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(Citation)
Urology,149:161-167

(Issue Date)
2021-03

(Resource Type)
journal article

(Version)
Accepted Manuscript

Histological investigation of the female vesicourethral junction and adjacent tissues for nerve-sparing radical cystectomy

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Word count of text: 2,780 words

Word count of abstract: 246 words

Key words: radical cystectomy; vesicourethral junction; urinary bladder; urethra; nerves; smooth muscle

Financial Disclosure

The authors declare that they have no relevant financial interests.

INTRODUCTION

Orthotopic urinary diversion with radical cystectomy (RC) provides excellent oncologic outcomes with minimal risk of urethral recurrence for female patients with muscle-invasive non-metastatic bladder cancer.[1-3] However, the procedure may result in postoperative urinary or sexual dysfunction. Nerve-sparing RC has been reported to improve outcomes.[4,5]

Nerve fibers from the pelvic plexus run beside the lateral wall of the vagina to the bladder neck and urethra.[4] However, the dissection plane for nerve-sparing RC is not always clear at the level of the vesicourethral junction, owing to a thick tissue mass lateral or posterior to the junction. Although optimal postoperative results for continence and voiding are obtained by preserving the entire lateral vaginal wall,[4] other landmark structures for nerve-sparing procedures during RC have also been reported.

Understanding the structures around the vesicourethral junction is essential for improving functional outcomes after RC. However, relevant anatomical findings in adults have been identified only macroscopically, with histological findings only in fetuses.[6] Furthermore, interindividual anatomical variations are frequently observed in this region, yet these variations have not been investigated systematically. Smooth muscle sphincter and striated sphincter innervation also has not been histologically confirmed yet in women.

Here, we histologically elucidated the fibromuscular construction and nerve

distribution around the vesicourethral junction, especially in the posterior and lateral region, to improve the functional results of nerve-sparing RC. We also examined whether the thick muscle mass around the junction could be confirmed intraoperatively and preserved.

MATERIALS ANS METHODS

The study was performed in accordance with the Declaration of Helsinki.[7] Pelvic specimens containing all the pelvic viscera were obtained from 33 donated female cadavers aged 68–88 years at death. All cadavers were postmenopausal, uni- or multiparous, and had experienced vaginal delivery. The cause of death had been ischemic heart failure or intracranial bleeding. None of the individuals had undergone pelvic surgery including caesarean section, confirmed by medical documentation and abdominopelvic cavity observation. These cadavers had been donated to Tokyo Dental College for research and education on human anatomy, and their use for research had been approved by the university ethics committees.

The donated cadavers had been fixed by arterial perfusion with 10% v/v formalin solution and stored in 50% v/v ethanol solution for more than 3 months. Before bisecting them, we recorded the total vaginal length, most distal portion of prolapse, and level of hymen. Stages of pelvic organ prolapse (POP) were recorded,[8] and we defined POP as stage ≥3. From each cadaver, we obtained the entire pelvic tissue mass surrounded by the obturator internus and levator ani muscles. From this, we made five macroslices (15 mm thick) that included the urethra, anterior wall of the vagina, and inferomedial edge of the levator ani muscle. We performed paraffin-embedded histology to obtain large frontal sections (50 × 70 mm).

We stained all sections using hematoxylin and eosin and Masson trichrome staining. Some sections were used for immunohistochemistry and elastica Masson (a variation of Masson-Goldner) staining to identify smooth muscle, striated muscle, collagen fibers, and elastic fibers.[9] Primary antibodies were 1) mouse monoclonal anti-human S100 (1:200 dilution; Dako Z0311; Dako, Glostrup, Denmark) for nerves; 2) rabbit polyclonal anti-human neuronal nitric oxide synthase (nNOS) (1:100 dilution; #4231; Cell Signaling Technology, Danvers, MA, USA); 3) mouse monoclonal anti-human vasoactive intestinal polypeptide (VIP) (1:00 dilution; sc25347; Santa Cruz Biotechnology, Dallas, Texas, USA); 4) rabbit polyclonal anti-human tyrosine hydroxylase (TH) (1:100; ab152, Merck Millipore, Temecula, CA, USA); and 5) mouse monoclonal anti-human smooth muscle actin (1:100; Dako M0851, Dako). Antibodies 2, 3 and 4 covered most pelvic autonomic motor nerves. Secondary antibodies were labeled with horseradish peroxidase, and antigen-antibody reactions were detected by a horseradish-peroxidase-catalyzed reaction with diaminobenzidine. Samples were counterstained with hematoxylin. Each specimen had a negative control (no primary antibody). An Eclipse 80 microscope (Nikon, Tokyo, Japan) was used for observation and photography. Ultralow-magnification images (less than ×1 at the objective lens) were obtained using a high-grade flat scanner with translucent illumination (GT-X970, Epson, Nagano, Japan).

We then investigated whether our anatomical findings could be confirmed in

female patients undergoing robot-assisted nerve-sparing RC (institutional review board approval No.170098). All three patients were postmenopausal, multiparous and had no history of prior pelvic surgery.

Statistical analysis

All analyses were performed in JMP 13.2 (SAS Institute Inc., Cary, NC, USA). Pearson's chi-squared test was used to compare frequencies of POP in cadavers with or without HSM. $p<0.01$ was considered statistically significant.

RESULTS

Interindividual differences in the muscular construction around the vesicourethral junction

The smooth muscles of the bladder were consistently divided into the detrusor muscle (a group of thick muscle bundles running along the superoinferior axis) and the neck muscle (a cluster of thin muscle fibers arranged circularly or randomly) (figs. 1BC, 2AB, 3A, S1AB, S2A). The latter became unclear in the posterior region of the urethra. In contrast, smooth muscles of the urethral wall gave off associated collagenous and elastic fibers laterally (figs. 1B, 3A, S1B,). In 15/33 (45.5%) cadavers, there was a clear border between the bladder smooth muscle and the urethral wall smooth muscle in the lateral region of the urethra. Notably, the border met intrapelvic loose connective tissue immediately medial to the upward reflection of the endopelvic fascia (figs. 1A, S1AB). The thick endopelvic fascia was reflected from the upper surface of the levator ani muscle to the external surface of the urinary bladder. In the remaining 18/33 (54.5%) cadavers, a mass of smooth muscle and collagen fibers in the lateral side of the urethra occupied a space between the urethra and the inferomedial edge of the levator ani. We termed this mass as hiatal smooth muscle (HSM) (figs. 2, 3, 4). The HSM connects the medial levator margin to 1) an elastic fibrous tissue mesh containing striated muscle fibers of the rhabdosphincter and 2) a longitudinal anal muscle running between the internal and external anal sphincters. It covered the lateral aspect of the bladder neck (figs. 2B, 3A). However, in all cadavers, the

border between the HSM and bladder neck muscle became unclear behind the urethra, and the bladder detrusor merged with the smooth muscles of the anterior vaginal wall (figs. 1C, 2A, S2AC). The vaginal smooth muscles were easily identified by their transversely arranged courses. Conversely, the bladder muscles were clearly separated from the urethral wall smooth muscles in the anterior and lateral sides of the urethra. In cadavers with HSM, POP was observed in 8/18 (44.4%) cadavers. On the other hand, POP was not observed in 15 cadavers without HSM ($p<0.01$; table S1).

The urethral rhabdosphincter was embedded in a fibrous mesh from the urethral smooth muscles (figs.S1BD). Laterally, this fibrous mesh joined the elastic fiber-rich perineal membrane that extended along the supero-medial aspect of the vestibular bulb (figs. 1B, 2B, S1B, S2A). Medially, the perineal membrane extended beneath the mucosal layer of the external orifice of the urethra as well as the vaginal vestibule. Laterally, it was connected by the inferior fascia of the levator ani, containing a wide venous plexus (figs. 1B, S2A). The deep transverse perineal muscle was embedded in the lateral part of the perineal membrane (figs. 2B, S2A).

Nerves around the vesicourethral junction

Abundant intrapelvic nerves (pelvic plexus branches) ran through and near the endopelvic fascia (figs. 1AD, 3B, S1F, S2DE). Among them, the cavernous and sphincter nerves (CSNs) and detrusor nerves (DNs) ran beneath the inferomedial edge of the levator

ani in a small area lateral to the bladder neck and the urethra. There were two groups of nerves. One comprised DNs entering the bladder detrusor (figs. S1F, S2D); another comprised the CSNs entering the clitoral body and urethral sphincter (figs. 1D, S2E). We found no distinct difference in nerve fiber composition between the DNs and CSNs (fig. S3). Among the CSNs, we detected pelvic nerve branches that innervated the urethral sphincter (arrowheads in figs. 1AD, 3B, S2DE). They entered the urethral sphincter posterolaterally and comprised sympathetic and parasympathetic fibers. The anteroposterior relationship between DNs and CSNs varied between specimens: the DNs were located anterior to the CSNs (figs. 1D, sup1F) in 18/33 cadavers (54.5%) and vice versa (figs. S1DE) in 15/33 cadavers (45.5%). All were located anterior to the anterior vaginal wall.

Clinical confirmation of the HSM

In three female patients undergoing nerve-sparing RC, the boundary between HSM and urethral smooth muscle was easily distinguished intraoperatively during dissection of the vesicourethral junction. Fig. 4D and E shows intraoperative finding of one of these cases (a 71 years old woman).

DISCUSSION

A mass of smooth muscle and collagen fibers occupied the space between the urethra and the inferior edge of the levator muscle in 55% of the elderly cadavers. We termed this mass the HSM. It was more frequently observed in cadavers with POP than without POP. Autonomic nerve fibers innervating cavernous tissue and sphincter muscles ran between the HSM (where present) and vesicourethral junction. The ventrodorsal relationship between CSNs and DNs varied between cadavers. To our knowledge, this is the first report describing the HSM and histologically confirming the nerves that innervate the urethral sphincter in women.

The connective tissue in the urogenital hiatus was comprehensively called the "hiatal ligament" by Shafik et al.[10] They reported that the levator ani is connected to the bladder neck along the hiatal ligament and to the striated urethral sphincter through the suspensory sling. We previously reported that the female detrusor extends posteriorly at the interface of the urethra and vagina in the hiatal ligament,[11] but we did not observe the posterior or posterolateral parts of the vesicourethral junction. In the present study, the HSM was present in 55% of the cadavers, was located in the posterolateral corner of the vesicourethral junction, and was not the vagina, detrusor, or striated sphincter. The HSM was mostly composed of smooth muscle, not connective tissue as reported by Shafik et al. However, 15 of our cadavers had nothing other than fibroadipose tissue in this region. We

also found no striated muscle component in this area, consistent with the findings of Colleselli et al.[12] The tissues surrounding the female urethral sphincter have not been investigated in studies that describe the urethral sphincter itself,[12,13] but some reports have described interindividual differences in the morphology as anatomic variations in the levator ani, endopelvic fascia, and urethra.[14-16]

Some degree of POP is nearly ubiquitous in older women.[17] We found that the presence of HSM was significantly associated with POP. The HSM might therefore be related to POP. We previously reported that the collagenous fiber connections to the pubis and the vagina are important against abdominal pressure.[11] From this, we speculate that the HSM relates to POP because the HSM connects not to the hard collagenous fiber ligaments but to the rhabdosphincter and the longitudinal anal muscle, which are soft tissues. In addition, the presence of HSM may also be related to age or hormonal environment. The HSM is likely missing in 45% of the cadavers because it can degenerate when the levator ani muscle decreases its contraction force due to hormonal environment or age. The HSM might correspond to the structure in men known as Muller's ischioprostatic ligaments or Walsh's pillars, an inconstant fibromuscular structure flanking the rhabdosphincter at the prostatourethral junction.[18] The ischioprostatic ligaments come from the parietal pelvic fascia at the level of the ischial spine and radiate into the anatomical capsule and prostate.[19] We speculate that the HSM provides an interface to avoid injury

caused by a rapid and strong contraction of the levator ani muscle rather than simple transduction of muscle contraction force.

Although the nerve fibers from the pelvic plexus to the bladder neck and urethra have been shown macroscopically,[12] we could not identify them in the present study. Zvara et al. reported that the nerve supply to the urethral sphincter consists of branches from the pudendal nerves and from the pelvic splanchnic nerve in male cadavers.[20] Yucel et al. used reconstructed three-dimensional images from human fetuses to demonstrate that the cavernous nerves originating from the autonomic nerve plexus around the vagina continued their course to the proximal urethra.[21] However, the nerves that innervate the urethral sphincter in women had not previously been confirmed histologically, meaning that controversy persisted around the topographical relation between the nerves, urethra, and bladder detrusor in women. The present study provided histological confirmation of the autonomic nerves that innervate the urethral sphincter in women and demonstrated the topographical relationship between the DNs and the CSNs. Although nerves entering the detrusor at and near the ureterovesical junction have already been described,[22] the CSNs along the endopelvic fascia revealed here run a different course from them. Our immunohistochemistry findings showed that the autonomic nerves in this region comprised mixed sympathetic and parasympathetic fibers, contrary to the traditional theory that the DNs largely emerging from the sympathetic hypogastric nerve should be predominantly

sympathetic, and that the CSNs might be branches of the parasympathetic pelvic splanchnic nerves. The nerves innervating the urethral smooth muscle sphincter are peripheral autonomic nerves and were too thin to be observed macroscopically.

Some female patients who undergo orthotopic neobladder reconstruction suffer from chronic urinary retention or incontinence, particularly if urethral innervation is not preserved, and this leads to a lower quality of life compared to healthy populations.[23,24] Furthermore, autonomic nerve preservation is important for sexual function and for voiding (better continence and less catheterization) after orthotopic reconstruction.[5] RC affects proximal urethra sensitivity, which is an important factor for achieving continence postoperatively,[25] and better sexual function has been reported after nerve-sparing RC than after non-nerve-sparing RC.[26] We found that the cavernous nerves run along the edge of the HSM. Distinguishing peripheral nerves intraoperatively is difficult, even with a clear magnified view. However, in our patients, the boundary between HSM and urethral smooth muscle was easily distinguished during dissection of the vesicourethral junction, allowing the HSM to be easily preserved (fig. 4D and E). Although complete resection of the cranial two-thirds of the vagina, with the caudal border of resection just below the bladder neck, is believed to result in the dissection of most autonomic nerves to the urethra,[4] our study shows that the CSNs run anterior to the lateral vaginal wall. The depth of the CSNs from the vesicourethral junction has not been reported; this information would enable surgeons

to achieve sufficient nerve sparing. Positional relationships of the structures found during procedures change according to the traction applied, so deep dissection of structures other than the bladder neck could damage nerves even if the lateral vaginal wall were preserved. Incising the endopelvic fascia in female nerve-sparing cystectomy remains controversial. Some authors have suggested that it should not be disturbed,[27] whereas others reported successful nerve sparing despite its incision.[5,28] Our findings support the view that avoiding incision of the endopelvic fascia benefits sparing of the CSNs.

When the area around the vesicourethral junction is separated during female RC, the tissue around the urethra is often dissected as "thick connective tissue" without being properly identified. However, to optimize nerve sparing in cystectomy in female patients with recognizable HSM, the correct dissection plane should be inside the HSM, providing that oncological safety is guaranteed. Confirmation of the HSM under direct vision could help identify the vesicourethral junction. Even if the lateral vaginal wall is resected to ensure oncological safety, nerve preservation is possible if the inner layer of the HSM is followed. In cases of vaginal sparing, blunt dissection as well as a sharp cut in direct vision is required for optimal nerve sparing because the surgical planes between the bladder and vagina are intermingled. In any case, the optimal depth of periurethral detachment varies between patients, like the presence or absence of HSM, which is why there is a risk of failed nerve preservation. We advise surgeons to be aware of interindividual differences in

the HSM, and knowledge regarding the relationship between HSM and POP should improve the accuracy of nerve preservation.

The present study is not devoid of limitations. Among the 33 cadavers examined, only one cadaver was 68 years old, and all other cadavers were over 70 years old (median 81 years old) at the time of death. For this reason, in the present study we could not examine whether young patients have HSM. Further investigation will elucidate the role and function of these muscles and nerves. Nevertheless, our results may help improve outcomes for female patients undergoing RC.

CONCLUSIONS

We found that 55% of elderly women have HSM between the urethra and inferior edge of the levator ani. Autonomic nerve fibers, including those innervating the urethral sphincter, run between the HSM and vesicourethral junction in female cadavers with HSM. When performing RC before neobladder reconstruction, if the layer for nerve sparing is difficult to find lateral to the vesicourethral junction, nerve sparing can be reliably performed using the HSM as a landmark.

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Figure Legends

Figure 1

Topohistology of the bladder detrusor and nerves in frontal sections from a 79-year-old woman. (*A*) S100 immunohistochemistry showing cavernous and sphincter nerves (CSN) running between the inferomedial edge of the levator ani muscle (LA) and urethral smooth muscles (UR). (*B*) Elastica Masson staining. (*C*) Masson trichrome staining. (*B*) and (*C*) show hiatal smooth muscle (HSM) between the urethra and the inferomedial edge of the levator. Inset of (*B*) corresponds to the square in panel *B*. (*D*) S100 immunohistochemistry corresponding to the square in (*C*) shows abundant nerves (cavernous and sphincter nerves) running inferiorly along the posterolateral corner of the urethral smooth muscles. Toward the inferolateral wall of the urethral smooth muscle, the CSNs ran inferomedially through the HSM. (*A*) and (*C*) show the most anterior and posterior sides, respectively. The distance between sections in the panels (*A*–*B*, *B*–*C*) is 2 mm, and the section in (*A*) is located 2 mm posterior to that in fig. 1 *B*. In these posterior sites, the bladder neck muscles were not clearly identified (stars), although the inferior margin of the detrusor is clear (dotted line). Arrowheads: nerves entering into the urethral sphincter. Scale bars: (*A*–*C*) 10 mm; (*D*) 5 mm.

Figure 2

Topohistology of the bladder detrusor and endopelvic fascia in frontal sections from a 76-year-old woman. (*A*) Masson trichrome staining. (*B*) Elastica Masson staining. (*A*) is located 2 mm posterior to (*B*) and contains a part of the posterior wall of the vagina (VAG). (*B*) shows the perineal membrane (PM) identified as a thick, elastic, fiber-rich membrane. (*C*) corresponds to the square in (*B*). The inferior end of the bladder detrusor was clearly identified (dotted line) but the bladder neck muscle was not (stars). The endopelvic fascia (EPF) makes a thick reflected lamina (arrows) extending upward. (*B*) and (*C*) show hiatal smooth muscle (HSM) between the urethra and the inferomedial edge of the levator. Scale bars: 5 mm.

Figure 3

Topohistology of the bladder detrusor and nerves in frontal sections from a 76-year-old woman (same specimen as in fig. 2). (*A*) SMA immunohistochemistry. (*B*) S100 immunohistochemistry. (*A*) and (*B*) show the central part of fig. 3 *B*. In (*A*), hiatal smooth muscle (HSM) occupies a large space between the urethra and the inferomedial edge of the levator ani (LA). According to smooth muscle configurations, a stripped line corresponds to the upper border of the urethral smooth muscles (UR). The bladder neck muscle was not clearly identified (stars). In (*B*), the CSN runs inferomedially through the HSM. Arrowheads: nerves entering into the urethral sphincter. Scale bars: 10 mm.

Figure 4

Schema and intraoperative finding showing HSM and CSNs.

HSM is highlighted in green. Schematic drawings showing (A) Lateral view through the bony pelvis. (B) Transverse section at the level of the proximal urethra. (C) Intraoperative view from the cranioventral side. Intraoperative findings of the left HSM during left nerve-sparing female robot-assisted radical cystectomy are shown in (D) and (E) (same picture with the HSM highlighted in green). The image was captured after the bladder neck was transected. HSM: hiatal smooth muscle, CSNs: cavernous and sphincteric nerves, EPF: endopelvic fascia

Supplementary Figure 1 (Figure S1)

Topohistology of the bladder detrusor and endopelvic fascia in frontal sections from a 79-year-old woman(same specimen as in fig. 1). (*A, C*) HE staining; (*B, D*) elastica Masson staining; (*E*), smooth muscle actin immunohistochemistry; (*F*), S100 immunohistochemistry. The section in (*A*), which includes the anterior wall of the urethra (UR), is located 2 mm anterior to the section in (*B*). In (*A*) and (*B*), there is a clear border (dotted line) between the bladder neck and urethral smooth muscles (UR). The endopelvic fascia (EPF) makes a thin reflected lamina (arrows) extending upward. (*C*) and (*D*) correspond to the circles in (*A*) and

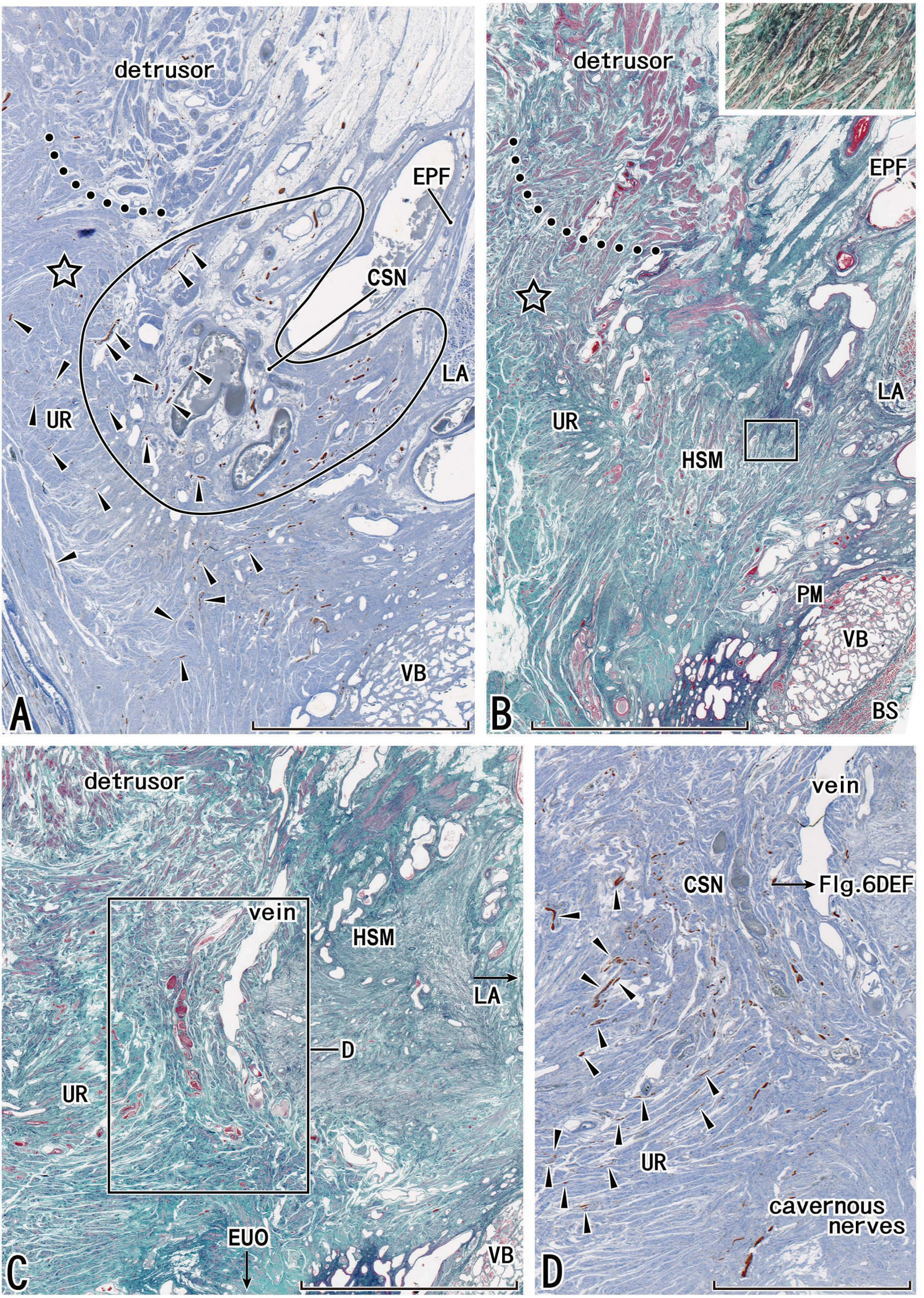
(*B*), respectively, and show muscle fibers (deep red) of the rhabdosphincter (RS). (*E*) and (*F*) are higher magnification views of the squares in (*B*) and show urethral smooth muscles and detrusor nerves, respectively. Where the bladder neck meets the intrapelvic loose connective tissue, the upward course of DNs can be identified in the medial side of the upward reflection of the endopelvic fascia. The perineal membrane (PM) is identified as a thick, fiber-rich, elastic membrane (*B*). Scale bars: (*A, B*) 10 mm; (*C*) 0.1 mm; (*D, E*) 1 mm.

Supplementary Figure 2 (Figure S2)

Topohistology of the bladder detrusor and vaginal wall in frontal sections from a 75-year-old woman. (*A*) Elastica Masson staining shows the topographical relationship between the inferomedial edge of the levator ani (LA), the bladder detrusor, the external urethral orifice (EUO) and the anterior vaginal wall (VAG). (*B, C*) Images correspond to the small squares in (*A*) and show the bladder detrusor inserting into the vaginal wall. (*D, E*) S100 immunohistochemistry. (*D*) corresponds to the large square in (*A*) and shows abundant nerves running in and along the endopelvic fascia (EPF), some of them direct to the bladder detrusor. (*E*) is 1 mm anterior to (*D*). Toward the inferolateral wall of the urethral smooth muscle, CSNs run inferomedially through the venous plexus between the rhabdosphincter area and the levator ani. Arrowheads: nerves entering into the urethral sphincter. Scale bars: (*A*) 10 mm; (*B, C*) 1 mm; (*D, E*) 5 mm.

Supplementary Figure 3 (Figure S3)

Immunohistochemistry of the detrusor nerves (DN) and the cavernous and sphincter nerves (CSN). (*A*–*C*) and (*D*–*F*) show the nerves indicated in figs. 1 *F* and 2 *D*, respectively. (*A, D*) Neuronal nitric oxide synthase (nNOS) immunohistochemistry. (*B, E*) Vasoactive intestinal polypeptide (VIP) immunohistochemistry. (*C, F*) Tyrosine hydroxylase (TH) immunohistochemistry. Both nerves contained abundant TH-positive sympathetic fibers and nNOS-positive parasympathetic fibers, as well as a few VIP-positive parasympathetic fibers.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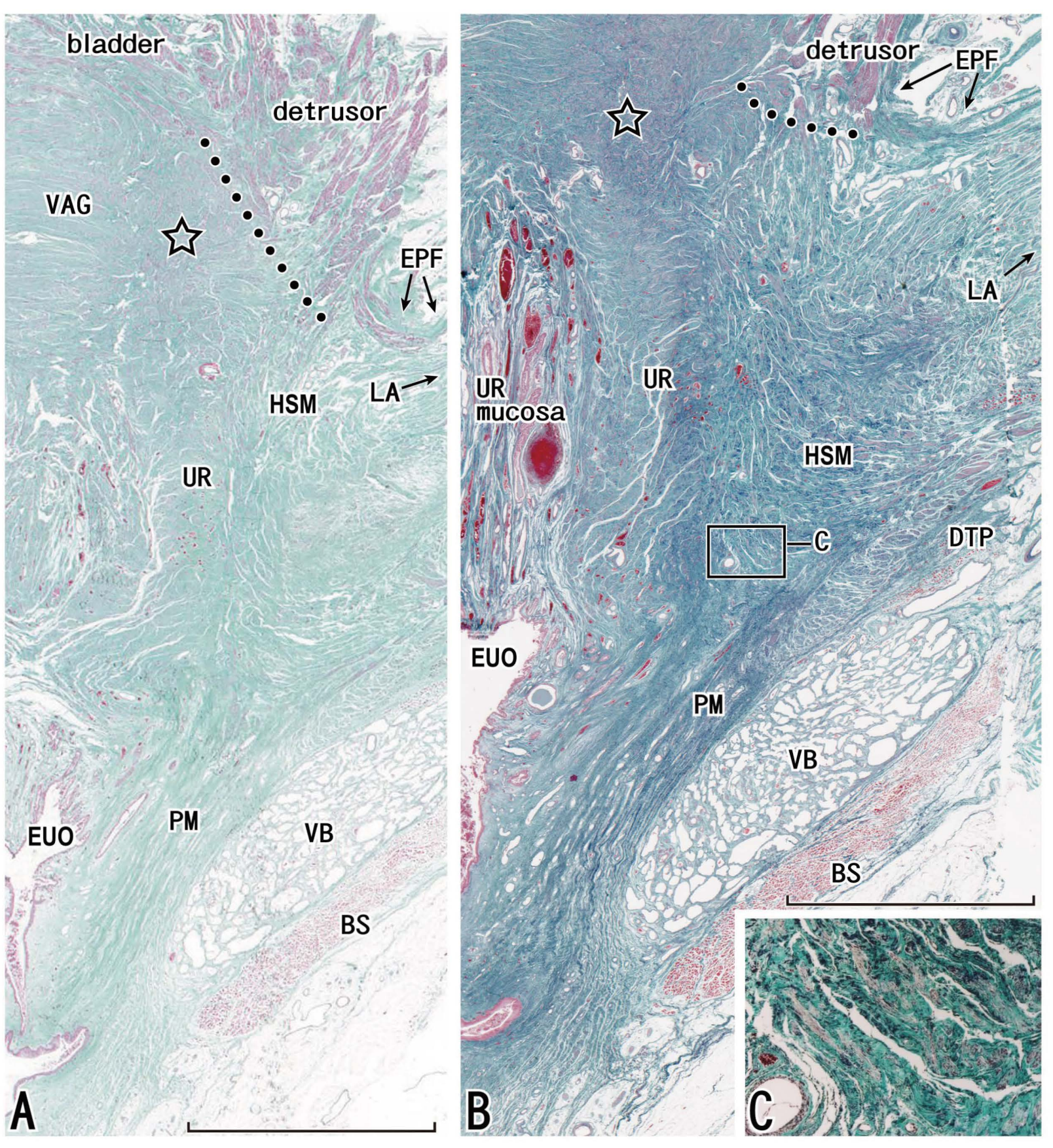
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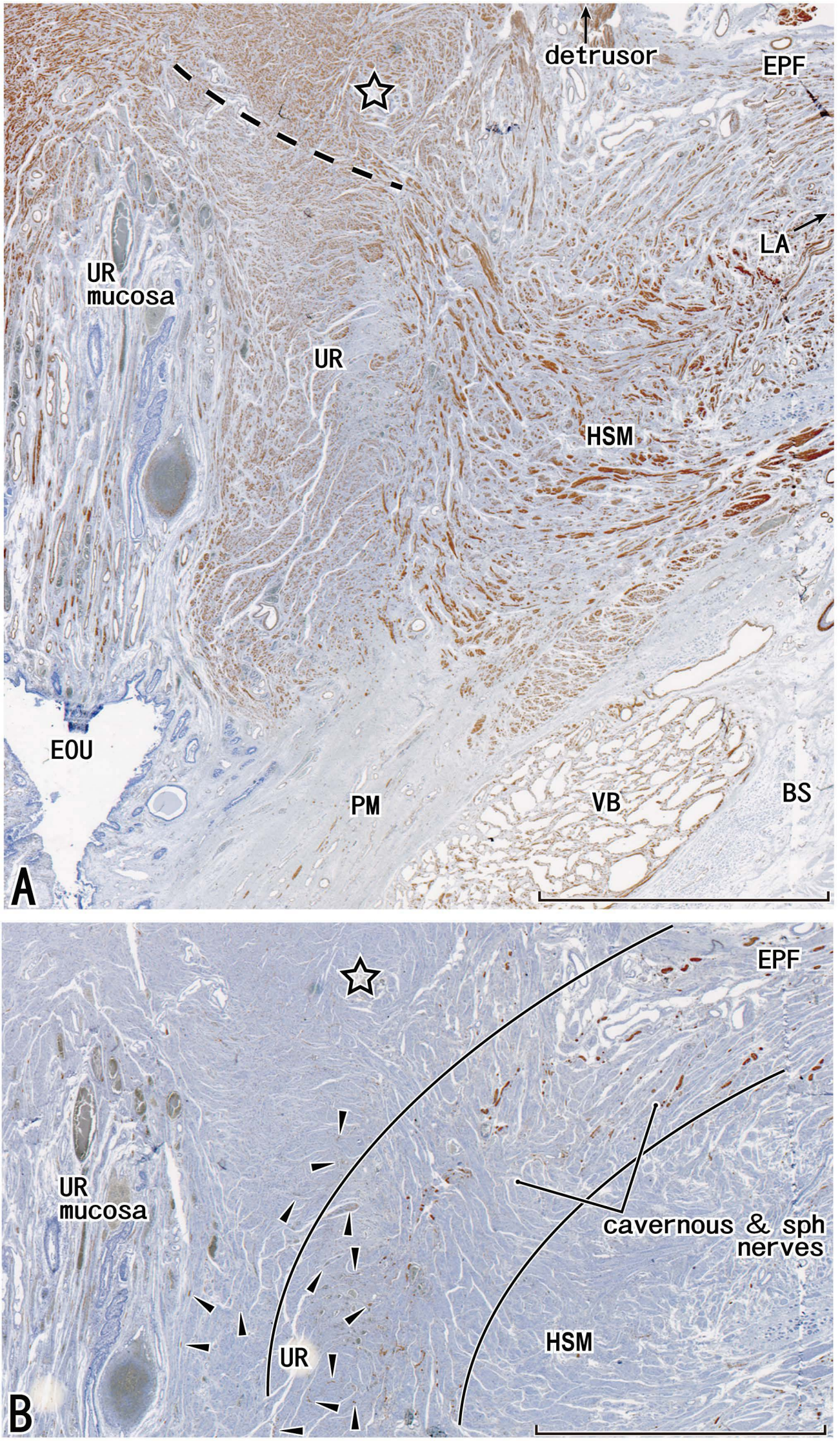
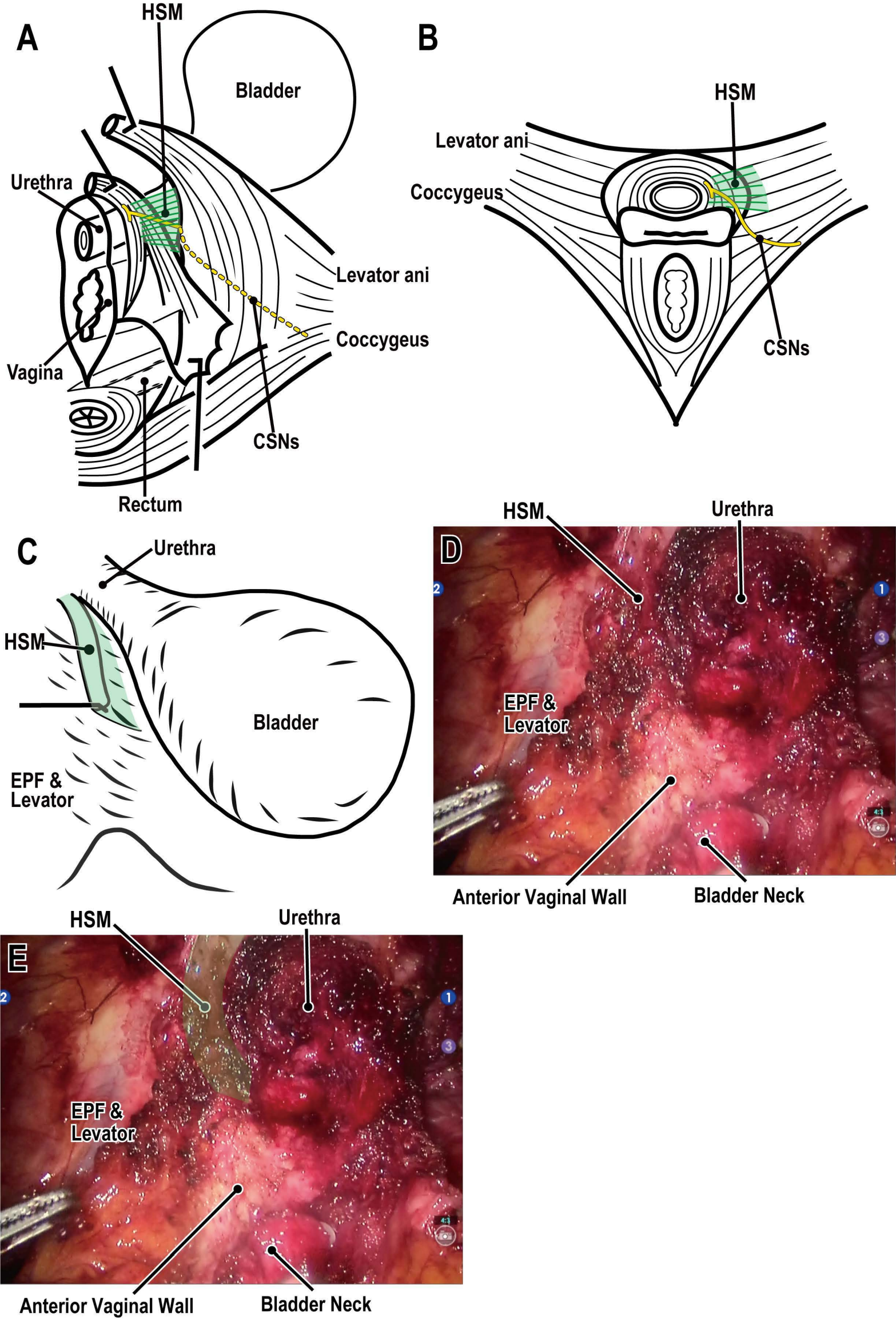
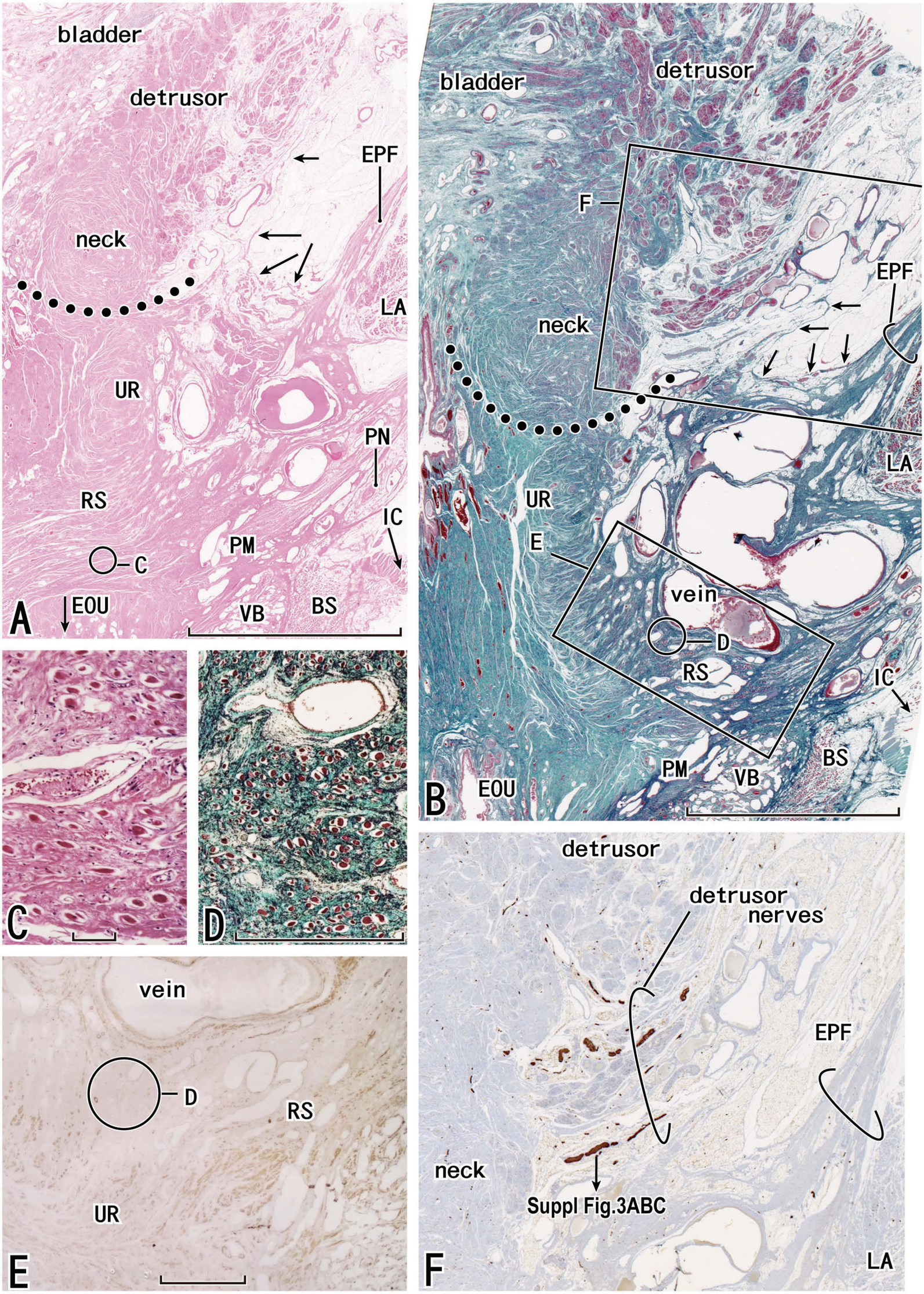
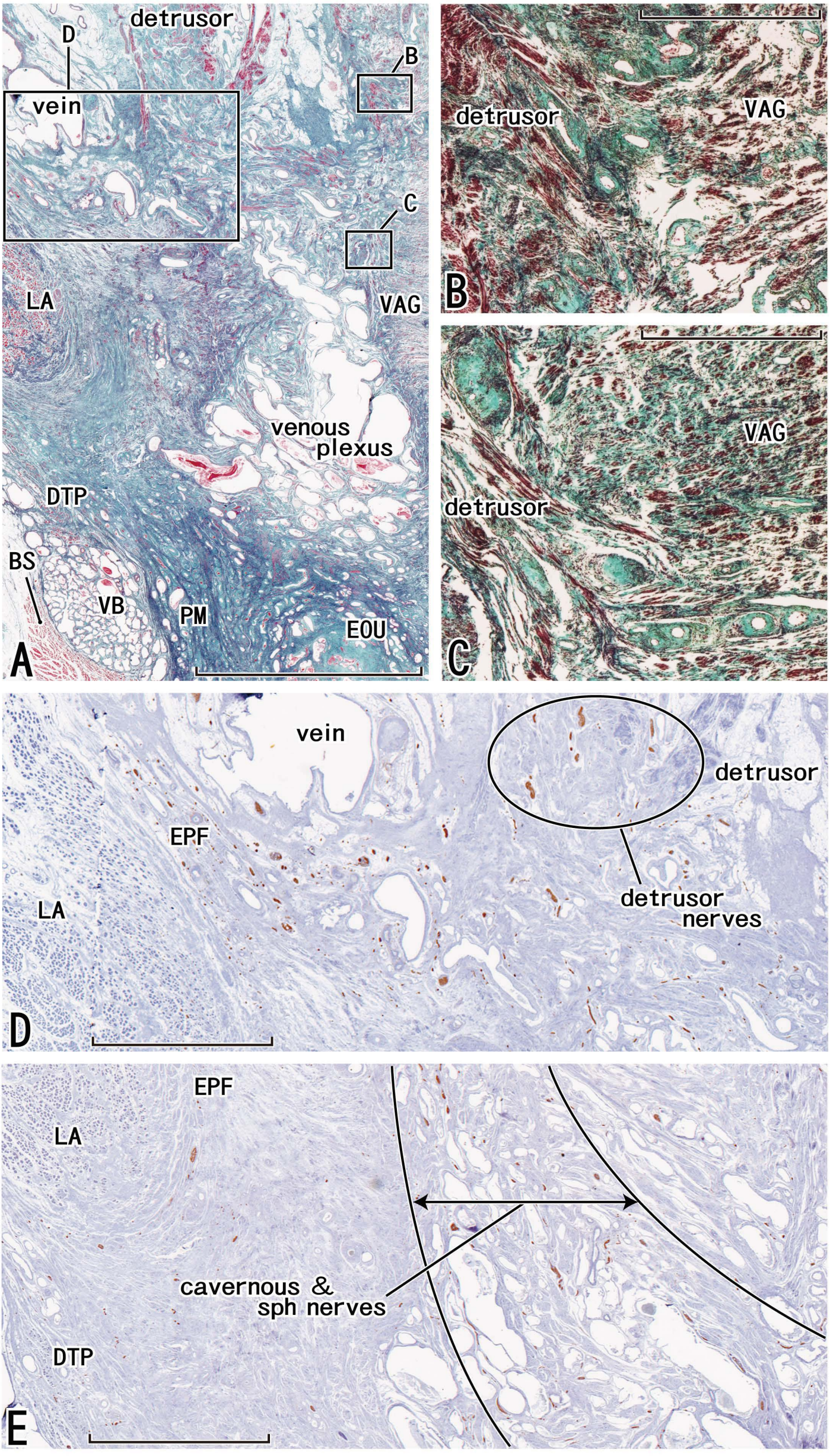
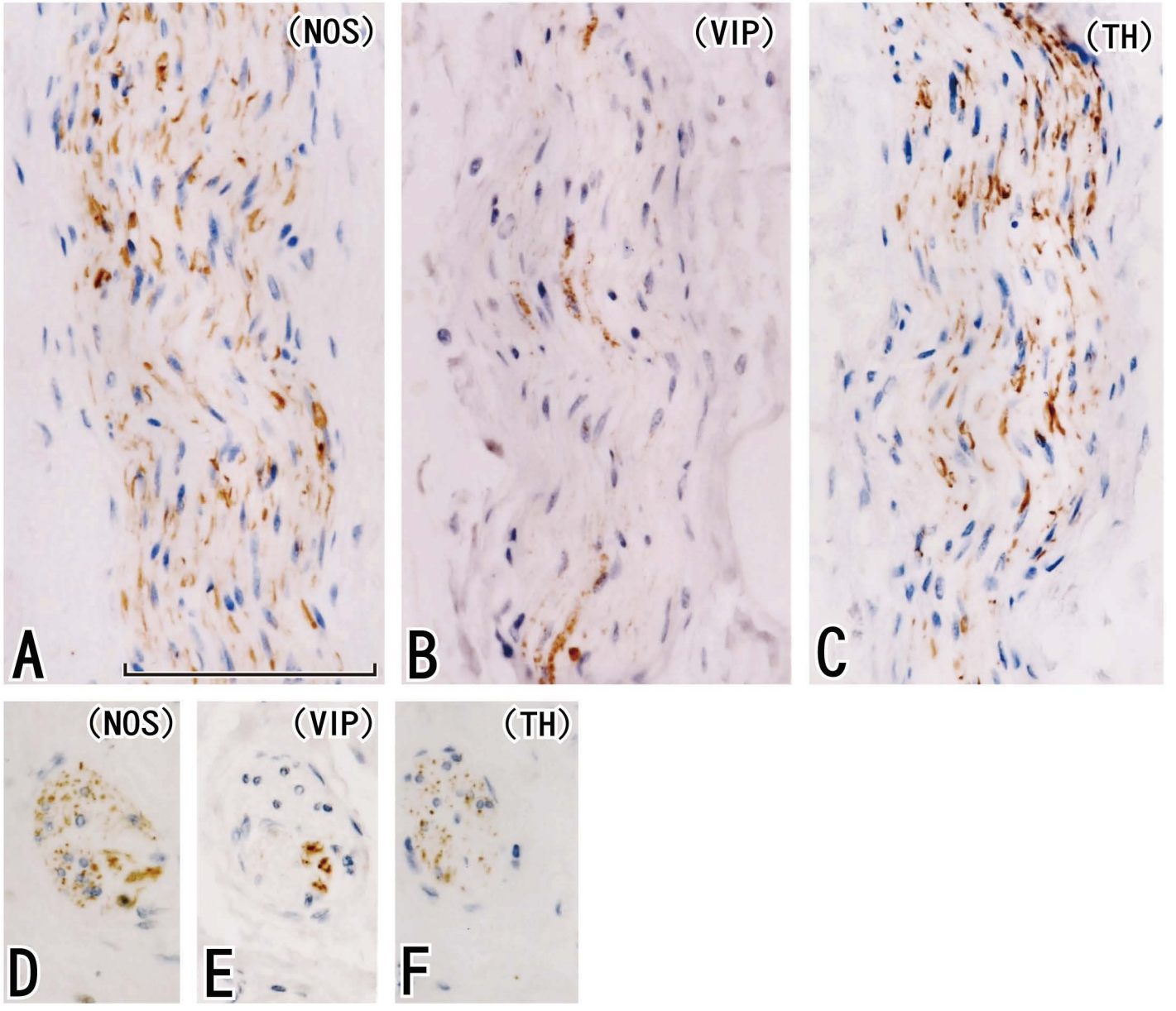

detrusor
EPF
LA
UR
mucosa
UR
HSM
EOU
PM
VB
BS
A
EPF
UR
mucosa
cavernous & sph
nerves
HSM
UR
B


A
HSM
Bladder
Urethra
Levator ani
Coccygeus
Vagina
CSNs
Rectum
B
HSM
Levator ani
Coccygeus
CSNs
C
Urethra
HSM
Bladder
EPF &
Levator
D
HSM
Urethra
EPF &
Levator
Anterior Vaginal Wall
Bladder Neck
E
HSM
Urethra
EPF &
Levator
Anterior Vaginal Wall
Bladder Neck


bladder
detrusor
EPF
neck
LA
UR
PN
RS
IC
PM
C
EOU
VB
BS
A
bladder
detrusor
F
EPF
neck
LA
UR
E
vein
D
RS
IC
BS
PM
VB
EOU
B
C
D
vein
D
RS
UR
E
detrusor
detrusor
nerves
EPF
neck
Suppl Fig.3ABC
LA
F


detrusor
D
vein
B
C
LA
VAG
venous
plexus
DTP
BS
VB
PM
EOU
A
detrusor
VAG
B
VAG
detrusor
C
vein
detrusor
EPF
LA
detrusor
nerves
D
EPF
LA
cavernous &
sph nerves
DTP
E


(NOS)
(VIP)
(TH)
A
B
C
(NOS)
(VIP)
(TH)
D
E
F

Supplementary Table 1 (Table S1)

Relationship between presence of hiatal smooth muscle (HSM) and pelvic organ prolapse (POP).

HSM	No POP	POP	Total
Present, number (%)	5 (55.6)	4 (44.4)	9
Absent, number (%)	13 (100)	0 (0)	13

P<0.01 (Pearson's chi-squared test)