study enables the structure-function correlation of collagen fibrils in a fibril-specific fashion to empower a nanodiagnostic method for sensitive and early detection of POP as well as other health conditions associated with the dysfunction of soft, loose connective tissues.

Collagen fibrils in POP samples were thicker, in agreement with other reports [48]. We speculate that the thicker fibrils arise from the increased COLI/COLIII ratio in the hybrid fibrils. It was reported that the amino propeptide of COLIII is excised far slower than that of COLI in the extracellular space prior to the assembly of triple-helical collagen molecules into fibrils [49]. Thus, COLIII plays an essential role in limiting the fibril growth and ultimately, the fibril width [50]. In a defined and constant extracellular milieu of aorta, where the synthesis and degradation of COLI and COLIII are steady, fibrils were found uniform in diameter across a tissue [51];

when the extracellular milieu was modulated to be COLIII deficient, thicker fibrils were formed [52]. An *in vitro* fibrillogenesis study [53] as well as a preliminary *in vitro* study in our lab showed that collagen fibril diameter decreased with the decrease of COLI/COLIII ratio. These results further imply the role of COLIII in regulating collagen fibril's width.

Notably, the POP fibrils were also significantly stiffer than non-POP fibrils. This can also be ascribed to the increased COLI/COLIII ratio. Due to the high tensile strength of COLI fibrils and the flexibility offered by COLIII, a higher COLI/COLIII ratio (i.e., a less COLIII content) in POP could generate more rigid and stiffer fibrils. This is supported by the results of our *in vitro* experiments using the mixture of COLI and COLIII, where an increase in fibril stiffness is concomitant to an increase in COLI/COLIII ratio (Figure 6). We did observe that the post-M collagen fibrils are slightly softer than the pre-M fibrils while the COLI/COLIII ratio is slightly higher. The hypo-estrogenic state in post-M women may impinge upon the biomechanical state of collagen fibrils by altering the collagen composition, synthesis, cross-linking and degradation [54, 55]. While it remains unclear how the multiple effects interplay to yield the softer fibrils, it is evident that the level of estrogen deficiency in post-M is trivial to the dramatic increase of collagen fibril stiffness in POP. The high COLI/COLIII ratio in POP is not the sole factor that causes the significantly high fibril stiffness (Figure 6). POP fibrils are loosely packed and stressed extensively along one direction (Figure. 5 G-I), representative of aged and mature fibrils. The higher proportion of such aged fibrils indicate irregularities in the turnover of collagen within POP tissues, hence, imbalanced kinetics of protein processing and transportation of COLI and COLIII to the extracellular space. This may lead to occasional, abnormal collagen accumulation at local regions of a fibril, as demonstrated in Figure 2E. Consequently, these morphological irregularities, such as stunted ends of POP fibrils, were found to associate with

substantially high stiffness as evidenced in Figure 4. Additionally, higher extent of glycation in mature fibrils also makes them stiffer than young fibrils [12].

The collagen fiber network is ultimately accountable for the tissue's resistance to rupture and fracture relevant to the failure in load bearing of pelvic organs in POP. Collagen fiber network is a hierarchically organized system. Its elasticity and toughness depends on both the property of individual fibrils and the way the fibrils weave to form the network. The analysis of immunofluorescent intensity of collagen within the tissue samples reveals that the total amount of collagen dropped whereas the percentage of COLIII was significantly lower in POP samples (Figure 5, Table 1). The results suggest the bulky and rigid POP fibrils presumably form a loose and less flexible matrix. The distinctive, braided collagen fibril bundles are present in pre-M samples, but are largely absent in post-M and POP samples. According to MacKintosh et al [56], a fiber network elastic modulus is linearly proportional to the mesh node density (frequency of fiber crossing). Hence, the braiding of collagen in pre-M samples represents an additional organizational level in the hierarchy of collagen assembly, which enhances the mechanical strength to adequately support the pelvic organs. Conversely, in the absence of such reinforced collagen framework (as in post-M and POP samples), the tissue is weaker. In the case of POP, taking the loose and less flexible fiber network into consideration, the tissue matrix is brittle and cannot sustain critical stress, hence, prolapse occurs.

Our conclusion that POP tissues possess a non-stretchable and fragile collagen matrix with reduced load-bearing ability is based on the observation of stiffer and bulkier collagen fibrils, increased COLI/COLIII ratio, decreased total collagen amount, as well as decreased braiding of collagen fibrils. Others have found that the total collagen amount is higher in POP tissues compared to normal tissues, but the amount of COLIII is much less [57]. Contrasting results in

terms of the ratio of COLI/COLIII were also reported by other researchers [58]. In addition to the inconsistency in the sample numbers, the patients' medical history, the family medical history and the tissue handling techniques, controversies among biochemical, biophysical and biomechanical studies arise from the lack of correlation with identified tissue components under study as well as the discrepancy in the scale of measurements. Our direct and *in situ* analysis of tissue samples allows us to choose specific tissue components within the tissue for investigation at nanoscopic and/or microscopic scales. The systematic nature of our approach enhances the reproducibility in future studies. Thus, the method is more suited to clarify the role of collagen and other extracellular matrix components in POP.

This study provides evidence that the collagen fibrils in POP are altered at different levels of organization- from individual fibrils to fiber network- as well as in the relative composition of collagen types. Further investigation undertaken by the group will identify whether the changes arise from mutation or/and defective cross-linking. The nanoscale features, including collagen D-period, fiber morphology, alignment and organization relevant to collagen composition, can be employed as biomarkers to enable pre-symptomatic or early diagnosis of POP. We plan to assess other structural components of the female pelvic floor in ongoing studies to confirm the findings. Our research marks the first work to acquire valuable diagnostic information by distinguishing nanoscale morphological and biomechanical differences, which also sheds light on the pathological cause of POP. It lays the groundwork for tracking the progressive changes that occur in the extracellular matrix components at different stages of a disease or health condition, and will potentially improve the diagnostic efficacy for POP and other connective tissue disorders.

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Figure Legends:

Figure 1. Scheme of Gomori staining assisted AFM imaging and elasticity measurement at a targeted collagen-rich mucosa region (blue stains - collagen) in vaginal wall connective tissues. A, B and C are images of the identical region from three adjacent slices of the same tissue specimen. D, E and F represent the smaller scale images of A, B and C, respectively, at the identified area marked by the rectangular frame in A. The yellow circles identify the same area in all images. Gomori staining of the first slice (A,D) provides the guidance for positioning the AFM probe at a collagen-rich region in the second slice (B,E) from fresh, unfixed tissue; Gomori staining of the third slice (C,F) verifies the area that AFM measurements were carried out.

Figure 2. AFM images (fluid tapping mode) illustrating the nanoscopic features of native collagen fibrils in the mucosa region of vaginal wall connective tissues. (A,B) Pre-M; (C,D) Post-M; (E,F) POP. The white arrows in (E) highlight the abnormal regions on a fibril.

Figure 3. Statistical analysis of collagen fibrils in pre-M, post-M and POP samples. (A) Histogram of fibril width distribution; (B) Histogram of D-period distribution; (C) Scatter plot illustrating the correlation between fibril width and D-period in individual fibrils; (D) Histogram of fibril elasticity distribution; (E) Scatter plot illustrating the correlation between fibril width and E-value in individual fibrils.

Figure 4. Fibril-specific elasticity measurements of collagen fibrils from a POP sample. (A) High-resolution topographic image; (B) Force map at the corresponding area. The Table shows E values measured at the pixels highlighted in (A) and (B).

Figure 5. Immunofluorescent images of collagen in vaginal wall connective tissues of pre-M (A-C), post-M (D-F) and POP (G-I) samples collected at the mucosa region staining against COLI (green) and COLIII (red). Bar size: 50 μm .

Figure 6. Comparison of E-values of native collagen fibrils from pre-M, post-M and POP tissue samples vs. that of reconstituted hybrid fibrils at different COLI/COLIII ratios.

Table 1. Structure, stiffness and composition of native collagen fibrils in vaginal wall connective tissues. The data were summarized based on more than 40 measurements of each sample type.

Table 1. Structure, stiffness and composition of native collagen fibrils in vaginal wall connective tissues. The data were summarized based on more than 40 measurements of each sample type.

	Pre-M	Post-M	POP
D-period (nm)	68.2±0.1	68.0±0.1	65.0±0.3
t- Test (p value)		0.2	< 0.0001
Width (nm)	55±2	57±2	130±6
E-value (MPa)	0.55±0.02	0.37±0.01	1.33±0.12
COLI amount, IF	1.0±0.2	1.2±0.2	0.5±0.1
COLIII amount, IF	0.7±0.1	0.7±0.2	0.2±0.1
COLI /COLIII, IF	1.4±0.2	1.7±0.2	2.6±0.1
COLI/COLIII, qPCR	1.0±0.2	-----	0.9±0.2

* The values are indicated as median ± standard error. The p value evaluates the statistical significance of the difference in D-period with respect to that of the pre-M samples (based on more than 50 measurements). The amount of COLI and COLIII are relative values evaluated based on the intensities measured in immunofluorescent images, normalized with respect to the intensity of COLI in pre-M samples.

Figure 1

Figure 2

Figure 3

Figure 4

Position	E value (MPa)	Position	E value (MPa)
1	1.39	6	2.19
2	1.45	7	1.99
3	4.07	8	0.88
4	3.67	9	1.45
5	1.26	10	1.33

Figure 5

Figure 6

Graphical Abstract: Text

In this study, nanoscopic structures and biomechanics of native collagen fibrils in surgical, vaginal wall connective tissues from healthy women and pelvic organ prolapse (POP) patients have been examined to identify nanoscale alterations in collagen as potential diagnostic markers for POP.