Pelvic Floor: Foundational Science and Mechanistic Insights for a Shared Disease Model

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Chapter 1: Pelvic Floor Structural Anatomy and the Mechanism of Disease: State of the Science and Future Directions

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Introduction

Engineers use their knowledge of structural components and material properties to create complex systems. If one considers the brick, mortar, and architectural design involved in building a skyscraper, malfunction of any one of these components could lead to structural failure and catastrophic consequences. However, even in an optimally designed skyscraper, forces of nature, such as an earthquake, can result in structural failure. The pelvic floor is a biologically complex system that is subject to structural failure of its components leading to prolapse and incontinence. Additionally, forces of nature, such as vaginal delivery, can alter way in which the pelvic floor structural system functions.

Currently, women have a 20% chance of undergoing surgery for either pelvic organ prolapse (POP) or stress urinary incontinence (SUI) by age 80, with re-operation rate as high as 29%.[1] Thus, mechanistic and translational research into the anatomical etiology of pelvic floor disorders (PFDs) is critical to refining our understanding of these conditions, improving treatments and developing novel preventative strategies. The purpose of this review is to advance such endeavors by sharing what is known and what questions are yet to be answered regarding the structural causes of POP and SUI. In

the following sections, the pelvic floor components have been organized into the structural engineering categories of brick, mortar, infrastructure, and applied forces.

The Bricks

Bony Pelvis

Like bricks in a wall, the integrity and alignment of the pelvic structures will dictate function, stability, and strength of the pelvic floor. The bony pelvis serves as a frame to which the coccygeus and the levator ani muscles, and connective tissues attach. Pelvimetry has been utilized for comparisons of the bony pelvis between women with and without POP by means of different imaging modalities. One computed tomography pelvimetry study of multiparous women with $\geq$ Stage II vaginal prolapse[2] and matched controls showed a mean transverse diameter of the pelvic inlet to be significantly greater in women with prolapse.[3] A larger intraspinous diameter has also been observed in women with POP.[4] Magnetic Resonance imaging (MRI) pelvimetry corroborated the findings of a wide transverse pelvic inlet in women with PFDs. Another study demonstrated that PFDs, and especially POP and SUI, are associated with narrow obstetrical conjugate, the distance between the sacral promontory and the upper medial border of the symphysis pubis.[5] Further exploration of these relationships has lagged, leaving many important questions unanswered such as "Does the wider transverse inlet or narrow obstetrical conjugate result in more strain placed on the pelvic floor or make recovery from an event

such as childbirth, less successful?" A more thorough understanding of how the configuration of the bony pelvis affects pelvic connective tissues and pelvic floor muscles, and to what degree the architecture of the bony pelvis is affected by the genetic and environmental factors is warranted.

Skeletal Muscles

The skeletal pelvic floor muscles (PFMs), which include levator ani and coccygeus, support the pelvic and abdominal viscera. Understanding the origins and insertions of these muscles helps explain their function and role in maintaining normal pelvic support. The levator ani consists of three paired muscle groups: iliococcygeus, pubococcygeus, and puborectalis (**Figure 1**), with the latter two also referred to as the "pubovisceralis" muscle. The pubococcygeus has several subdivisions, including pubovaginalis, puboperinealis, and puboanalis, which attach ventrally to the pubic rami and dorsally to the pelvic soft tissue. Iliococcygeus is a thin muscle that originates from the arcus tendineus levator ani and inserts into the anococcygeal raphe. Puborectalis originates at the pubic ramus and courses behind the rectum, fusing in the midline with the deep portion of the external anal sphincter. The orientation of the pubococcygeal and puborecatalis muscles quantified within the sagittal plane of MR images differ by 60 degrees, while the pubococcygeal and iliococcygeal muscles differ by 8 degrees.[6] However, studies of cadaveric PFMs in which muscle orientation vectors were used to generate 3D vector fields have noted smaller differences (generally < 50 degrees), on average, between PFM subdivisions (in both 3D angles and 2D angles in both the axial and sagittal planes), greater variability, and asymmetry between contralateral PFMs.[7] Therefore, PFMs differ in their mechanism of action within the pelvis and specific mechanics

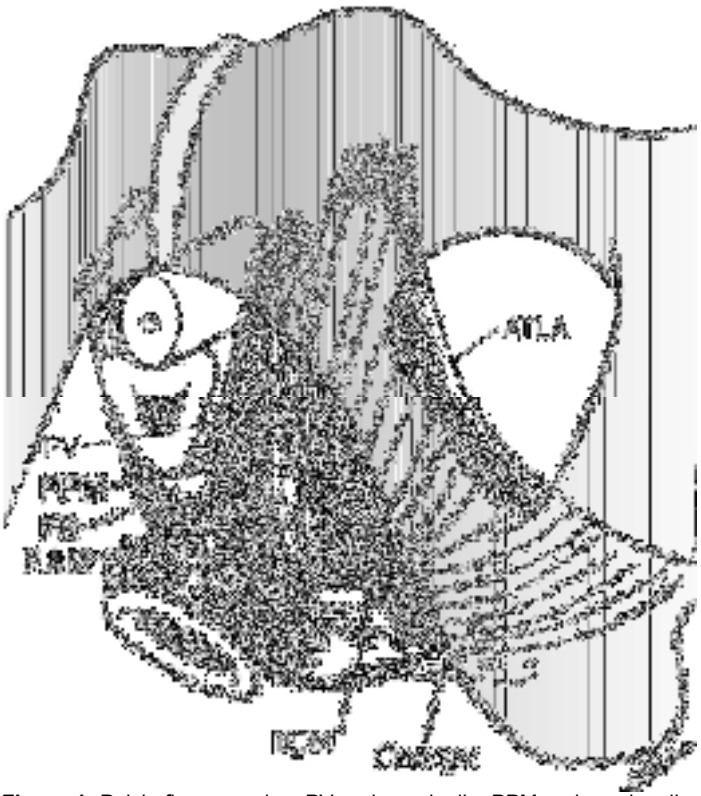

Figure 1. Pelvic floor muscles. PV: pubovaginalis, PPM: puboperinealis, PAM: puboanalis, PB: perineal body, ICM: iliococcygeus, PRM: puborectalis, ATLA: arcus tendineus levator ani, EAS: external anal sphincter

may vary across individuals due to muscle fiber orientation variability. The puborectalis muscles form the lateral borders of the "levator hiatus."[8] More distally, at the level of the introitus, the pubococcygeus muscle contributes to the size of the "genital hiatus." The coccygeus muscle overlies the inferior aspect of the sacrospinous ligament and runs from the ischial spine to the distal sacrum and coccyx. The architectural design of this muscle is conducive to its function as a stabilizer of the bony pelvis and the coccyx.[9]

The levator ani are important postural muscles and accordingly contain a high proportion of slow twitch fibers. In a study by Heit et al., aiming to compare levator muscle fiber types between women with and without prolapse, levator ani biopsies were taken during surgery and analyzed histologically. In both groups, slow twitch (type I) skeletal muscle fibers were predominant, comprising 2/3rds of the fibers.[10] Normally, the continuous action of the levator ani helps to maintain a closed genital hiatus which provides support to the pelvic viscera.[8,11] Levator ani dysfunction can result in impaired genital hiatus closure, with force generated during maximal pelvic floor contraction 40% lower in women with relative to women without POP. In the same study,[12] genital hiatal size, measured on clinical exam, was found to be 50% larger in women with versus without POP (4.7±1.4 cm vs 3.1±1.0 cm, p<.001). One long-standing question is whether prolapse is the result or the cause of an enlarged genital hiatus. In a longitudinal study of ~1,200 parous women, Handa et al. discovered that increased genital hiatus size *preceded* prolapse. Furthermore, women who developed POP had a more rapid increase in genital hiatus size (4x that of controls) in the five years preceding the diagnosis. These data demonstrate that failure of genital hiatus closure is a significant causal factor in prolapse development.[13]

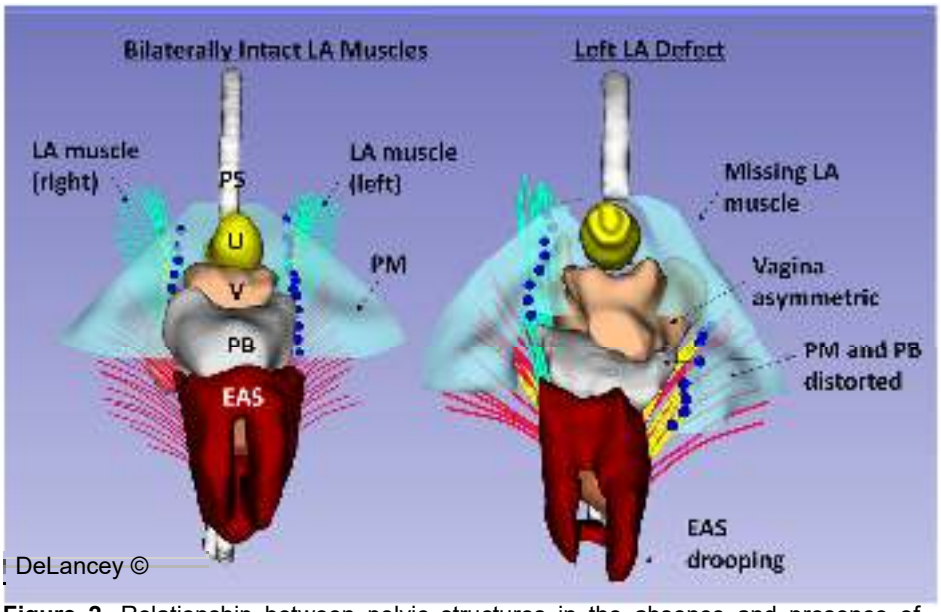

Figure 2. Relationship between pelvic structures in the absence and presence of unilateral levator ani (LA) defect. PS: pubic symphysis, U: urethra, V: vagina, PB: perineal body, EAS: external anal sphincter, PM: perineal membrane.

One factor associated with increased hiatus size is childbirth-related levator ani radiological defects, which can be seen on MRI or ultrasound. **Figure 2** shows a comparison of pelvic organ support with and without a unilateral levator ani defect using 3D reconstructed MR images from living women. The presence of a levator ani defect significantly distorts the alignment and orientation of the pelvic organs. This structural failure is also associated with POP. A landmark study by DeLancey et al. found major levator ani defects infer a 7-fold increased odds of POP compared to normal radiological appearance.[12,14] Other levator ani changes visible on pelvic imaging that are associated with POP, include enlargement of the levator and genital hiatuses and increased levator area and levator bowl volume.[15-17] Despite these associations, our mechanistic understanding of why these changes occur and their role in POP development is incomplete. Specifically, we currently lack a complete understanding of the functional or histologic changes in the levator ani muscles, such as denervation, sarcopenia, or degeneration that may lead to prolapse in women without major levator ani defects.

While levator ani defects have a strong association with POP, the association with SUI is less clear.[18,19] In considering the anatomical building blocks responsible for urinary continence, attention should be paid to the urethral structures. The urethral lumen and vessels are surrounded by a thin inner longitudinal smooth muscle wrapped in a circular smooth muscle layer that is, in turn, enveloped in striated muscle. The circular and striated muscles close the urethra and the longitudinal layer is theorized to aid in opening its lumen, though this is not entirely known.[8] The urethra is supported by the anterior vaginal wall and the levator ani muscles. Continence depends on the ability of both intrinsic urethral function and its external supportive structures to resist increases in abdominal pressure. Maximal urethral closure pressure (MUCP) is a critical measure of urethral function and has been shown to be 43% lower in women with SUI compared to asymptomatic controls.[20] Although loss of urethral support, evident by enlarged Q-tip angle, anterior vaginal wall descent, are associated with SUI, the effect sizes of these parameters range from 0.5-0.6. However, the clinically measurable contributions of the supportive structures appear to be less important for maintaining continence than MUCP, which has an effect size of 1.6. The above indicates that while extrinsitic support of the urethra is important, intrinsic sphincteric function appears to be the most critical factor in maintaining continence.

Apical Ligaments

The PFMs work collectively with pelvic connective tissues and apical ligaments to maintain normal pelvic support. To ask the question of whether the connective tissue or the muscle is more important is like asking which of two blades of a pair of scissors is most important. Apical support is established by the cardinal and uterosacral ligaments which, unlike their names imply, are not true ligaments comprised of dense connective tissue. Histologically, these ligaments are mesenteries containing nerves, vessels, and loose areolar connective tissue.[21] The cardinal ligaments originate from the upper border of the greater sciatic foramen and mesentery of the hypogastric vessels and insert into the cervix and upper vagina. The uterosacral ligaments originate from the upper posterior vagina and/or cervix and insert onto the sacrospinous ligament/coccygeus muscle complex.[21,22] The apical ligaments establish Level I pelvic floor support and impairments in these structures are associated with apical, anterior, and posterior vaginal wall prolapse.[23,24] Some *in vitro* studies suggest that ligament stiffness plays a significant role in maintaining pelvic support, while *in vivo* studies have shown that ligament stiffness only accounts for 19% of variation in cervix location, a proxy for apical support. Ligament length may be a more important factor in apical support.[25-28] In one MRI-based study, cardinal ligament length during maximal straining was found to be 30% longer in women with prolapse compared to controls.[29] A major knowledge gap is the role of these ligaments in the development of different types of prolapse. Specifically, it is unknown whether ligament changes are the cause of POP versus result of traction forces from vaginal prolapse, and whether ligament changes are reversible and therefore potential therapeutic targets.

Smooth Muscle

Smooth muscle plays an important role in the pelvic floor and is abundant in the vagina. Many studies looking at vaginal smooth muscle utilize small animal models as rodent vaginal histology is analogous to the human, consisting of four distinct histological layers – epithelium, subepithelium (fibrillar and non-fibrillar components of the ECM), muscularis (smooth muscle), and adventitia.[30,31] Animal studies have shown that regional variation in smooth muscle content and contractility in the vagina exists, which may correspond with different embryological origins of the proximal and distal vagina. For example, Oh et al. found a greater proportion of smooth muscle concentrated in the proximal versus distal vagina of rabbits.[31] These variations may reflect different support needs in those regions. In a study by Skoczylas et al, smooth muscle contractility in the rat vagina induced by electrical field stimulation was greatest in the proximal region, corresponding to the area with the

highest smooth muscle concentration.[32] Regional variation in neuroreceptors in the rat vagina have also been identified and may correlate with differences in function. For example, the smooth muscle in the proximal vagina contains predominantly cholinergic receptors while the distal vaginal contains mostly adrenergic receptors.[32] Animal models also allow studies of smooth muscle adaptations and during pregnancy and degeneration following simulated birth injury. Assessments of vaginal response to distention are possible in the pregnant rodent model. *Ex vivo* studies have shown a decrease in stiffness and contractile properties during pregnancy and delivery of any route with eventual recovery of the baseline stiffness postpartum.[33,34] However, an *in vivo* study in the rat model by Alperin et al[35] found that vaginal distensibility did not recover to pre-pregnancy baseline four weeks after spontaneous vaginal delivery suggesting a permanent change in this biomechanical factor which may be due to histologic changes to smooth muscle.

Human studies of vaginal smooth muscle have mostly been *in vitro.* Excised tissue taken from the anterior vaginal cuff at the time of hysterectomy showed significantly decreased non-vascular vaginal smooth muscle in patients with prolapse versus controls.[36] The contractility of vaginal smooth muscle is also altered in women with prolapse and fails to respond to phenylephrine, suggesting impaired function. In another study looking at smooth muscle content with prolapse, tissue from the anterior vaginal cuff was excised at the time of hysterectomy from 28 women with prolapse and 12 controls. Smooth muscle content was determined using the fractional area of α-smooth muscle actin staining cells in the muscularis. Consistently with the other studies, women with prolapse were found to have significantly decreased smooth muscle content compared to the controls.[37] *However, whether prolapse is a result or cause of altered vaginal smooth muscle remains a critical gap in our understanding of the pathophysiology of pelvic organ prolapse.*

Similar to other sphincters in the body, the urethral sphincter is comprised of an inner ring of smooth muscle and an outer ring of striated muscle. Numerous studies exist on the striated urethral sphincter; however, few studies have focused on the smooth muscle component. In a cadaveric study of 109 women, Clobes et al found that the urethras from older versus younger women (70-89 vs 20-39 yo) had a lower density of smooth muscle fibers and greater thickness, although the latter did not reach statistical significance.[38] Perucchini et al performed urethral histology on 25 female cadavers ages 15-80 and found aging to be associated with a decrease in total number of striated urethral muscle fibers resulting in a 2% loss of striated muscle fibers per year.[39] This age-related change may help explain the increased prevalence of stress urinary incontinence with age. In a study of 82 nulliparous

women ages 21-70, Trowbridge et al[40] found increasing age to be strongly negatively correlated with maximal urethral closure pressure (MUCP) ($r= -0.76$, $p<.001$). For each decade, MUCP decreased by 15 cmH_2O. However, our understanding of the role of the smooth muscle internal urethral sphincter in maintaining normal continence and pathologic changes involved in stress urinary incontinence remains incomplete.

The Mortar

Mortar is a mixture of water, fine sand, and cement. While it adds some structural support, its main role is to bind the other elements of the structure. In humans, the extracellular matrix (ECM) is a combination of water, fibrillar components, proteoglycans and polysaccharides. The ECM is an integral component of the pelvic floor muscles, ligaments, organs, endopelvic and visceral fascia.[41] The ECM maintains tissue homeostasis, adapts to mechanical stresses, and plays an integral role in the regeneration of damaged tissues.[42] Collagen or "glue producer" (Greek kola = **glue**, and the suffix -gen = **producer),** is the most frequently encountered protein in the ECM, followed by elastin, another major component that contributes to the elasticity of pelvic structures.[42] There are at least 16 different types of collagen, with type I and type III being the most abundant in the pelvic floor.[43] In one of the earliest studies evaluating collagen fibrils in PFDs, Jackson et al found a reduction in total collagen content and a decrease in collagen solubility in premenopausal women with $\geq$ Stage 2 POP compared to controls. This study also found a four-fold increase in matrixmetalloprotinases (MMP) 2 and 9, the two main proteinases that degrade collagen and play a major role in collagen turnover and tissue remodeling.[44] Several additional studies have evaluated collagen content, isoform ratios, and protease activity with varying and sometimes conflicting findings. Changes in collagen amount, type, and mechanical properties have all been considered important to POP development. However, inconsistencies in total collagen amount and ratios of type I to type III collagens in POP versus no POP tissues have been reported.[45,46] Perhaps, this highlights an overemphasis in prolapse literature regarding the functional contributions of various collagen isoforms. Kim et al proposed that these inconsistencies may have resulted from the lack of association between tissue structure and function in earlier studies. Using atomic force microscopy (AFM), their group compared the collagen fibrils in sectioned vaginal tissue with Gomori trichrome to identify collagen from four pre- and five postmenopausal women without POP to five women with POP, aged 51-73. AFM is a high-resolution technique that allows for the evaluation of tissue integrity on a nano to micro scale. Using this method,

collagen fibrils from women with POP were found to be stiffer, bulkier, and correlated with immunofluorescent images showing increased type I/type III ratios. Braided bundles of collagen fibrils were absent in both women with POP and postmenopausal controls, while distinctive braids 2-3 μm in width were a standard motif in fibrils of premenopausal controls. In addition, collagen fibrils were thicker and stiffer in POP compared to both control groups. While the overall amount of collagen was decreased on histological analysis, the amount of type I to type III was doubled in POP.[47] This could lead to the thicker fibrils as type III collagen tends to limit fibril width. The findings from Kim's study were consistent with Jackson's study, and when taken collectively suggest that a loss of collagen, an essential component of connective tissue, likely contributes to functionally relevant alterations in the pelvic floor supportive structures. While it is commonly accepted that women with connective tissue disorders have higher prevalence of PFDs, few studies exist on this topic and those that do, rely on patient survey data often with small sample sizes.[48] Owing to small sample sizes, heterogeneity of the populations and tissues studied, and variation in outcome measures and laboratory techniques used, data regarding whether collagen changes are a cause or result of POP development are inconclusive.

Elastin is another component of the ECM that has been studied in the context of PFDs. While most studies suggest POP is associated with a decrease in mature elastin, Karam et al. found that POP was associated with lower elastin content.[49] In this quantitative immunohistochemistry study, full-thickness biopsies of anterior vaginal wall were obtained from 33 postmenopausal women undergoing prolapse repair and 10 controls of similar age, undergoing radical cystectomy. In the specimens from women with prolapse, elastin content was marginally statistically significantly lower (10.6% vs 14.4%, $p = .049$) and elastin fiber diameter was significantly smaller (0.9 μm vs 1.8 μm, $p<.001$) relative to the controls. However, using a similar analytic technique, Lin et al. found a significant increase in elastin in anterior vaginal wall specimens from 23 women with prolapse compared to 15 controls.[50] Groups in Lin et al. study were not matched for age or menopausal status and this may account for the discrepancy in findings compared to the Karam et al. study. In addition, small sample sizes and limited statistical power preclude definitive conclusions in either study. In another study by Moon et al., increased expression of the elastolytic proteases in uterosacral ligament tissue from postmenopausal women with POP was found supporting the findings of Karam et al. that prolapse is associated with decreased elastin.[51,52] Lysyl oxidase-like-1 (LOXL1) is a protein linked to postnatal elastin deposition. Lui et al found that mice with LOXL1-deficiency had impaired ability to replenish elastic fibers after parturition which led to prolapse and lower urinary tract dysfunction including

SUI.[53] Other studies have reported changes in elastin quantity and remodeling with prolapse and animal studies demonstrating an association between loss of elastin and POP, suggest that elastin plays an important role in the maintenance of pelvic floor support and continence; however, elastin-related factors that distinguish pathologic and physiologic remodeling in the pelvic floor, remain unclear.

The aforementioned ECM components are key players in the supportive function of the pelvic floor. Specifically, the endopelvic fascia which largely establishes mid-vaginal (Level II) support consists of both collagen and elastin that form a continuous unit of connective tissue to support the bladder, uterus, vagina, and urethra.[54] In the urethra, loosely woven connective tissue and elastin in addition to longitudinal muscle bundles are present in the urethral submucosa.[55] Few studies have examined connective tissue changes in the urethra with SUI, despite treatments like periurethral collagen injections and midurethral slings largely relying on connective tissue for their therapeutic effects. The perineal membrane is a connective tissue structure, composed of both elastin and collagen, that serves as a substrate for attachment of the compressor urethrae, distal vaginal, and levator ani muscles, which function together to establish Level III pelvic support. The two dorsal halves of the perineal membrane attach medially to the perineal body, which is a collagen-rich area between the distal vagina and external anal sphincter. While a few cadaveric studies exist on the perineal membrane,[56,57] our understanding of this structure's role in prolapse or SUI pathogenesis is limited as we lack direct tissue studies in living women.

In mortar materials, small scale defects like dislocation, cracks, voids, and impurities influence their physical properties.[58] Recent advances in microscopy have revealed previously unknown anatomic structures, such as a potentially active interstitial space between cells that adhere directly to the underlying collagen bundles. These spaces, supported by a collagen lattice, may serve as shock absorbers.[59] However, little is known about their function in the pelvic floor. Given the many different types and forms of the proteins in the ECM, continued study of what is normally present and what changes with pelvic floor dysfunction will need to continue.

Infrastructure

Infrastructure contained within the brick and mortar anatomy of the pelvic floor consists of communication channels (nerves) and delivery systems (vasculature). This provides the tissues with neurological connectivity, hormones, nutrients, waste removal, immune support, and gas exchange.

In SUI, the pudendal nerve becomes a focus since it provides motor function to the urinary sphincter and sensory innervation to the pelvic floor. It arrives there from the 2nd, 3rd, and 4th anterior sacral rami coursing behind the sacrospinous ligament, the lesser sciatic foramen, over the obturator internus fascia, in Alcock's canal.[60] In an electrophysiology study using stimulating surface electrodes on a urethral catheter, women with SUI (n=28) had decreased urethral sensitivity, prolonged reflex latency, and prolonged motor response at the urethral sphincter compared to age- and parity-matched controls (n=28).[61] Comparison of single fiber pelvic floor electromyography of the pubococcygeus muscle in 69 asymptomatic women to 105 patients with SUI and/or POP showed that women with SUI have significantly more denervation of the pelvic floor compared to those who are asymptomatic.[62] This leads one to think there is acquired damage underlying this condition with aging and childbirth. However, a longitudinal study of primigravidae assessing pelvic floor neurophysiology found that a prolonged motor unit potential duration was not associated with SUI at 7 or 15 years postpartum. Here, Dolan et al. postulate that prolonged motor unit potential, signaling denervation/reinnervation, may be evidence of injury repair rather than permanent deficit.[63] Whether SUI persists in these patients or not, may depend on the healing process. Nerve healing, more specifically axon regeneration, is modulated by neurotrophins. One important neurotrophin in this process is brain-derived neurotrophic factor (BDNF). Studies performed in a rat model of pudendal nerve crush injury show its importance in nerve repair. Electrical stimulation in these models increases BDNF expression, direct therapy with BDNF accelerated recovery of the neuromuscular continence mechanism, and its inhibition decelerated recovery.[64-66] In contrast, when pudendal nerve terminal latency was used as a proxy for pelvic denervation, no significant differences have been identified between women with and without prolapse.[67] The above could be due to the fact that, as opposed to the sphincteric skeletal muscles, pelvic floor muscles are innervated by the coccygeus and the levator ani nerves.[68] Unfortunately, almost nothing is known about the effects of parturition or aging on the pelvic floor muscle innervation.

Intact vascularization of the pelvic floor structural components is another necessary component of normal function. The importance of healthy vasculature to the urinary continence mechanism is well-demonstrated by diabetes, a small vessel disease, which increases both urge and stress incontinence.[69] The urethral sphincter contains a prominent vascular plexus within the mucosal surfaces that is thought to be a key factor in the urethral closure mechanism.[8,70] In a small study of five continent women who underwent urethral pressure profile measurements before and after

neuromuscular blockade ("curarization") in addition to clamping the arterial supply to the urethra, it was determined that 1/3rd of intraurethral pressure is determined by the vasculature.[71] However, in a larger study comparing five Doppler flow parameters of urethral vasculature between 244 continent and 111 incontinent women, no significant differences were seen in any of the parameters[72] between groups. In another Doppler study by Hall et al, Doppler resistive index was not significantly correlated with maximum urethral closure pressure or Incontinence Impact Questionnaire-7 scores in 53 women with SUI.[73] The conflicting results of these studies indicate that our understanding of the role of urethral vasculature in urinary continence and incontinence is still incomplete.

The role of impaired vasculature in prolapse is even less well-elucidated. Few studies exist on vascular changes with POP. In one study using sidestream dark-field imaging, a novel stroboscopic LED ring-based imaging modality built into a handheld microscopy device, Weber et al showed no difference in vaginal microcirculatory architecture, capillary tortuosity, and microvascular flow between vaginal walls of women with (n=17) vs without (n=10) prolapse.[74] Findings from this study suggest that vascular pathology does not play a major role in prolapse development; however robust conclusions cannot be made due to a paucity of data.

Forces of Nature

PFDs have root causes that are multifactorial, with ethnic and genetic predispositions, increasing body-mass index, aging, functional abnormalities, and vaginal birth, which can disrupt the levator ani complex and its connective tissue attachments to the vagina.[75]

Race/Ethnicity

A cross sectional analysis of the Women's Health Initiative (WHI) showed anterior vaginal wall prolapse to be most common and present in more than one third of the women.[76] While 80% of WHI subjects were Caucasian, there were differences in prolapse noted by ethnicity when controlling for age, BMI, and other health factors. When comparing with Caucasian women, African American women demonstrated a lower risk of uterine prolapse. Compared with Caucasian women, Hispanic women had a higher rate of uterine prolapse. Asian women in this study had a higher risk of cystocele and rectocele when compared with Caucasian women, but a lower risk of uterine prolapse.[76] This finding was not supported by the study conducted in Australia, which found that East Asian women presented more commonly with uterine prolapse, while Caucasians had more posterior compartment

prolapse.[77] In another population study, Hispanic and Caucasian subjects had increased risk of symptomatic prolapse compared to African-American women, with Caucasians more likely to have prolapse at or beyond the hymen.[78] There can be several reasons for discrepancies among populations, but from an anatomical standpoint, studies have reported differences in the bony pelvis which may translate to differences in forces on the pelvic supportive structures. An MRI study with self-reported race categories from 234 participants in the Childbirth and Pelvic Symptoms Imaging Study uncovered such differences. The authors found Caucasian women to have a wider pelvic inlet and outlet with a shallower anteroposterior outlet compared to African-American women. Caucasian women also had less pelvic floor mobility post vaginal delivery.[79] In another study on bony pelvic dimensions and race, African American women were found to have a 5.1% smaller pelvic floor area than their European American counterparts.[80] The authors postulate that the larger size increases the force on the pelvic floor and in turn, the risk for prolapse.[80] In contrast, a study of 96 disarticulated pelvises from the Cleveland Museum of Natural History found no significant differences between races for any pelvic dimension.[81]

Regarding SUI and race, higher urethral closure pressures have been reported in African American women compared to Caucasians and correspondingly, African American women were found to have a lower prevalence of stress incontinence.[82] In a population based study of 2,109 women, prevalence of self-reported daily stress incontinence was highest among Hispanic women, followed by white, black and Asian-American women (36%, 30%, 25% and 19%, respectively, $p < 0.001$).[83] African American women were found to have less SUI than all other groups studied but had the highest prevalence of urgency urinary incontinence, which was also reported by Brown et al.[84]

However, a discussion about race and PFDs would be incomplete without recognizing the impact that systemic racism and cultural biases may have on diagnosis and treatment of these conditions. In a study assessing PFD knowledge among 416 community dwelling women, African American women were significantly less likely to recognize risk factors for PFDs or correctly identify common preventative and curative strategies compared to white women.[85] In truth, the role of race may have less importance in the actual pathophysiology of PFDs than it does in the inherent socioecomonic and educational disparities that lead to fewer non-white women seeking medical care for these conditions or participating in pelvic floor research. As a field, we should intentionally prioritize equitable provision of treatment for PFDs and also strive to improve representation of racial/ethnic minorities in our research.

Genetics

Several studies also suggest a genetic component to POP. Women with first degree family members, such as a sister or mother, with prolapse, have a 2-3 fold increased odds of POP themselves.[86] Twin studies have revealed genetic linkage in both prolapse and stress incontinence, but environmental factors also played an important role.[87] A review on genetic polymorphisms associated with POP showed a variation in COL1A1 (collagen type I, alpha 1) with prolapse.[88] After comparing the alleles for thousands single nucleotide polypmorphisms (SNPs), a genome-wide association study (GWAS) showed that those with POP and their family members have 6 SNPs (4q21 (rs1455311), 8q24 (rs1036819), 9q22 (rs430794), 15q11 (rs8027714), 20p13 (rs1810636), and 21q22 (rs2236479)) that are significantly associated with POP.[89] The above association could be due to the effect of these genetic variants on pelvic floor connective tissues. Connell et al. demonstrated that the homeobox (HOX) gene HOXa11 expression is significantly reduced in the uterosacral ligaments (USLs), a main supportive structure of the uterus and upper vagina, in women with POP compared to controls.[90] Furthermore, comparisons of the collagen type I, collagen type III, MMP2, and MMP9 expression in the same tissue revealed that decrease in HOXa11 expression was accompanied by the dramatically reduced expression of both collagen types, but an increased expression of MMP2 in the USLs of women with POP compared to the USLs of women without POP.[90] HOX genes are highly conserved genes that encode transcription factors that orchestrate tissue-specific differentiation during embryonic development of the urogenital tract. The same group also showed that HOXa11 is critical for the development of the USLs by demonstrating the absence of a USLs in Hoxa11-null mice.[90] This suggests a genetic predisposition to aberrant ligament development, however, variations in HOXa11 expression have never been correlated with inferior biomechanical properties of USLs.

With respect to SUI, a recently published GWAS study that included ~9,000 European women in the discovery cohort with additional 4,000 subjects in the replication cohort, identified replicable genetic risk locus for SUI (rs138724718) located on chromosome 2 near the macrophage receptor with collagenous structure (MARCO), a scavenger receptor, associated with SUI.[91] While the mechanisms by which these SNPs predispose to the development of POP or SUI remains undetermined, the use of genetics in screening at risk patients is an important avenue to pursue in the future.

Repetitive Pelvic Loading

Acquired forces on the pelvic floor also increase the risk for anatomic failure. Obesity has been shown to be a risk factor for PFDs.[76,92] For SUI, the high intravesical forces generated in obese women with cough have been shown to override the continence mechanism even in the presence of normal urethral function.[93] The mechanism by which obesity leads to POP is less clear but likely due to high intraabdominal forces, the effects of which accumulate over time. Chronic straining related to constipation, a form of repetitive pelvic loading, can also impact the pelvic floor, perhaps creating neurological damage. Seventeen women, with increased perineal descent by position of the perineum relative to the ischial tuberosities, split into 11 with long standing constipation (mean 26 years) and 6 with short term (mean 6-8 years) were studied with pudendal nerve terminal motor latency. The long standing constipation group had an increase in this parameter versus the short term group[94] Repetitive physical strain on the pelvic floor in certain occupations such as farm and factory workers is also be associated with POP.[86]

Childbirth

Perhaps the most impactful force of nature on the pelvic floor is vaginal childbirth. The pelvic floor brick and mortar structure undergoes a seismic event during vaginal delivery. Vaginal delivery is a significant risk factor for POP and SUI, with each additional delivery increasing a cumulative risk for these PFDs.[86,92,95] Magnetic resonance imaging (MRI) has been used to describe levator ani muscle abnormalities in women after their first vaginal birth compared to nulliparous women. While there was no levator ani defects in nulliparous women, 20% of the primiparous did have them.[96] These injuries were mostly to the pubococcygeal portion of the levator ani and to a lesser extent, the iliococcygeal portion.[96] In a study of 68 primiparous women at high risk for levator ani injury who underwent MRI at 7 weeks and 8 months postpartum, 41% had a visible levator ani tear at 7 weeks, with no improvement observed by 8 months postpartum.[97] Conversely, bone fractures and muscle edema visualized at 7 weeks showed almost complete resolution by the 8 months MRI. When comparing functional and POP-Q data changes from 7 weeks to 8 months postpartum, women with major levator ani defects had the smallest improvement in levator ani muscle strength and also significantly increased posterior vaginal wall descent compared to women with minor or no defects. While functional measures of levator strength were assessed, infering information on neurologic status, neural innervation of the levators was not directly evaluated. Levator ani neuropathy with childbirth was assessed in a study by Weidener et al in which 58 primiparous women underwent

electromyography of the levator ani antepartum, and at 6 weeks and 6 months after the delivery.[98] This study reported neuropathy in 24.1% of women at 6 weeks, with 64% of them recovering by 6 months. The pattern of acute neuropathic injury causes loss of motor units with resultant action potentials of low amplitude at 6 weeks but once recovered and reinnervated, those action potentials displayed high amplitude at 6 months. The authors report that this pattern suggests a neuropathic muscle injury as opposed to a myogenic injury. However, this study did not report data on the prevalence of levator ani tears and it is possible that some women with impaired neurogenic recovery may have had a structural tear in the muscle. Conversely, permanent denervation of the levators would result in atrophy of the muscle with time, which may appear as a defect on imaging. Neuropathy and structural injury to the muscles themselves likely both negatively affect pelvic floor function; however, the relationship between the two and relative importance of each in the development of PFDs remains unclear.

Cesarean delivery has often been questioned as a means to prevent pelvic floor dysfunction. A study on PFDs and associations with parity and mode of delivery, showed a protective effect of cesarean delivery, but 7 cesarean sections would be needed to avoid one instance of pelvic floor dysfunction.[95] A study of 1,500 women showed that when compared with spontaneous vaginal delivery, cesarean delivery was associated with significantly lower SUI and POP.[99] Viewed another way, changes seen in pelvic muscle strength, often associated with these PFDs, has also been compared between vaginal and cesearean delivery. In this study of 1,100 patients, women with vaginal delivery were more likely to have low peak pressures (<20 cm H_2O) on perineometry during voluntary pelvic muscle contraction compared to women with cesearean delivery. Furthermore, low peak pressures were associated with a significantly faster onset of POP and SUI.[100] In a landmark longitudinal study of 1,528 women recruited 5-10 years after their first delivery and followed for nine years, cesarean delivery significantly decreased the risk of developing POP, SUI, and overactive bladder compared to vaginal delivery. Operative vaginal delivery carried the highest risk of POP and anal incontinence. While cesarean section is clearly protective of pelvic floor injury, it does not entirely eliminate the risk of PFDs. Even among women with elective cesarean without labor, levator ani neuropathy has been reported.[98] Furthermore, not all women who deliver vaginally go on to develop prolapse and stress incontinence. This again leads us to consider the building blocks of the pelvic floor as they weather this force of nature, which leaves some structures unscathed and others in ruins. Therefore, it is incumbent upon our field to develop evidence-based strategies to identify women at high risk for

PFDs prior to pregnancy and delivery and to develop early intervention and rehabilitation strategies to aid in postpartum recovery.

Conclusion

Having taken an inventory of the building blocks assembled in the pelvic floor, one can appreciate the parallels to structural engineering. Structural failures in the pelvic floor are multifactorial and complex, and impairments in one component can compromise the structural integrity of the entire functional unit. Pathophysiology of PFDs is multifactorial and to uncover the root causes, one can think of the material building blocks and their assembly in the pelvic floor anatomy. How does the mortar (collagen, elastin, smooth muscle) interact with the bricks (skeletal muscles, bony pelvis) and infrastructure (nerves and vasculature) to produce support under the forces of nature that produce tension, compression, shearing, and torsion. Where it succeeds and where it fails may guide successful prevention and repair. It is imperative that basic and translational research write these blueprints to the pelvic floor structural anatomy.

This review summarizes our current understanding of the pelvic floor anatomy and how structural impairments lead to pelvic floor dysfunction. In doing so, we have also highlighted several important knowledge gaps, as outlined below. Fortunately, more attention is being given to closing these gaps through research initiatives by organizations such as National Institute of Child Health and Human Development.[101] In addition to levator ani injury, priority should be given to identifying all structural injuries resulting from vaginal delivery including those that occur in pelvic floor connective tissues and smooth muscle, determining the degree to which these injuries recover postpartum, and quantifying the relative contribution of each identified structural impairment to prolapse development and stress incontinence later in life. We should prioritize the development of novel biomarkers and non-invasive imaging techniques to accomplish this goal. In addition, determining the genetic and epigenetic contributions to these disorders should also be prioritized. Once we have a more comprehensive understanding of the factors leading to structural impairments in prolapse and incontinence, preventative strategies can then be developed to target high risk women and novel therapeutic targets can be identified.

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Chapter 2: Biomechanics of the Female Pelvic Floor

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Introduction

Biomechanics is the study of forces and motion in a biological context. Human beings are fundamentally biomechanical organisms since, like many creatures, we must locomote to acquire food and resources to live. Our bodies are constantly acting in response to gravity, friction, pressure, inertia, etc., and yet most of our activities are so common that we often do not recognize the significant complexity of the mechanics that are involved. Because our very livelihood requires that we perform activities successfully, we have evolved in ways that allow for optimal and efficient mechanical performance. Generally, it is only when our bodies are unable to meet the mechanical demand of our daily lives that we realize that something is wrong. Female pelvic floor disorders (PFDs) are symptomatic because of this exact scenario.

Though organs and tissues of the female pelvis are not often thought of in a biomechanical context similar to that of orthopaedic and cardiovascular tissues, this likely reflects our biases as a culture more than the actual functional role of these tissues. In fact, the processes of urination, defecation, intercourse, and childbirth are all fundamentally biomechanical. Certainly, these processes are intimately orchestrated with other mechanisms (e.g. cellular and biochemical events) in the same way that a biceps contraction or the regulation of blood pressure are; however, if you consider the critical functions that these organs and tissues are performing, they are fundamentally biomechanical. Whether it is the storage and evacuation of urine or feces that require tissue stretching and force generation, the flow of urine through the ureters that require coordinated peristaltic motion, or the delivery of a baby that is the result of coordinate uterine contractions with semi-voluntary pushing on the part of the mother, biomechanics is critical.

Considering that human childbirth is one of the most significant and demanding biomechanical event in parous women's lives, there has been comparatively little attention paid to the biomechanics of this event and its long-term consequences for mothers compared to biomechanical processes in other areas of medicine. Unlike the fields of orthopedics and cardiovascular medicine, where biomechanical concepts have been embraced to the point of being incorporated into residency training, female pelvic medicine remains decades behind in its appreciation for the relevance of these same concepts to clinical and research endeavors. Nevertheless, significant

strides, made primarily by biomechanical engineers, have enabled a better understanding of the mechanics of the female organs and their support. The following chapter will highlight where we, as a field of female pelvic medicine and reconstructive surgery (FPMRS), stand in terms of our current biomechanical knowledge and discuss some of the significant gaps in knowledge that are a high priority for researchers and clinicians alike.

Mechanics emerges based on the structure, composition, and anatomy of the human organism. Just as forces and motions can be studied macroscopically for an entire organism or tissue, the study of mechanics is also highly relevant at the level of individual cells and proteins. Thus, this chapter will first revisit some of the fundamental anatomy and tissue constituents that play critical biomechanical roles. For a more detailed description of pelvic structural anatomy, please see Chapter 1. This chapter will then describe some of the biomechanical interactions and responses of proteins and cells that give rise to and regulate macroscopic level mechanics, i.e. at the level of tissues and organs. Indeed, the so-called field of mechanobiology is vast and will only be described with enough background in the context of this chapter to appreciate the mechanical principles and findings that are being discussed. While the work at this scale remains limited as it relates to female pelvic floor dysfunction, it is likely an area that will be critical to understanding these disorders in the future. Next, the chapter will review fundamental concepts of tissue mechanics and describe some of the work that is providing insight into the tissue level changes that are associated with pelvic organ prolapse (POP) and stress urinary incontinence (SUI). This will form a basis for the next section that will cover how computational modeling uses image analyses and experimental data to help us develop hypotheses and predictive simulations that, if validated, have the potential to lead to patient-specific simulations that are used for clinical diagnoses and surgical planning. Finally, the chapter will conclude with a short summary of the significant amount of work that remains to be done in terms of understanding the biomechanics of pelvic floor disorders. It is hoped that the reader will come away with an appreciation of the interconnectedness of biomechanical research from the smallest of scales all the way up to macroscopic simulations of organ systems and why it is critical to support these areas of research in the field of FPMRS.

Anatomy and Composition Related to Pelvic Organ Prolapse (POP) and Stress Urinary Incontinence (SUI)

The female abdominal and pelvic organs are mechanically supported by the coccygeus, the levator ani (LA) muscles, and connective tissue attachments that join the uterus and vagina to the pelvic sidewalls. These structures are divided into three distinct levels (I, II, III, in order of superior to inferior locations within the body). Level I attachments, such as the uterosacral and cardinal ligaments, connect the upper third of the vagina and cervix to the coccygeus muscle-sacrospinous ligament complex and epimysium of obturator internus; Level I structures resist gravity and prevent the downward displacement of the pelvic organs ("Biomechanics of the Female Pelvic Floor," 2016; DeLancey, 1992). Level II structures attach the middle third of the vagina laterally to the arcus tendineus fasciae pelvis. The lateral connections of the vaginal wall prevent the pelvic organs from moving ventrally with increased abdominal pressure that occurs with coughing or pregnancy ("Biomechanics of the Female Pelvic Floor," 2016; Couri et al., 2012; DeLancey, 1992). Level III connects the distal third of the vagina to the pubis and anterior aspect of the ischium. It is generally thought to include the perineal membrane, perineal body, and the superficial perineal muscles (DeLancey, 1992). It should be noted that these Level III structures also provide mechanical support to the distal urethra and external anal sphincter.

LA muscle injury during childbirth is an established contributor to POP development (DeLancey, 1993; Harris & Bent, 1990; Peschers et al., 1997), while vaginal delivery is the greatest epidemiologic risk factor for the development of PFDs (Handa et al., 2011; Kepenekci et al., 2011; Mant et al., 1997; Viktrup et al., 1992). Thus, POP is classically thought of as resulting from defects in the pelvic floor musculature, primarily the LA, connective tissues, or a combination of the two (Raizada & Mittal, 2008; Visco & Yuan, 2003). The current research on the role of skeletal muscle in POP centers on vaginal delivery related damage to the LA. In the case of injured LA, the change in normal anatomical position of the vagina and LA causes widening and opening of the genital hiatus that predisposes the pelvic viscera to prolapse (Schaffer et al., 2005). Vaginal delivery can also lead to muscle necrosis and eventually atrophy and fibrosis (Schaffer et al., 2005; Snooks et al., 1990). Through the evaluation of 319 primigravid women prospectively, the LA showed poor performance and decreased strength (measured via digital palpation and perineometry) in women with POP 6 months after vaginal delivery as compared to before delivery and to women without POP (Diez-Itza et al., 2011). Diez-Itza et. al. also found that instrumental delivery was an independent risk factor for LA dysfunction postpartum, likely because instrumental deliveries increase the trauma incurred by the LA (Diez-Itza et al., 2011). Indeed,

radiologically visible defects of the LA are associated with a greater incidence of POP (Saunders, 2017). Additionally, recurrence of POP after surgical repair is reported at twice the rate in women with radiological LA defects (Lammers et al., 2012). However, when evaluating 89 primiparous women postpartum via magnetic resonance imaging, LA injury resulting from delivery was not associated with SUI, though it was associated with POP and fecal incontinence (Heilbrun et al., 2010). The latter result should be considered with caution because SUI can be masked by POP, so the relationship between LA injury and SUI may be more complicated than we currently appreciate ("Committee Opinion: Evaluation of Uncomplicated Stress Urinary Incontinence in Women before Surgical Treatment," 2014; Sussman et al., 2020). Thus, there are likely structural defects that may influence SUI more than POP and vice versa; but the interconectedness of support and biomechanical interplay of pelvic organs within this space should not be ignored.

Interestingly, not all women with LA tears develop POP, and not all women with POP are diagnosed with LA tears (DeLancey et al., 2007). In addition to physical injury, weakness of the LA may result from genetic factors, oxidative stress, or abnormal mitochondrial activity (Visco & Yuan, 2003; Yiou et al., 2009). Differential expression of myosin fibril and H-protein related genes in women may result in abnormal length and quality of myofilaments with poor contractile function, which creates inadequate pelvic floor musculature and eventually causes POP. It was found utilizing reverse transcriptase polymerase chain reaction analysis that myosin-binding protein H expression is down-regulated up to 6-fold in the pubococcygeus muscle (part of the LA) of patients with POP (n=17, biopsies obtained during pelvic reconstructive surgery) relative to those without (n=23, biopsies obtained during other gynecologic surgery) (Hundley et al., 2006). In another study performed on pubococcygeus biopsy specimens, myosin related proteins (quantified via microarray analysis) were significantly downregulated and inhibitory actin-binding proteins, which reduce the actin-myosin interaction, were upregulated in patients with stage III/IV POP (n=5, biopsies obtained during pelvic reconstructive surgery) relative to controls (n=5, biopsies obtained during non-prolapse related abdominal surgery) (Visco & Yuan, 2003). These differential gene and protein expressions might alter the biomechanical stability provided by the pelvic floor musculature and contribute to the pathogenesis of POP.

Microarray analysis has also been used to assess the vaginal tissue of women with SUI. Genes involved in elastin metabolism, including elafin and keratin 16, were differentially expressed in the periurethral vaginal wall samples from women with SUI (n=5, biopsies obtained during surgery

for UI) and asymptomatic women (n=5, biopsies obtained during benign gynecological surgery) (B. Chen et al., 2006). Their increased expression in women with SUI suggests altered cellular responses in the pelvic tissues of women with SUI and that elastin remodeling likely plays an important role in SUI pathogenesis (B. Chen et al., 2006). Since then, the literature has identified genes involved in intermediate filament cytoskeleton and extracellular matrix organization that are overexpressed (11 genes) or underexpressed (fibromodulin and glucocerebrosidase) in women with SUI (Isali et al., 2020). Significant molecular level alterations have the ability to alter the macroscopic tissue and organ system level mechanical behavior. These changes in gene and protein expression in women with POP and SUI likely negatively contribute to the mechanical integrity of the female pelvic tissues, which could either be a cause or consequence of these PFDs.

POP develops decades after childbirth, indicating that remodeling of the connective tissues continues to evolve and may be altered by factors such as aging and menopause (Blomquist et al., 2018; Word et al., 2009). Additionally, women with connective tissue disorders, such as Marfan Syndrome, are at high risk for developing POP due to defects in elastic fiber synthesis (Carley & Schaffer, 2000; Kerkhof et al., 2009). Connective tissue composition also likely contributes to urinary continence. Periurethral tissue biopsies collected from pre- and postmenopausal women with and without SUI (n=8 per group) during gynecologic surgery and analyzed using immunohistochemistry, reveal significant decrease in collagen content and disruption of collagen fibrils in premenopausal patients with SUI, indicative of altered connective tissue remodeling that may alter the tensile strength of these tissues contributing to SUI pathogenesis (Trabucco et al., 2007). While connective tissue dysfunction may therefore contribute to the development of PFDs in conjunction with acute injuries sustained during childbirth, the underlying mechanisms remain unknown.

Another important contributor to the biomechanics of the pelvic organs and tissues is smooth muscle. Despite its known role in women's sexual and reproductive health, this constituent is largely understudied in the context of PFDs (Huntington et al., 2020; Mei et al., 2013; Northington et al., 2011). Anterior vaginal wall smooth muscle morphology has been evaluated in multiple studies where specimens were taken from the apex of the anterior vaginal cuff of women with POP (n=11 to 28, tissues obtained during POP surgery) and controls (n=8 to 12, tissues obtained during

hysterectomy for benign gynecologic conditions other than POP). Comparisons of vaginal smooth muscle content demonstrated that the fractional area of smooth muscle was significantly decreased in women with POP (Badiou et al., 2008; Boreham et al., 2002). Meanwhile, a rodent model for parturition-associated incontinence revealed that smooth muscle inhibition genes are upregulated in the urethras of female Sprague-Dawley rats with experimentally induced SUI (n=10) compared to those that did not develop SUI after intravaginal balloon dilation within 24 hours after spontaneous vaginal delivery (n=14) (Lin et al., 2009). More research is necessary to determine if this change in gene expression has functional implications on the organ level. Smooth muscle is also found in supportive connective tissues such as the uterosacral and cardinal ligaments. More than one third of the uterosacral ligament is comprised of smooth muscle cells and can contract quite significantly, as identifieed in various species, including rat, swine, and human (Baah-Dwomoh, Alperin, Cook, & de Vita, 2018; Donaldson et al., 2021; Drews et al., 2012a, 2012b). Moreover, several studies have indicated that contractile function of these ligaments may have implications in the pathogenesis of POP (Gabriel et al., 2005; Takacs et al., 2009, 2010). The histomorphology and immunohistochemistry of the uterosacral ligaments (biopsies of which were obtained during abdominal or vaginal surgery) of postmenopausal women with POP (n=25) were compared to controls (n=16) (Gabriel et al., 2005). While no differences in smooth muscle cell quantity were found between these groups, the considerable number of smooth muscles cells suggests that the smooth muscle of the uterosacral ligaments contributes to the mechanical support of pelvic organs (Gabriel et al., 2005). In another study, expression of smooth muscle regulatory proteins in the uterosacral ligaments (biopsies of which were obtained during abdominal or vaginal hysterectomy performed for benign conditions) of women with (n=9) and without (n=9) POP was assessed via real-time polymerase chain reaction. The ratio of caldesmon-smooth muscle actin gene expression was significantly larger in women with POP, signifying decreased smooth muscle contractility in the presence of POP (Takacs et al., 2010).

We still understand very little about how structure, composition, and function of the pelvic floor and lower urinary tract components are interrelated. There are many open questions about the in vivo load bearing capacity of the pelvic organs and supportive structures and how these tissues remodel in response to factors such as underuse/overuse; hormones and aging; regenerative therapies; and biologic and non-biologic materials. Unfortunately, female pelvic medicine lags

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significantly behind other areas of medicine on these fundamental questions. Since the researchers working in the filed of FPMRS now have to play catch-up, so to speak, some of the fundamental work that needs to be done to help answer these questions is no longer seen as innovative or mechanistic enough to be fundable in the current highly competitive research environment. Additionally, many in the field have yet to embrace the concept that, like the blood vessels in the cardiovascular system or tendons in the musculoskeletal system, the primary function of these tissues is biomechanical. All of this contributes to the perception that research in this area is not fundable, which discourages junior investigators, especially engineers, from entering this space and perpetuates a derth of PFD-related applications being submitted to funding agencies. Thus, it has become something of a negative feedback loop.

Mechanosensitivity

All tissues and organs throughout the body are mechanosensitive and depend on specific mechanical loads to maintain their structure and function, i.e. mechanical homeostasis. Changes in load create alterations in the cellular- and molecular-level responses as the tissues adapt to a changing environment. Cell shape, growth, proliferation, differentiation, and the extracellular matrix proteins that cells produce are influenced by the local mechanical environment (Chiquet et al., 2009; Ruiz-Zapata et al., 2013). The most visible example of this process is represented by skeletal muscles. Endurance exercises like running or cycling strengthen muscles (Matthews, 1931), while lack of use (e.g. a limb being in a cast or prolonged bed rest) results in atrophy (Bain et al., 2001; Rittweger et al., 2005). What is less appreciated is that all soft tissues respond to mechanical stimuli and those that are load-bearing follow this same paradigm.

Unfortunately, these concepts have only received limited attention in the context of PFDs relative to other fields. This is curious since the vagina, for example, along with its muscular and connective tissue support, is constantly adapting to a changing mechanical environment. The vagina and other pelvic tissues are exposed to changes in force due to intra-abdominal pressure resulting from activities of daily living, filling and emptying of the bladder and rectum, sexual function, pregnancy and parturition, injury to supporting muscles and connective tissues, POP, as well as reconstructive surgeries for POP and SUI (Moalli, Shand, et al., 2005; Ruiz-Zapata et al., 2013). As a result, the extracellular matrix proteins remodel through a process modulated by mechanosensitive cell types (Ewies et al., 2008; Wang et al., 2015; Zong et al., 2010). In the pelvic

floor muscles, the consequence of such changes in load is fiber elongation via sarcomerogenesis, which has been determined via quantification of pelvic floor muscle plasticity of late-pregnant (n=10) and nonpregnant (n=10) Sprague-Dawley rats (Alperin et al., 2015). Though, in the case of pregnancy, it is difficult to delinate the contribution of hormone-induced remodeling versus the mechanosensitive remodeling resulting from alterations in the mechanical environment due to the growing fetus.

With respect to vaginal fibroblasts, we know that they are indeed mechanosensitive. Under healthy, steady state conditions, these cells maintain the extracellular matrix— and ultimately the tissue's overall structure and function—through deposition, rearrangement, and removal of extracellular matrix components (Humphrey et al., 2014). Vaginal fibroblasts from women with POP are responsive to increased mechanical load with greater upregulation of matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9) compared to vaginal fibroblasts from women without POP (Jackson et al., 1996; Moalli, Shand, et al., 2005; Wang et al., 2015). This demonstrates that POP likely reduces the ability of these cells to maintain extracellular matrix homeostasis, negatively impacting the tissue's mechanical integrity. However, whether these changes are causal or secondary to the development of prolapse remains unclear (Hendrix et al., 2002; Zong et al., 2010).

Evidence suggests that the vagina also remodels in the presence of SUI. Vaginal tissue from women with POP/SUI (n=7) and continent controls (n=15), analyzed using quantitative competitive reverse transcription polymerase chain reaction (RT-PCR), demonstrated increased MMP-1 and decreased inhibitor TIMP-1 gene expression in the presence of SUI and POP, indicative of increased collagen degradation (B. H. Chen et al., 2002). These findings are corroborated by another study that found significantly reduced Type 1 collagen content in periurethral vaginal wall specimens and vaginal fibroblasts of women with SUI (n=12, biopsies obtained during SUI surgery) with respect to controls (n=12, biopsies obtained during transvaginal gynecologic surgery) using RT-PCR (Liu et al., 2018). There is a common phrase in bioengineering that potentially explains this phenomenon - use it or lose it. The likely reason for collagen degradation in the presence of POP and SUI is the mechanosensitive response of the extracellular matrix to underuse. This does not refer to sexual function per se; rather, a loss of vaginal support means that the forces associated with daily living are no longer being transferred through the vagina. In these pathologic states, this may result in specific regions of the vagina

being mechanically understimulated. The body responds by degrading the underutilized collagen in those regions—collagen is degraded because it is metabolically inefficient to maintain the integrity of a tissue when that integrity is not required. It is analogus to muscle and bone atrophy in an astronaut that undergoes a long space flight or a patient that undergoes a long period of bedrest. The organ/tissue is not lost completely, but much of its mechanical integrity has been. However, there is also a potential silverlining here. If tissues in the pelvis behave like other loadbearing tissues in the body, it is possible that women with POP and SUI may be able to recover vaginal mechanical integrity if physiologic loading to the vaginal wall is restored. If proven true, this would be very exciting as it would open up a whole new way to look at POP and SUI surgery and highlight a need for rehabilitation protocols after surgery. However, this remains an open question. Thus far, most surgical treatment for SUI and POP are not restoring or replacing an antomical structure and, therefore, do not restore normal physiologic loading.

One of the commonly used surgical treatments for POP and SUI are synthetic mesh augmented repairs. While these interventions are intended to provide mechanical support, they can lead to deterioration of the vaginal and urethral fibromuscular layers. The lack of mechanical stimulus to specific regions of the vagina and subsequent atrophy has been potentially attributed to stress-shielding, i.e. under-stimulation, that occurs due to a mismatch in material stiffness between the native tissue and implanted mesh (Feola et al., 2012). This phenomenon was evaluated in an animal study where the active and passive mechanical properties of mesh-tissue complexes were tested. Mesh-tissue complexes from the vagina were procured and compared between rhesus macaques who underwent hysterectomy and sacrocolpopexy with meshes of various stiffnesses (n=34) and sham controls (n=11) (Feola et al., 2012). Consistent with the theory of stress shielding, tissue deterioration was observed in the animals implanted with the stiffest mesh, as both vaginal contractility and the estimated tissue contribution to the mesh-tissue complex's passive mechanical behavior decreased the most in the presence of the stiffest mesh. Following implantation, the stiffer material, in this case surgical mesh, 'shields' the softer native tissue from the physiological forces and pressures (Majima et al., 2003). This finding was further supported by a later study showing that the histomorphology of mesh-tissue complexes from rhesus macaques (sacrocolpopexy; n=38, versus sham controls; n=12) also demonstrated more severe maladaptive remodeling with the stiffer meshes Stiffer meshes were also associated with the thinnest smooth muscle layers, increased apoptosis, decreased collagen and elastin content, and increased total collagenase

activity as evaluated via Masson's trichrome staining, immunofluorescent labelling of mouse anti-α-smooth muscle actin, *in situ* TUNEL labelling of cell apoptosis, hydroxyproline assay, desmosine crosslink radioimmunoassay, 1,9-dimethylmethylene blue assay, and collagenase activity assay (Liang et al., 2013). A study in rats found similar effects (Skoczylas et al., 2013). The implantation of mesh with a higher stiffness resulted in a greater loss in smooth muscle thickness, as well as a decrease in smooth muscle and nerve function. Collectively, these findings suggest that a stiffer mesh likely results in regions of the vagina that experience greater stress shielding, and smooth muscle atrophy and disorganiziation (compared to remodeling in other constituents, such as collagen and elastin) appear to be early markers to indicate that the vagina is experiencing non-physiologic loads.

While the previous paragraphs highlighted some of the theory and evidence for negative tissue remodeling resulting from underuse/reduced mechanical stimulation, the processes of negative tissue remodeling can also occur in response to high forces or excessive repetitive mechanical loads that may rupture collagen or elastin fibers or induce smooth muscle cell apoptosis, all of which contributes to inflammatory signaling (Dobrin & Mrkvicka, 1994; Frost et al., 2002; Shaw & Xu, 2003). While the goal of the inflammatory response is tissue recovery, the immune response may become maladaptive (Dunn et al., 2015), especially if high forces or excessive repetitive mechanical loads occur before the body successfully repaired the damage resulting from the prior insult. Pelvic floor-specific examples of overuse injuries include the high loads experienced during vaginal childbirth and with persistent straining (Castelucci et al., 2019; Dunn et al., 2015; Fenner et al., 2003; Zong et al., 2010). Inflammatory cytokines were studied in 153 women following vaginal delivery and compared across varying degrees of perineal lacerations (Dunn et al., 2015). Markers of inflammation (e.g., IL-6) were significantly higher in women with severe perineal lacerations 2 weeks to 2 months postpartum, indicative of a sustained inflammatory response after the acute injury that was sustained during vaginal delivery (Dunn et al., 2015). It is possible that these mechanically compromised tissues might be experiencing persistent microdamage events resulting from activities of daily living that would not be considered excessive to non-mechanically compromised tissues. The subsequent reparative process may be insufficient to remodel the tissues before another microdamage event occurs. In such a scenario, previously tolerable loads would now be able to result in additional tissue injury, which results in a positive-feedback loop that can

be called a maladaptive inflammatory response (Dunn et al., 2015; Vaginal Childbirth and Pelvic Floor Disorders, 2013).

Hormones further complicate our understanding of tissue responses to over- and underuse. It is likely that their presence or absence can directly alter the way cells respond to stress or the inflammatory processes that result from microdamage. Using *in vitro* cyclic stretching protocols in which cells are cultured on a flexible substrate or membrane that can then undergo controlled amounts of stretch (frequency, amplitude, and duration), reseachers can determine the relationships between hormone levels (estrogen or progesterone) and mechanical loading. This has been done in separate studies with fibroblasts obtained via vaginal wall biopsies (Wang et al., 2015; Zong et al., 2010). In both studies, fibroblasts were sensitive to cyclic stretching as measured by either cytoskeletal changes or increased MMP activity. However, the presence of hormones was able to obviate those changes, suggesting that they might provide a protective effect to overuse.

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In addition to impacting MMP activity, estrogens have also been shown to induce fibroblast proliferation (Ewies et al., 2008), observed via cDNA microarray of cardinal ligaments from asymptomatic women (n=8). Hormonal status also affects the mechanosensitivity of vaginal fibroblasts differently between women with (n=8) and without (n=7) POP, quantified by immunocytochemistry of vaginal biopsies from women during hysterectomy or repair surgeries (Kufaishi et al., 2016). Specifically, human vaginal cells from controls vs women with POP attached to collagen IV more efficiently and, when seeded on collagen I, expressed lower levels of cell adhesion molecules, demonstrating a potential impact of POP on the way these cells may respond to mechanical and hormonal stimuli (Kufaishi et al., 2016). Collectively, these findings suggest that understanding the alterations in hormone levels experienced during menopause between women with and without POP and/or SUI could help elucidate some of the mechanisms behind the development of PFDs and allow for identification of at-risk women before symptoms develop. This type of mechanistic research could also provide a scientific understanding of some of the observed clinical benefits of hormone therapy with regard to tissue quality.

While there are currently no clinical targets that can be gleaned from the mechanobiology research in FPMRS, the existing knowledge points towards direct parallels to the well-studied tissue responses in other systems. Thus, we can propose a similar mechanical conceptualization of the impact of tissue loading within the pelvis that has already been proposed for other load-bearing

371 tissues (Woo et al., 1982, 1987). It introduces the concept of a "physiologic window" of
372 mechanical stimulation for which tissue type has a range (magnitude, frequency, and duration) of
373 mechanical stimulation that is necessary to maintain its structure and function, i.e. homeostatis. If
374 these tissues are mechanically under-stimulated, i.e. experience mechanical stimulation below the
375 lower limit of the "physiologic window", they will atrophy, which reduces their load bearing and
376 active force generating capacities (Woo et al., 1982, 1987). On the other hand, if these tissues are
377 over-simulated, i.e. experience mechanical stimulation above the upper limit of the "physiologic
378 window", it can lead to micro or macro tissue damage and inflammation. These changes, in turn,
379 would result in reduced passive or active force generating capacity, or completely loose structural
380 integrity and rupture—concepts that will be discussed in more detail in the next section. The
381 "physiologic window" and its adaptability to forces of various magnitudes, frequencies, and
382 durations, is tissue specific and is likely to change with age, exercise/inactivity, hormonal status,
383 and other genetic, demographic, and life-style factors. Importantly, because pelvic soft tissues are
384 largely interconnected, it is likely that mechanical changes in one structure will lead to remodeling
385 of the entire supportive complex.
386 Knowing the tissue-specific details about their response to mechanical stimulation is critical
387 to our understanding of the mechanisms that drive the development of PFDs and how to best treat
388 these complex conditions. For example, if a surgical approach for POP or SUI utilizes autologous
389 tissues, it is important to assure that these tissues will not be continually loaded outside of their
390 "physiologic window", when placed in a new position/configuration, and unable to adapt.
391 Otherwise, it is likely only a matter of time before the repair fails due to tissue compromise. This
392 fundamental knowledge revolutionized treatment paradigms in the field of orthopeadics and
393 cardiovascular medicine. In the early 1980s, the standard of care for an anterior cruciate ligament
394 injury was immobilization, reconstructive surgery, and then additional immobilization (Bilko et
395 al., 1986). Subsequent studies showed that such treatment regiments were extremely detrimental
396 to non-injured surrounding tissues, and precluded adequate remodeling of the anterior cruciate
397 ligament replacement graft (Woo et al., 1987; Noyes et al., 1987; Roth et al., 1988). As a result,
398 these injuries were often career-ending for athletes.. Today, anterior cruciate ligament injury is
399 treated with early reconstructive surgery, followed by supervised rehabilitation therapy. As a
400 result, many athletes can compete within a year after injury (Bien & Dubuque, 2015). While the
401 benefit of rehabilitation protocols following vaginal delivery and surgery for PFDs have shown

mixed results, it could be argued that this is more a consequence of our lack of the fundamental knowledge regarding the injury mechanisms and the "physiologic windows" of pelvic tissues rather than the benefits of rehabilitation. The female pelvis is a complicated system and, thus, deserves appropriate attention in order to achieve similar paradigm-shifting changes in clinical management as were achieved in other fields.

Tissue-Level Biomechanics

Uniaxial Mechanical Testing

Whether it is overt tissue failure or mechanobiological remodeling, the etiology of PFDs, without a doubt, is intimately related to the mechanical properties of pelvic organs and surrounding supportive tissues. Thus, it is important for the readers to gain an understanding of mechanical concepts to appreciate how biological tissues are tested and the information that mechanical testing provides. Consider a uniaxial tensile test—one of the most fundamental biomechanical tests, where forces are applied to a specimen in one direction. The applied forces cause the material to elongate, providing a load (typically measured in Newtons) versus elongation (typically measured in millimeters) curve (Figure 1).

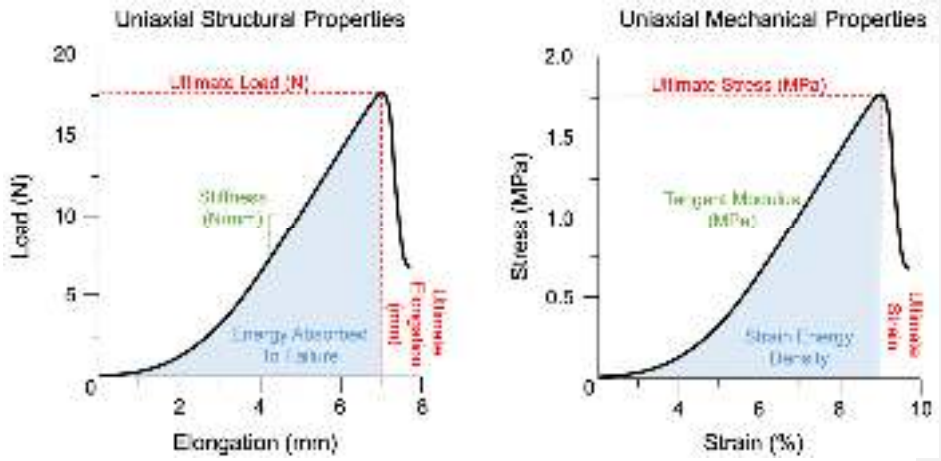

Figure 1: An illustrative example of a load-elongation curve (left) and a stress-strain curve (right) that would be generated from a uniaxial tensile test. To highlight associations between structural and mechanical properties, comparable parameters (e.g., ultimate load and ultimate stress) are denoted similarly.

These data describe the *structural properties* of the sample, which, importantly, are not the same as the mechanical properties of the material/tissue. Structural properties are represented by such parameters as ultimate load (i.e., force at failure), ultimate elongation (i.e., length at failure), stiffness (i.e., the slope of the load-elongation curve that measures the specimen's resistance to being elongated), and the energy absorbed to failure (i.e., area under the load-elongation curve, a measure of the work or energy put into the structure to cause failure). With regard to the latter, the greater the energy absorbed the more the structure has the ability to accommodate the loads applied by either changing its shape or dissipating the energy in other ways. The shock absorbers on a car, for example, absorb energy by both changing shape and converting mechanical energy into heat that is then dissipated. Tissues can also change shape and dissipate energy via collagen realignment and the displacement of water. Structural properties of a specimen are impacted by extrinsic factors, like the size of the specimen. For example, a thicker piece of steel is going to provide more resistance to being elongated and require more force to break it, so its stiffness and ultimate load will be higher. Such tests can be performed on nearly any biological specimen and do not necessitate the specimen to consist of just one material or be of a specific shape.

In order to make apples to apples comparisons between materials, however, it is standard to cut out an isolated piece of a single material in order to measure its *mechanical properties*. Unlike structural properties, which can be influenced by extrinsic factors, e.g., the amount of material that is present, mechanical properties are intrinsic to that specific material in the same way that its density or ability to conduct electricity are. They result from the chemical composition and internal structure (atomic, molecular interactions, and other microstructural elements) of the material. For biological tissues, these properties generally can be attributed to tissue composition and organization in terms of collagen, elastin, and water that is generally associated with glycosaminoglycan molecules. They are physical properties of the material itself and will not change if more or less material is present. For example, a large piece of steel will have the same mechanical properties as a smaller sample that is isolated from that larger piece. This enables us to compare the mechanical properties of steel, for example, to those of silk even though we might not have the same quantities of those materials when testing is performed.

Confusion often arises because, if done correctly, we can obtain data on the structural properties and mechanical properties from the same uniaxial test (Figure 1). For example, if the

structural test described above is performed by applying force along the length of the specimen consisting of a single tissue, the known geometry of the sample can be utilized to perform normalizations that allow for the mechanical properties of the material to also be determined. By normalizing the force by the cross-sectional area of the sample, the stress (typically in units of Pascals, Newtons/meter^2) within the sample can be calculated. In addition, measuring the change in length in the middle portion of the sample and normalizing it by its initial length, strain (unitless) can be measured. This allows for the generation of a stress versus strain curve, which describes the mechanical properties of a material. Parameters describing the stress-strain curve include ultimate stress (i.e. maximum stress before failure), ultimate strain (i.e. the strain achieved at failure), tangent modulus (commonly referred to as Young's modulus for a specific class of materials, representing the resistance of the material to being deformed), and the strain energy density (i.e. toughness of the material).

It should be noted that the tangent modulus is also commonly referred to as the "material stiffness"; however, this should not be confused with the stiffness mentioned when describing the structural properties above. Often engineers will simply use the term "stiffness" because the context is implied (i.e., material versus structural); however, this is often a point of confusion for non-engineers who may not appreciate these differences in context. Another point of confusion is why the term "tangent modulus" is being used here as opposed to "Young's modulus", which many have heard about in their high school or college physics courses. This is because "Young's modulus" refers only to a special class of materials that are common in engineering. These materials are referred to as "LEHI" materials, which stands for: Linear, Elastic, Homogenous, and Isotropic. Biologic tissues are generally non-linear (the stress-strain curve is not linear), viscoelastic (they dissipate energy), inhomogeneous (their composition is not uniform), and anisotropic (they display different mechanical properties depending on the direction in which they are pulled or compressed). Thus, they are not "LEHI" materials. Nevertheless, many publications will still use "Young's Modulus" when referring to biological tissues. By doing so, they are indicating that they are assuming that the tissue is behaving as a "LEHI" material, but this is generally an incorrect assumption for biological systems.

It is important to note that measurements of the mechanical properties of a tissue can be highly sensitive to a number of experimental factors (e.g., hydration, temperature, methods used for measuring strain and cross-sectional area, freezing and thawing, etc.). This makes reproducibility

of even a simple uniaxial tensile test very difficult. Because of a lack of experience that many traditional engineers have with biological tissue, it is not uncommon to find a wide array of mechanical properties being reported in the literature for the same tissue. This underscores the important role of bioengineers who are specifically trained to deal with biological tissues and why it is critical to report detailed methodology when performing these tests.

Biological tissues also display mechanical properties that depend on time. These so-called viscoelastic properties are very important in terms of tissue function (e.g., tissue has the ability to stretch more the longer a force is applied to it). However, a rigorous description of these behaviors is beyond the scope of this article, but can be found in previous literature discussed within the context of female pelvic tissues (Abramowitch et al., 2009; Baah-Dwomoh et al., 2016).

While significant work remains in characterizing the mechanical properties of the female pelvic soft tissues, several experimental studies have been published demonstrating changes in some tissues associated with POP and SUI (Baah-Dwomoh et al., 2016). The majority of these studies have been performed *ex vivo* using the uniaxial testing methods described above. Due to differences in experimental protocols, methods, animal models, and the conditions of the tested tissues, current findings on the effect of POP and SUI on the mechanical properties of the pelvic organs and supportive tissues are conflicting. While some suggest that pelvic tissues become more complaint (i.e. lower tangent modulus) (Epstein et al., 2007; Lei et al., 2007; Rahn et al., 2008) in the presence of POP, others suggest that pelvic tissues become stiffer (i.e. higher tangent modulus) (Jean-Charles et al., 2010). A vacuum probe was used to estimate vaginal wall stiffness *in vivo* in women with (n=25) and without (n=23) POP (Epstein et al., 2007). Similar to a uniaxial tensile test in which the load is known and the resulting elongation is measured, the force of the vacuum is known so that once elongation is measured the stiffness can be quantified. The vaginas of women without POP were significantly stiffer (Epstein et al., 2007). In another study, vaginal tissue samples obtained during transvaginal hysterectomy from women with (n=21) and without POP (n=22) were tested uniaxially, revealing a possible contridiction to the above results - the tangent modulus (reported as Young's modulus in the paper) was significantly lower in women with POP (Lei et al., 2007). This trend has also been observed in fibulin-5 knockout mice. Intact vaginal wall samples from nulligravid fbln5$^{-/-}$ mice without POP (n=4) and nulligravid fbln5$^{-/-}$ mice with severe POP (n=7) were compared via *ex vivo* ring expansion tests—in which a ring-shaped apparatus

515 expands the tissue radially (Rahn et al., 2008). Resulting stress-strain curves demonstrated that the
516 vaginal tissues from mice with POP were less stiff.
517
518 SUI could also be associated with decrease in vaginal stiffness. Utilizing an intravaginal device
519 to increase the transverse diameter of the vagina, the vaginal stiffness of women with (n=21) and
520 without (n=24) SUI was measured *in vivo* and was, on average, less stiff in women with SUI
521 (Verelst & Leivseth, 2007). However, not all studies agree with the above. In the study by Jean-
522 Charles et al, vaginal tissue was collected from patients with POP (n=30, samples acquired during
523 reconstructive surgery) and fresh cadavers without prolapse (n=10) and compared via uniaxial
524 tensile testing (Jean-Charles et al., 2010). Both the anterior and posterior vaginal walls were stiffer
525 in women with POP (Jean-Charles et al., 2010). The aforementioned challenges in measuring
526 mechanical properties accurately are further complicated by the differences in medical and surgical
527 history, mode of delivery, overall parity, and age of the study subjects; as well as specific
528 anatomical regions from which the specimen was procured.
529
530 The uterosacral ligaments (USLs) are commonly tested uniaxially utilizing cadaveric samples
531 (Baah-Dwomoh, Alperin, Cook, & De Vita, 2018; Chantereau et al., 2014; Martins, Lopes Silva-
532 Filho, et al., 2013; Rivaux et al., 2013). USLs, which play an important supportive role,
533 demonstrate superior mechanical properties (i.e. higher stress at failure, tangent modulus, and
534 strain energy density) compared to other similar pelvic "ligaments", such as the round or broad
535 ligaments (Martins, Silva-Filho, et al., 2013; Rivaux et al., 2013). Despite their superior
536 mechanical properties, biaxial mechanical testing has revealed that USLs' tangent modulus in the
537 main loading direction decreased as a function of POP severity (n=10 no POP, n=8 stage II POP,
538 and n=6 stage III/IV POP), suggesting that the USLs of women with POP are less able to resist
539 comparable *in vivo* loads while supporting the vaginal apex (Danso et al., 2020). With all other
540 things being equal (i.e., the applied tension, cross-section of the ligament, etc.), this means the
541 vaginal apex would descend lower in the pelvis than it would if the tangent modulus was higher.
542 Furthermore, since mechanical properties are intrinsic to the material, changes in mechanical
543 properties are a reflection of a change in tissue composition and/or organization. In this same study,
544 for example, collagen organization in the USLs quantified via histology differed between samples
545 from stage II vs stage III/IV POP (Danso et al., 2020). In this case, changes in mechanical

properties were correlated with collagen content; however, this is not always the case since many constituents and organizational changes can have a similar impact. Nevertheless, a change in the mechanical properties of a tissue is always indicative of a change in tissue quality. Thus, it is not surprising that POP severity was correlated with reduced tissue quality (i.e., decreased collagen content) of the uterosacral ligament; but it begs the question of whether this change is causally related to prolapse or is secondary to it. In other words, the reduction in tissue quality could be promoting prolapse development, however, based on what we know about tissue remodeling in response to mechanical changes, it is possible that a defect in Level 2 or 3 support has changed the loading conditions on USL, resulting in degenerative remodeling. If we are eventually going to move clinical practice towards regenerative approaches that treat the causal defect, the answers to these questions will need to be further elucidated. This would require prospective longitudinal studies of women who may or may not develop POP or SUI to identify if tissue quality is altered before or after the disease, in conjunction with *in vivo* imaging to diagnose and functional tests to assess tissue mechanical integrity. In addition, experimental animal models are essential to relate findings of the *in vivo* non-invasive imaging with tissues' mechanical and structural properties, in order to better understand and validate relationships between tissue-level alterations and organ system biomechanics of the female pelvis.

Finally, as previously discussed here as well as the preceding chapter on *Pelvic Floor Structural Anatomy,* pelvic organs and supportive tissues contain a significant amount of smooth and/or skeletal muscle. Thus, in addition to passive properties, which have been the focus of this section, the active mechanical properties that reflect the contractile behavior of these tissues need to be examined as well (Clark et al., 2019; Huntington et al., 2019, 2020). The human vaginal muscularis has been tested via active uniaxial testing, with one end of the specimen fixed to a stationary pole and the other secured to a force transducer to measure forces and displacements generated by active muscle contraction (Northington et al., 2011). Longitudinally tested anterior vaginal muscularis samples obtained during hysterectomy for benign indications from premenopausal women with and without POP (n=6 per group) did not differ in their responses to potassium chloride stimulation that involve activation of voltage-operated Ca^{2+} channels, but contractile responses to phenylephrine, which acts through alpha-adrenergic receptors on the smooth muscle, were detected only in control tissues (Northington et al., 2011). This could be

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related to the reduced expression of $\propto_{1a}$ adrenergic receptors in the vaginal smooth muscle, quantified via analysis of digital fluorescent images in this study, and possibly contribute to the pathophysiology of POP. The differential response to various stimulation mechanisms of the vaginal smooth muscle samples procured from women with POP is an important consideration for future experiments.

Although we are currently unable to determine the active mechanical properties of human pelvic floor muscles *in vivo*, muscle architecture of cadaveric tissues—the greatest predictor of active muscle function—has been evaluated (Alperin et al., 2016). Parity (assessed by comparing 11 vaginally nulliparous and 12 vaginally parous donors) was associated with increased fiber length—likely demonstrating the muscles' need to maintain dynamic force production in response to the increased mechanical loads created by the altered postpartum mechanical environment (Alperin et al., 2016). Meanwhile, aging uncoupled from parity (assessed by comparing 11 donors ≤51 years old and 12 donors >51 years old, difference of 40 years between groups) was associated with dramatically decreased physiological cross-sectional area, indicating up to 50% reduction in predcited force generating capacity of these muscles with aging. The above is a possible mechanism contributing to POP and SUI development (Alperin et al., 2016). The above studies suggest compromised active muscle mechanical properties in the presence of POP and SUI, although, in comparison to passive mechanical properties, the active properties of most female pelvic tissues remain very much understudied.

Collectively, active and passive mechanical properties provide insight into tissue structure and composition, help differentiate diseased from non-diseased tissues, inform constitutive and computational models, and are fundamental to the development of new diagnostic tools and treatment modalities. For example, the tissue mechanical properties most predictive of POP and SUI could become the focus of diagnostic and preventative strategies, while treatments could be made more efficient by focusing on those tissues verified as having the greatest impact on the biomechanics of the urethra and vagina specifically. Thus, this research area requires significant attention in order to advance the field of FPMRS.

Biaxial Mechanical Testing

Pelvic tissues undergo various multidirectional forces in the body. The extracellular matrix composition and organization enable pelvic tissues to resist these forces in order to maintain their

608 shape. Thus, the mechanical behavior of pelvic organs and tissues is mainly anisotropic. This term
609 means that the mechanical properties of the tissue depend on the direction in which the force is
610 applied. While uniaxial tests are useful and physiologically relevant for a number of outcomes,
611 especially when examining tissue failure, their application to anisotropic tissues is limited. A
612 uniaxial test, as implied by the its name, only measure tissue's response to forces applied in one
613 direction at a time (Basha et al., 2009; Northington et al., 2011; Skoczylas et al., 2013). To fully
614 characterize the mechanical properties of anisotropic tissues, biaxial testing methods are needed.
615 These include planar biaxial tensile testing, ring tests, and extension-inflation tests (Huntington et
616 al., 2019; Pack et al., 2020; Robison et al., 2017). Like uniaxial tests, biaxial tests can focus on
617 either passive or active mechanical properties.
618 Biaxial extension-inflation mechanical testing is excellent for tubular tissues such as the
619 vagina and urethra. Pressures can be applied to the lumen while the organ is stretched
620 longitudinally, which helps maintain native geometry and matrix orientation (Akintunde et al.,
621 2019; White et al., 2019). To highlight why this is important, one must appreciate that a tissue's
622 consistuents, especially collagen, can rotate within a tissue if not constrained. In other words,
623 simply pulling along the longitudinal direction without providing pressure within the lumen would
624 allow more circumfentially oriented fibers to rotate and ultimately become recruited to resist
625 longitudinal elongation. A similar scenario would occur circumfrentially if only pressure were
626 applied without any longitudinal stretch. Thus, to more accurately measure tissue properties, it is
627 important to load the tissue using conditions that mimic those applied *in vivo*. This enables
628 experiments that elucidate how composition, structure, and function of tissues are interconnected.
629 For example, using biaxial extension-inflation tests of murine vaginal samples (n=8) before and
630 after intraluminal exposure to elastase, it was determined that the elastase exposure decreased the
631 tissue's collagen-associated stiffness (Akintunde et al., 2019). Further, increased basal smooth
632 muscle tone contributed to a decrease of the vaginal tangent modulus. In other words, elastin and
633 smooth muscle appear to "protect" collagen from experiencing the stresses and strains that it would
634 experience if these other consistuents were not present (or not functional in the case of smooth
635 muscle). This is significant since a common observation in the literature is that the smooth muscle
636 layer—and concomitantly the contractile function of vaginal tissue—is greatly diminished in
637 women with POP (Boreham et al., 2001; Northington et al., 2011). Specifically, immunoblast
638 analysis performed on vaginal muscularis samples from women with (n=15, samples obtained

639 during prolapse reconstructive surgery) and without (n=11, samples obtained during hysterectomy
640 for benign conditions) POP showed that caldesmon,which mediates Ca^{2+}-dependent inhibition of
641 smooth muscle contractility, is substantially more abundant in women with POP (Boreham et al.,
642 2001).
643 Similar mechanical evaluations of tissue constituents of the female urethra with respect to SUI
644 have been conducted, though not probing the same questions as above. Extension-inflation testing
645 was used on the urethras of female Sprague-Dawley rats (Jankowski et al., 2004; Prantil-Baun et
646 al., 2010) to measure the biomechanics and adrenergic responses of the urethral smooth muscle
647 between control animals (n=8) and those that underwent vaginal distension to simulate vaginal
648 birth injury (n=9). In addition, histology was performed on proximal, middle, and distal urethral
649 segments. The urethral smooth muscle tone was higher for controls, indicating that damage during
650 vaginal delivery may reduce urethral smooth muscle function—possibly contributing to
651 postpartum SUI. The histological assessment revealed decreased nerve density after vaginal
652 distension, suggesting that adrenergic nerves are likely damaged during vaginal delivery, altering
653 adrenergic responses in the proximal and mid-urethral smooth muscle (Prantil-Baun et al., 2010).
654 It has also been shown that the implantation of sacrocolpopexy mesh in rhesus macaques
655 (n=27) causes a significant reduction in smooth muscle fraction and contractile function in the
656 vagina compared to sham controls (n=7) when active mechanics were assessed via uniaxial testing
657 (Jallah et al., 2016). Thus, there needs to be further investigation into why smooth muscle seems
658 to be particularly sensitive to changes in the mechanical loading environment. Whatever the reason
659 for this mechanism *in vivo*, this work clearly demonstrates that there is an important interplay
660 between active and passive mechanical properties that are likely critical for normal function and
661 the maintenance of mechanical homeostasis (Clark et al., 2019).
662 It would be ideal if we could conclusively demonstrate how changes in mechanical
663 properties of tissues within the pelvis inform clinical decisions or our scientific understanding of
664 pathological mechanisms as they have for other fields. However, female pelvic medicine is still at
665 its infancy when it comes to this work. Much of the progress that has been made was the result of
666 serendipitous tissue availability from a series of cadavers or surgical patients by various
667 investigators. Thus, the experiments often include small sample sizes, lack appropriate
668 inclusion/exclusion criteria, and/or lack sufficient controls to make definitive conclusions. Animal
669 models have been used to overcome these issues and are critical for the type of basic science

discoveries that guide clinical studies, but many still argue about their relevance to humans. This is why these animal models must be optimized and validated for the specific conditions/mechanisms that are being studied, which will further increase their utility and better steer more costly and risky human studies to improve research efficiency. Thus, until more funding is made available to develop robust study designs that focus on mechanical testing endpoints and/or there is more investment to uncover the appropriateness of specific animal models for research questions that have mechanical testing endpoints, the impact of mechanical testing focused research cannot reach its full potential. However, that is not to say that studies simply characterizing the properties of pelvic tissues are not of value—far from it! These data are essential to serve as inputs into computational models (discussed later in this chapter) that allow for the development of hypotheses and predictions that have immediate clinical relevance. Thus, such work has significant value even when the clinical relevance is not immediately apparent.

Constitutive Modeling

Translating the results of mechanical testing into computational models that can provide hypotheses regarding pathogenises of PFDs and make clinically relevant predictions requires the use of constitutive models. Constitutive models are mathematical descriptions of mechanical behavior that are used in a variety of contexts. They are equations built or selected to best describe tissue's stress-strain data obtained via mechanical testing, as described earlier in this chapter. The simplest constitutive models have few parameters that may have some physical significance, where a high or low value for a specific parameter provides some insight as to what is going on inside the tissue or provides some intuitive sense for how the tissue will behave from a mechanical perspective. However, those models are generally only applicable for a very specific set of experimental conditions, i.e. strain rates, strain ranges, boundary conditions, specimen orientations, etc. A robust model should be able to describe the mechanical behavior of tissues for a large number of experimental conditions. The trade-off is that these models become mathematically complex with many parameters whose physical significance is often lost. In addition, these more robust models, when used in finite element simulations (discussed later in this chapter), add to computational complexity and time. Thus, there is no "best" model for all situations. The "best" model tends to be the simplest that allows the researcher to answer a specific question of interest.

By coupling mathematical models with experimental data in systematic ways, researchers have the potential to uncover relationships between tissue composition, structure, and function. In addition, when utilized within simulations, these models help demonstrate complex interactions between organs and tissues that are not easily measured experimentally. For example, constitutive modeling of pelvic connective tissues has been used to generate finite element simulations of straining as a way of comparing the connective tissues of women with (represented by assuming reduced tissue stiffness due to collagen degradation) and without SUI (Bhattarai & Staat, 2018). This model predicted urethral hypermobility and greater levator plate angulation in women with SUI during increases in intraabdominal presssure, in agreement with clinical observations and imaging studies (Bhattarai & Staat, 2018). As only the connective tissues were altered between the healthy and pathologic models, this underlines the potential contribution of compromised connective tissue integrity to the pathogenesis of SUI. Constitutive models can be phenomenological, meaning there is no underlying physical or biological basis to the mathematical framework, or they can be based on physical and/or biological principles (Brieu et al., 2016; Jean-Charles et al., 2010; Peña et al., 2011). In terms of the latter, the passive uniaxial tensile properties of the vagina were described using the Holzapfel-Gasser-Ogden model for nulliparous, pregnant, and parous ovine samples (n=5 per group) (Rynkevic et al., 2019). This model, which was developed to describs passive mechanical properties of cylindrical multilayered striuctures (Holzapfel et al., 2000), was able to fit all sample groups, shedding light on the importance of collagen content throughout pregnancy and post-partum. Importantly, this study used constitutive modeling to build a bridge between nonlinear mechanical behavior evaluated via uniaxial testing and vaginal morphology evaluated via histology. This means that if vaginal tissue morphology is known, then the mechanical properties can be estimated without performing additional mechanical testing (Rynkevic et al., 2019). The Holzapfel-Gasser-Ogden model has been also used to characterize the biaxial mechanical properties of the murine vagina (n=8) before and after intraluminal exposure to elastase to recapitulate how decreased collagen-associated stiffness in the vagina, resultant from elastase exposure, may be a possible mechanism driving POP development (Akintunde et al., 2019).

While most constitutive modeling focuses on the passive tissue properties, recent investigations sought to model the contraction of the pelvic muscles and organs (F. S. Q. da S.

Brandão et al., 2016; Sharifimajd et al., 2016). A finite element model was generated using magnetic resonance imaging from a healthy volunteer and material properties obtained from previously published data of cadaveric tissues (F. S. Q. da S. Brandão et al., 2016). Yeoh and Ogden constitutive equations, which are more phenomenologically based, were fit to the mechanical data in order to describe the behavior of pelvic soft tissues (F. S. Q. da S. Brandão et al., 2016). The simulated straining with active pelvic floor muscle contraction reasonably matched dynamic magnetic resonance imaging, serving as validation of the model and a potential control simulation to compare to future simulations of POP and SUI (F. S. Q. da S. Brandão et al., 2016). Active contraction of uterine smooth muscle has also been simulated and predicted uterine electrical activity and intrauterine pressures reasonably matched those measured clinically and reported in the literature (Sharifimajd et al., 2016). In the few studies investigating contractility, not all pelvic ligaments and organs were considered due to imaging and current limitations of the model and they have not yet progressed enough to evaluate POP and SUI explicitly. Higher-resolution imaging and robust mechanical tissue data from women with POP and SUI may be incorporated to inform further constitutive models that consider both the contractile and passive components of pelvic floor muscles and organs in both healthy and pathologic states.

Constitutive modeling is also important for improving computational models, discussed below, and understanding complex mechanical behavior. If the eventual goal is to generate accurate patient-specific simulations of PFDs and their treatments, then constitutive modeling is an important step towards that end. Additionally, constitutive models allow for a greater quantity of and more specific parameters describing mechanical behavior. In cases where both increases and decreases in tissue stiffness have been associated with POP or SUI, this could be the key to resolving these contradictions and isolating trends in tissue mechanical behavior that are predictive of POP and/or SUI development. However, until more mechanical data are available to facilitate the improvement of constitutive models and their influence in computational models is explored more thoroughly, the gap between constitutive modeling and clinical impact will remain immense.

Organ and System Level Biomechanics

Clinical Tools and Measures

When biomechanical properties cannot be measured directly, other tools must be used to assess pelvic tissues. Though less robust and more limited than *ex vivo* mechanical testing, the ability to quantify mechanical behavior *in vivo* is invaluable and an important aspect to relating pelvic tissue biomechanics with POP and SUI. Importantly, such tools can often be utilized by clinicians and employed in patients during office visits or while undergoing imaging or surgery.

Pelvic floor muscle contraction can be quantified via electromyography. Utilizing a vaginal probe with built in electrodes to measure LA muscle activity during coughing, one study found no differences between women with (n=16) and without (n=8) SUI, suggesting that pelvic floor muscle contraction during coughing is insufficient to protect some women with SUI from leakage of urine (Madill et al., 2010). In another study, concentric needle electrodes were inserted transvaginally to measure LA and perianally to measure external anal sphincter electromyographic activity at rest and with moderate and maximum contraction in women with SUI (n=9), POP (n=11), and in controls (n=15). Contrary to the prior study, greater myographic activity was measured in controls, supporting that women with SUI or POP have motor unit loss in the LA and external anal sphincter (Weidner et al., 2000). This disparity between studies emphasizes the importance of reproducible and validated methods and underscores the complexity of urinary continence mechanism. Electromyographic activity of the LA measured with concentric needles inserted 2 centimeters laterally to the anus at rest and during contraction identified reduced activity during contraction in multipara (n=50) compared to nullipara (n=20) (Shafik & El-Sibai, 2002). These findings suggest that LA muscle dysfunction associated with parity is a likely contributor to the development of PFDs. Although, when it comes to SUI, it is important to recognize that there is independence between the urethral sphincter and LA, as shown via electromyographic evaluation of the urethral sphincters of 108 women (Kenton & Brubaker, 2002). Thus, both need to be studied in order to fully understand SUI .

Clinical evaluation of POP and SUI can be enhanced with the use of instrumented catheters to measure a variety of pelvic pressures, which are representative of organ and muscle strength. Compression of an instrumented catheter, such as a balloon or microtransducer catheter, by active contraction of an organ's smooth muscle or surrounding striated muscle and/or passive closure of the canal in which the catheter is inserted causes changes in pressure that are measured by a manometer. Increased squeezing of the structure reduces the space in the canal, resulting in pressure increases—just as manually compressing a balloon increases its internal pressure by

reducing the space in which the air in the balloon can reside. Manometers have been used in the vagina, in that case referred to as a perineometer, to measure changes in vaginal pressure and evaluate pelvic floor or perineal muscle strength. Using a perineometer connected to a balloon catheter, pelvic floor muscle strength was found to be lower during squeeze in women with SUI (n=51) compared to continent controls (n=50) (Amaro et al., 2005). This observation was due to the significantly smaller maximum pressures, i.e. the vaginal canal was not squeezed to the same degree in women with SUI as it was in continent women. Similarly, lower perineometer measures in 40 women, age 18-30, were correlated with increased UI and pelvic floor dysfunction symptoms (da Silva Borin et al., 2013). Both of these studies indicate that the pelvic floor muscles are weaker in women with SUI, evident from their reduced ability to constrict the vaginal lumen.

Catheters instrumented with manometers are also often used to assess bladder and urethra function as part of urodynamic studies. In the same way that contraction of the LA squeezes the vagina and increases intravaginal pressure in the area proximal to the contraction, pelvic floor muscle or urethral sphincter contraction will result in pressure increases at specific points throughout the length of the urethral lumen. Depending on the type of contraction or maneuver being evaluated, comparatively smaller pressures in specific regions may be indicative of compromised urethral sphincter or LA muscle strength. The exact number of locations evaluated in a urodynamic study depends on the number of sensors contained within the instrumented catheter. Common urodynamic measures include maximal urethral closure pressure, intravesical pressure at rest and during dynamic maneuvers, and urethral pressure profiles. Reduced maximal urethral closure pressures have been associated with increased SUI severity (n=124) (Yang et al., 2010) and the presence of SUI (n=52) (Swenson, Kolenic, et al., 2017). Additionally, this parameter has been determined to be a significant predictor of urodynamic SUI with these variables having an inverse relationship (n=341) (Wlaźlak et al., 2015) and has been used to evaluate the success of surgical treatments of SUI (n=26) (Kirby et al., 2015). Increased intravesical pressure at maximal cough has also been associated with SUI but only in obese women (n=52) (Swenson, Kolenic, et al., 2017). In general, these findings support that lower urethral pressures, indicative of reduced urethral smooth or striated muscle contractile strength, correspond with SUI.

The electromyographic and vaginal and urethral pressure studies demonstrate how indirect measures (i.e., those not measuring tissue/organ-tissue complex mechanical or structural properties directly) can be used to draw conclusions about the integrity and function of pelvic

organs and tissues. However, these findings are limited by the assumptions of the tools and measurement methods employed, which is why complementary tissue-level mechanical testing is also required to fully elucidate disease mechanisms. For example, the LA's ability to constrict the vaginal lumen with contraction may be influenced by both the active mechanical properties of the muscle and passive properties of the vaginal wall. One may assume a reduced intravaginal pressure is the result of less contraction, but maybe the vagina was stiffer, resulting in less deformation in response to the same amount of LA muscle contraction. Delineating these types of interactions and their contributions to SUI and POP requires additional research and the use of various methodologies simultaneously.

System Level Mechanical Testing

While not particularly common, system level biomechanics can be evaluated via *in situ* structural testing if connective tissue attachments are left intact. This allows for the evaluation of the combined behavior of multiple organs/tissues, which is useful in the study of POP and SUI as it is likely that the biomechanics of many organs and tissues contribute to disease development. This methodology has been used previously in a rodent model by fixing the excised pelvis in place while the distal vagina was pulled until the vaginal connective tissues failed. When performed on Long Evans rats to compare the vagina and connective tissues in nulligravid (n=12), primigravid (n=23), and primiparous postpartum (n=39) animals, this experimental setup quantified increased distensibility, i.e decreased stiffness, of the vagina connective tissue complex in pregnancy (Lowder et al., 2007; Moalli, Howden, et al., 2005). This is likely demonstrative of tissue remodeling during pregnancy that prepares the maternal pelvis for vaginal delivery and reduces the risk of stretch-related injury to the pelvic soft tissues. For *in vivo* experiments, tools have been developed to safely measure the biomechanical response of pelvic organs and tissues in living, sedated women. One such example is the novel computer-controlled linear servo actuator developed to measure the force-displacement behavior of the uterine cervix and suspensory ligaments (Smith et al., 2013). This device has been used to evaluate apical support stiffness in women with (n=38) and without (n=14) POP during preoperative clinical examinations and identified significantly lower apical stiffness in women with apical vaginal prolapse versus controls (Swenson, Smith, et al., 2017). This indicates that reduced stiffness of the uterine cervix and/or suspensory ligaments may be a mechanism or consequence of POP.

As these experiments are not isolating individual tissues, conclusions can only be drawn about the organ-tissue complex as a whole. However, additional animal studies could allow for the study of both organ-tissue complex structural properties and individual tissue mechanical properties evaluation. The ability to employ such methods in living women is valuable with the potential to draw connections between humans and relevant animal models, although currently limited to those women already undergoing surgery, even if for a non-PFDs reason. As many other topics discussed in this chapter, this methodology has many potential applications that have yet to be adequately explored in the field of FPMRS.

Biomechanical Applications of Medical Image Analysis

Medical imaging allows visibility of organs and tissues with minimal risk to the patient and without altering the *in vivo* environment. Though medical imaging was designed to visualize anatomy, it provides unique opportunities for clinicians and researchers to investigate the biomechanics of the female pelvic floor when performed dynamically or across multiple timepoints or patient groups. Additionally, the anatomy segmented from medical imaging is necessary to generate accurate computational simulations of female pelvic biomechanics and variation in anatomy has the potential to highlight biomechanical mechanisms of POP and SUI.

Magnetic resonance imaging (MRI) utilizes magnetic fields and radio waves to generate images and can be performed statically or dynamically and with or without contrast—which can be inserted into the vagina or rectum to improve visibility of those organs. MRI has been used to assess LA muscle function in the context of SUI and POP. For example, dynamic MRI with congruent urodynamics can help determine women with SUI who may or may not benefit from pelvic floor rehabilitation by identifying either slight or greater pelvic floor muscle atrophy (measured via MRI examination), respectively (Del Vescovo et al., 2014). Women with less initial pelvic floor muscle atrophy were more likely to benefit from pelvic floor rehabilitation and resolve their SUI symptoms (Del Vescovo et al., 2014). MRI performed during straining has been used to assess changes in apical ligament lengths and orientations, where cardinal ligament elongation was found to be greater in women with POP (n=10) compared to controls (n=10) (Luo et al., 2014), and to quantify the more caudal vaginal motion and increased posterior vaginal wall deformation

886 in women POP (n=37) compared to controls with normal vaginal support observed during straining
887 (n=35) (Lewicky-Gaupp et al., 2010). These studies suggest that the cardinal ligaments and vagina
888 are less stiff in the presence of POP, which allows more motion of the vagina during straining.
889 Static MRI can be used to help delineate potential biomechanical mechanisms of POP and
890 SUI. The cervix was found to be 36% longer in women with POP (n=51) compared to those with
891 normal support (n=46), and increased cervical length corresponded with increased uterine descent,
892 highlighting how both the cervix and uterus *en toto* are affected by changes in surrounding
893 connective tissue support with POP (Berger et al., 2012). Moment of inertia (measured in
894 milimeters^4), a geometric property of the cross-sectional area that allows definition of a structure's
895 bending or deflection properties, was measured to evaluate the biomechanical impact of POP on
896 the pelvic floor muscles (S. Brandão et al., 2013). In addition to being an effective parameter for
897 assessing pelvic floor damage, moment of inertia was significantly smaller in women with POP
898 (n=21) compared to those without (n=9). This suggests that the pelvic floor muscles, specifically
899 the pubovisceralis, of women with prolapse are less able to resist deformation (S. Brandão et al.,
900 2013). In women with SUI (n=22), bladder neck funneling at rest was more prevalent and the
901 posterior urethrovesical angle was larger than in continent women (n=22) (Pontbriand-Drolet et
902 al., 2016). The posterior urethrovescial angle of women with SUI at rest was actually more
903 comparable to that of continent women during straining, suggesting that the urethra and/or bladder
904 neck are less supported by surrounding tissues in women with SUI (Pontbriand-Drolet et al., 2016).
905 Diffusion tensor imaging and tractography use the diffusion (i.e, motion) of water
906 molecules to visualize muscle and collagen fibers. For example, ligaments generally have a
907 preferred collagen alignment. The assumption is that collagen influences how water can travel
908 through a tissue. In other words, water will follow the path of least resistance when displaced by
909 external forces, meaning molecules will not force themselves between tightly packed collagen
910 fibers and will instead travel along them. This is the concept behind fiber orientation quantification
911 with diffusion tensor imaging. The motion of the water along this path of least resistance provides
912 detailed information about the orientation of fibers within biological tissues. Quantifying the
913 muscle fiber configuration of the female pelvic floor muscles and collagen fiber orientations of
914 pelvic connective tissues increases our understanding of pelvic floor biomechanics by highlighting
915 preferred fiber orientations that will dictate mechanical behavior. Additionally, this provides
916 information that can improve biomechanical models and computational simulations by allowing

fiber orientations to be included in material descriptions (defined by constitutive equations) of female pelvic tissues. Diffusion tensor imaging, thus far, has been performed on the superficial perineal muscles, perineal body, external anal sphincter (Zifan et al., 2018; Zijta et al., 2013), urethral sphincter (Zijta et al., 2013), and portions of the LA (Rousset et al., 2012; Zijta et al., 2013). Notably, these studies have described external anal sphincter fibers crossing at the perineal body before continuing anteriorly as the transverse perineal and bulbocavernosus muscles (Zifan et al., 2018; Zijta et al., 2013), but, as this technique is still emerging, most studies have not been validated and associations with SUI and POP are yet to be established.

Ultrasound, or sonography, is widely available and more portable and affordable than MRI. Ultrasound can be performed in 2D, 3D, or 4D, utilizing high-frequency sound waves to generate images of internal organs and tissues. There are a variety of probes that can be used including those applied to the exterior of the body (i.e. abdominal, transperineal, and translabial) and those inserted into the body (i.e. endovaginal and endoanal). Transperineal ultrasound has been used to visualize the urethra and the changes it undergoes during coughing and straining. By tracking the displacements, velocities and accelerations of urethral segments, researchers determined that the urethras of women with SUI (n=9) displaced further and faster than those of continent controls (n=23) (Lovegrove Jones et al., 2010). This suggests that the urethras and surrounding connective tissues of continent women are stiffer, enabling them to better resist deformations, thereby reducing and slowing urethral motion. Software has also been developed to aid with biomechanical analysis of dynamic imaging. For example, semi-automated software has been used to track the bladder neck during straining and generate kinematic curves, which demonstrate significant differences between women with (n=20) and without (n=10) SUI (Czyrnyj et al., 2018). Larger anterior displacement of the anorectal angle in controls is suggestive of pelvic floor muscle weakness in women with SUI, as a weaker contraction of the LA would result in less anorectal angle displacement.

Basline muscle tone, contractile ability, and passive stiffness of the LA are important for resisting increases in intraabdominal pressure. When these muscles lack either active or passive mechanical integrity, they may deform excessively in response to increased intraabomdinal pressure. As these muscles support surrounding organs, this excessive deformation would result in the larger displacements noted in the uterus and vagina, and may contribute to that observed in the urethra. Ultrasound has been effective in making such measurements. For example, two

studies (Pirpiris et al., 2010 and Ling et al., 2020) used translabial ultrasound to evaluate urethral mobility during straining in women with SUI (n=198 and n=190, respectively). It was determined that increased motion/bending of the mid-urethra is strongly associated with SUI symptoms and urodynamic SUI, respectively. Thus, it appears that the displacement of the mid-urethra is key for passive closure continence mechanisms and important to consider in SUI treatments. Interestingly, ultrasound imaging also revealed that when contraction of the pelvic floors of women (N = 191) during coughing were stronger, those women were more likely to be continent. Though this was a retrospective study, it demonstrates that there may be an active role of the pelvic floor muscles in maintaining continence (Dietz et al., 2012). In terms of POP related ultrasound findings, studies concerning the effect of passive pelvic floor muscle mechanics on the uterus and vagina during straining showed that the uterus descended further (n=263 women with POP) (Shek & Dietz, 2015) and the vagina exhibited larger displacements (n=238 women with POP) (Dietz et al., 2018), indicative of reduced stiffness in women with increased severity and/or quantity of POP symptoms. It was proposed that this may result from diminished LA stiffness in women with POP, meaning the LA will deform further in response to the same applied force. Thus, the assecability of ultrasound and its ability to capture dynamic images of anatomic motion lends itself to biomechanical observations that have clinical relevance.

Strain and shear wave elastography utilize ultrasound to measure biomechanical properties of tissues *in vivo*. In *strain elastography*, the examiner uses the ultrasound probe to exert force that compresses the tissues below it. Tissue stiffness is then calculated by tracking tissue displacement in response to a known force. *Shear wave elastography* works similarly, but instead of exerting force, the ultrasound probe emits shear waves that displace the tissues. While many studies report their findings in terms of elasticity, as shear waves travel faster through stiffer tissues, one can think of this as a relative indicator of tissue stiffness (Gluskin, 2016). However, because individual tissues are not isolated and remain *in situ*, the measurements reflect more of a structural measurement, as opposed to mechanical. Furthermore, due to technical limitations and lack of validation, these are often only estimates.

Despite the above, elastography has been used to assess changes in the pelvic floor associated with SUI and POP. Using strain elastography, urethral mobility and paraurethral tissue stiffness were compared between women with (n=52) and without (n=47) SUI (Kreutzkamp et al., 2017). This study found that SUI was associated with increased urethral mobility and urethral

mobility, in turn, was influenced by paraurethral tissue stiffness (Kreutzkamp et al., 2017). Specifically, in women with increased urethral mobility, the paraurethral tissue at the mid-urethra was less stiff, allowing comparable forces to displace the urethra further than in women without SUI. When comparing women with (n=38) and without (n=20) POP via shear wave elastography, LA muscle stiffness was significantly higher in women with POP at rest, but lower in women with POP during straining compared to controls (Tang et al., 2020). This suggests that the composition of the LA is changing from muscle to connective tissue in the presence of POP, resulting in increased tissue stiffness (Tang et al., 2020). On the other hand, lower stiffness of the pelvic floor muscles identified in women with POP compared to controls during dynamic maneuvers, suggests potential muscle dysfunction that may result in hypermobility of surrounding tissues as observed during ultrasound imaging which was explained previously in this section. Elastography has also been used to estimate biomechanical properties of the perineal body (L. Chen et al., 2015), bladder neck (Ying et al., 2013), and urogenital sphincter (a structure composed of striated muscle located along the caudal two-thirds of the urethra) (Aljuraifani et al., 2018). Although these studies did not establish trends with POP and SUI, they highlight that this type of ultrasound imaging has potential in specific contexts.

Overall, various imaging studies have come to similar general conclusions: Organ and tissue displacements during increases in intraabdominal pressure are greater in the presence of POP and SUI, likely due to reduced stiffness of the urethra, vagina, and surrounding connective tissues and in the passive mechanical properties of the LA. Additionally, smaller displacements and pressures (described in the previous section) in women with POP and SUI during active muscle contraction of the urethra, vagina, and the pelvic floor indicate that reduced contractile ability (specifically in the pelvic floor muscles) corresponds with POP and SUI. As the results of clinical image analyses are visible, they are typically easier to interpret and, therefore, the potential for direct clinical impact is greater—especially in the assessment of conservative and surgical treatment strategies for POP and SUI. As stated previously, clinical imaging also provides the geometric data (i.e., patient-specific anatomy) necessary to develop computational models of female pelvic biomechanics, and, as such, is an important bridge between computational modeling and clinical outcomes.

Computational Modeling

Computational simulations, ranging from 3D reconstruction to virtual reality, have been used to create virtual physiological models leading to the construction of predictive tools that try to represent the complexity of distinct living systems. Conceptually, living systems can be modelled based on different structural levels, namely, molecular, cellular, tissue, organ, organ system, and complete organism. Computational simulations integrate combinations of biological information, tissue and system level biomechanics, constitutive modeling, and imaging.

The goal is to use computational modeling to establish predictions to support the diagnosis, prognosis, and treatment in specific areas of clinical intervention. For example, such models have been used to simulate thermal tissue remodeling in the endopelvic fascia, vaginal wall, and urethral wall during deeply penetrating Nd:YAG laser treatment of SUI in order to compare the transvaginal vs the transurethral approach (Hardy et al., 2017). Computational models have also been used to predict the magnitude of pore collapse of transvaginal mesh in response to multiaxial loading, which increases the risk of mesh erosion, to inform future surgical strategies and mesh design (Barone et al., 2019). Computational models can be linked to other innovative procedures, providing information that enables patient-specific decision-making. One example of such a model can be found in cardiology, where researchers have developed a workflow utilizing cardiac imaging and computational modeling to identify optimal infarct-related ventricular tachycardia ablation targets with the goal of minimizing the area of ablation clinically while maintaining procedure effectiveness (Prakosa et al., 2018). These models incorporate both patient- and region-specific myocardial fiber orientations and cell and tissue electrical properties to suggest ablation targets and this workflow has been validated in both retrospective and prospective human studies. The hope is that, in the future, treatments for POP and SUI can be catered to individual patients in a similar way using computational simulations based on their patient-specific anatomy and tissue mechanical properties. Although this patient-specific paradigm has yet to be achieved in FPMRS, computational simulations have become widely used in the study of PFDs.

There are two main ways that computational simulations can be used: 1) with rigorous experimental data, robust constitutive models, and excellent imaging for both model development and validation to accurately explain complex biomechanical phenomena and make predictions about what will happen when changes occurs (e.g. a surgical intervention) and 2) when there is a lack of imaging and/or experimental data, assumptions can be made and altered to generate hypotheses about how the system might behave and which factors might be most important to the

overall system's function. Either approach can be very informative and used to save both research time and money. It should also be appreciated that this type of work is very iterative. Highly complex computational simulations can require tremendous computer resources. Thus, if investigators can achieve sufficient answers via a simpler simulation that requires less resources, they will choose that route. Because there is less complexity, those simulations are often easier to troubleshoot during development and easier to interpret when results are obtained. Once simpler simulations are understood and validated, investigators can continue to build-in more complexity in order to increase the accuracy of the predictions or to address additional questions.

In regard to biomechanics, the most common type of computational model is the *finite element model*. In order to generate a finite element model, one needs to know or make informed assumptions to define appropriate geometric (i.e., anatomy and shape), material property (e.g., Neo-Hookean parameters), and boundary/loading conditions (i.e., how different parts/tissues interact with one another and their environment). The goal of the forward finite element method is to simulate specific tissues in order to predict resulting stresses and strains during a certain scenario—for example, simulating LA stresses and/or strains during vaginal delivery. For the reverse finite element method, the goal is to use known forces and displacements to calculate the tissues material properties.

It is expected that computational simulations will be able to determine how injuries to the pelvic floor muscles, pelvic connective tissues, perineum, and the urethral and anal sphincters, may contribute to the development of PFDs. With sufficient data and resources, these analyses can be performed on a patient-specific basis or to describe population-based trends. Since the publication of one of the first childbirth models in 2004 by Lien et al. (Lien et al., 2004), computational models are becoming promising tools to quantitatively analyze the biomechanics of the female pelvic floor, allowing structural hypotheses to be examined in ways that were not previously conceivable. For example, a finite element model was generated to simulate POP in order to describe how LA avulsion, levator hiatus enlargement, and increased vaginal length contributed to increased prolapse at maximum strain (Gordon et al., 2019), supporting long-standing clinically-formulated hypotheses concerning the development of POP.

Important steps have been taken to develop understanding of the biomechanics of the pelvic floor during vaginal delivery (Li et al., 2010). Many models have focused on the deformation of the pelvic floor muscles during the second stage of labor, intended to make reasonable stretch

predictions and/or evaluate potential mechanisms of birth-related injury (Hoyte et al., 2008; Lien et al., 2004; Parente et al., 2008). The majority of such models are still in their early stages and will require iterative updates in order to become clinically meaningful. Researchers are still deterimining the sensitivity of model outcomes to the material properties assumed to characterize the pelvic floor muscles (Jing et al., 2012; Li et al., 2011; Parente et al., 2009) and the impact of including/excluding specific pelvic floor muscles (Routzong et al., 2019). The goal of such studies is to determine how model assumptions may be impacting the predicted mechanical behavior of the pelvic floor muscles. Vaginal childbirth is currently a main focus in this field, as researchers hope to elucidate the mechanisms that associate parity with increased risk of POP and SUI development. As vaginal delivery cannot be easily studied *in vivo,* computational simulations are an advantageous tool for studying the biomechanics of childbirth.

In addition to studying injury during vaginal delivery, computational models are being used to simulate the urethra. Dynamic finite element simulations revealed that material properties assigned to the bladder and urethra do not significantly impact predicted vesical pressure and displacements during coughing (Spirka et al., 2013). In this study, models results were validated by comparing the simulated pressures to those measured via urodynamic study. Model validation is important to proving the ability of computational models to accurately predict *in vivo* biomechanics and is an important step towards generating models that can be used clinically. On one hand, the findings from this computational study indicate that vesical pressure cannot be used to validate future finite element models of the urethra, as it will not be indicative of the accuracy of the assumed material properties. On the other hand, this suggests that vesical pressures can be accurately simulated even if the material properties are not well characterized (Spirka et al., 2013). With regards to passive urethral biomechanics, finite element modeling has also been used to isolate which tissues have the greatest influence on urethral shape and motion during straining. Utilizing a sensitivity analysis composed of 50 simulations, one study found that the material properties of the urethra, perineal membrane, bladder, and paraurethral connective tissues were meaningfully impactful, indicating that the mechanical properties of these tissues need to be well defined for computational simulations of urethral passive closure to be reasonably accurate (Routzong et al., 2021). The surgical repair of SUI has also been evaluated with finite element models. By comparing midurethral slings of varying stiffnesses, one study found that stiffer meshes exert more force on the urethra, likely resulting in tissue erosion (S. Brandão et al., 2017).

This provides information that, once verified with additional experiments, can be used to guide future surgical treatment strategies and medical device designs.

Although computational models have made great advances in the past 15 years, improvements in the accurate definition of parameters describing material properties need to be made and the influence of anatomic variation understood before their outcomes can be applied clinically to a larger population of women. However, their potential is great. Computational studies can improve the efficiency and reduce the cost of animal and human experimental studies by identifying tissues worth focusing on via simulations of normal biomechanics or pathologic states; they can be used to test and rule out medical product designs before they've been physically created; and once verified in one scenario can be used to predict another that may be more difficult to image or acquire *in vivo* mechanical data for—such as vaginal delivery. With the improvement in computational capabilities and resources available to clinicians and engineers, computational simulations of female pelvic biomechanics will undoubtedly continue to grow in popularity and complexity in the coming years.

Gaps in Knowledge

Though biomechanical research in the field of women's health has certainly progressed in recent years, we still remain far behind other clinical biomechanics fields, such as orthopedics or cardiology. This chapter has already identified many understudied problems related to PFDs, such as how female pelvic tissues remodel in response to underuse and overuse and how muscle active mechanical properties are altered in the presence of POP and SUI. One goal should be to more aptly use biomechanical concepts to guide POP and SUI preventative, diagnostic, and treatment strategies. For example, just as engineers were able to guide the post-operative rehabilitation of the anterior cruciate ligament and surrounding tissues by critically evaluating the impact of immobilization on these tissues, we hope that biomechanical engineers in the field of FPMRS will be able to use biomechanical principles and studies to guide future pelvic floor rehabilitation strategies.

Thus, the most important conclusion is that the FPMRS field needs a much greater emphasis on biomechanics in general. The lack of fundamental work in this space in itself is an enormous gap in knowledge. For the engineers reading this e-book that are not already in the field of pelvic medicine, your expertise is sorely needed. There are many unsolved biomechanical

questions—too many for those currently working in the field—that can lead to significant changes in clinical practice if they can be answered. What contributes to a complicated vaginal delivery? Which injuries contribute to POP and SUI? How? Can they be prevented? For the engineers who are currently working in this space, we need to work harder in communicating biomechanical concepts to non-engineers. The fact that many female pelvic medicine providers lack an understanding of what biomechanical studies can offer the field is, in part, due to our lack of effective communication and failure to connect the dots between biomechanical experiments and analyses and clinical practice. To the health care providers who are treating PFDs, please maintain an open mind about the potential for this type of work. This major gap in knowledge can be overcome with better communication and an interdisciplinary approach to the basic science studies, the ultimate goal of which is to improve lives of women with pelvic floor disorders.

While this chapter did not focus much on this, the perceived lack of translation between animal models and humans continues to be a major obstacle in moving biomechanics research forward. Whether mechanical, biological, or molecular responses are being studied, many animal models can be validated to answer specific research questions and need not check every box (i.e. be a biped, have all of the same pelvic muscles, etc.) in terms of being a perfect analog for the human condition. More research is necessary to identify the specific roles that individual animal models can play and to understand their limitations. This is going to be a necessity since many *in-vivo* methods cannot be performed, for ethical or technical reasons, on pregnant women or healthy volunteers to obtain non-pathologic data. The more accurate a methodology is, the more invasive it typically is, and many of the most robust and precise protocols can only be performed on animal models. Better integration of computational studies in humans and experimental methods carried out in animal models can help to overcome these limitations.

Another area that needs further exploration is that of tissue damage and tear propagation in muscles and connective tissues that support the pelvic organs, e.g., that occurs during vaginal delivery, as this pertains to those acute injuries that likely change mechanical homeostasis within the pelvis and possibly lead to the development of POP and SUI. This is particularly important to improve our understanding of the vagina, perineum, and pelvic floor muscles during vaginal delivery. It is likely that collagen fibers have an important protective role in controlling the tear propagation process (McGuire, Abramowitch, et al., 2019; McGuire, Crandall, et al., 2019).

However, how tear propagation and subsequent healing is altered by pregnancy, menopause, and aging remains unknown and need to be further investigated.

In addition, tissue remodeling in response to changes in mechanical stimuli is likely an essential area of focus moving forward. This has already been mentioned in this chapter, but it should again be emphasized. There is a significant time delay between vaginal birth and the onset of pelvic floor disorder symptoms. While improvements in imaging, understanding tissue damage, and computational modeling will help us to uncover more details about the impact of vaginal childbirth, creating preventative strategies necessitates that we understand the mechanisms that relate birth injury with the onset of symptoms. It is very likely that pregnancy and delivery disrupt mechano-homeostasis and leads to degradative changes in other supporting tissues that take time to manifest. This process is likely exacerbated by changes in hormones, weight gain, and other factors that impact how forces are being transferred within the pelvis (e.g. repetitive heavy lifting, chronic straining).

In terms of computational research, we need to work to improve model validation and application. Simulations of vaginal delivery, for example, should ideally be able to mimic the vaginal delivery of specific patients. However, we should also avoid the pitfall of having models be so specific that they can only be reasonably applied to a few individuals. By utilizing diverse patient populations in model development and performing robust studies when determining appropriate model inputs (such as material properties, geometry/anatomy, and boundary/loading conditions), this issue can be overcome. These ideas lend towards a paradigm in which models can be adjusted in order to reasonably represent every individual.

Validation is a concern for clinical imaging studies as well. Though diffusion tensor imaging has been validated in specific striated muscles (Schenk et al., 2013), it has not been well validated for the female pelvic floor muscles. Even when the female pelvic floor was analyzed, validation was performed with a male cadaver (Zifan et al., 2018). As the chosen imaging settings and post-image processing can substantially alter diffusion tensor imaging results, rigorous validation is needed before conclusions can be drawn from these studies. In the case of ultrasound, the engineering community continues to debate what parameter is actually being measured by elastography: Are Young's moduli or other specific material parameters being measured or are resulting measures only relative? The reproducibility of strain elastography still needs to be determined, especially for female pelvic floor muscles and connective tissues (Gachon et al.,

2019), which are more geometrically complex and deeper than other tissues typically analyzed with elastography.

The search for global biomechanical tests and outcomes-tailored biomechanical diagnostic tools that can include patient characteristics and better representative models continues. This chapter mainly focused on biomechanics related to childbirth and the manifestation of POP and SUI; however, similar analyses and arguments can be made for the studies of their treatments. Indeed, some investigators are applying these approaches to gain a better understanding and advance the development and use of POP grafts and surgical repair for SUI. While challenges in identifying the biomechanical properties of pelvic tissues remain and given their need for complete functional recovery after restoration, the progress achieved over the years should be acknowledged. We are just scratching the surface of what the field of biomechanics has to offer the field of FPMRS, and we know this because of the innovative solutions that have emerged in other fields of medicine, which have embraced biomechanics and bioengineering. This is indeed a growing area of research and the potential for major improvements in the quality of life for women is extremely high.

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Chapter 3: The Impact of Hormonal Milieu on the Female Pelvic Floor Structure and Function

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Female pelvic organs and supportive structures of the pelvic floor are subjected to significant hormonal fluctuations during women's life span, including variations in the vaginal epithelium and continuous remodelling of the pelvic floor connective tissues throughout the menstrual cycle.[1] Later in life, major changes occur as a result of the deprivation of ovarian hormones following menopause. In this chapter, we present an overview of the effects of estrogen and other hormones on the pelvic floor structure and function and summarise major gaps in the current literature.

Molecular mechanisms of estrogen action

The term "estrogens" refers to a group of primary female sex hormones. There are four forms of estrogens: Estrone (E1), Estradiol (E2), Estriol (E3) and Estretrol (E4)[2]. Chemically, estrogens belong to the family of organic compounds known as steroids. In women, estrogens are primarily synthesized in the granulosa and theca cells of the ovaries, but also in smaller amounts by other tissues such as liver, pancreas, adrenal glands, adipose tissue, breast and placenta during pregnancy.

E2 (17β-estradiol) is the most common and potent form of estrogen during female reproductive years. [3] It plays an important role in the development of reproductive system and secondary sexual characteristics during puberty.[4,5,6] While females produce all estrogens throughout life, E3 and E4 are predominantly found during pregnancy, and E1 is usually present at higher levels during menopause.[7] The main source of E2 biosynthesis is dietary cholesterol. E2 is synthesised due to activity of multiple enzymes, the most important of which are aromatase (CYP19A1) and 17β-hydroxysteroid dehydrogenase (17-HSD). Aromatase is widely distributed in gonadal and extra-gonadal tissues including the bone, brain, adipose tissue, and blood vessels [8]. E1 that is mainly produced during menopause in peripheral extra-gonadal tissues, can be transformed to E2 by the 17-HSD enzyme in adipose and breast tissue, vascular endothelium, smooth muscle cells, brain and bone cells, where it is metabolized.[9] The bioactivity and levels of circulating estrogens is controlled by gonadotropins (FSH and LH) via hypothalamic-pituitary feedback. In humans, the bioavailability of estrogens is restricted by high-affinity binding to circulating sex hormone-binding globulin (SHBG). [10] Only 1–5% of circulating E2 (the free

fraction that is not bound to SHBG, albumin, or other proteins) is thought to be biologically active.[11]

Molecular mechanism of estrogen action in the female pelvic floor and genitourinary tract

At the cellular level, E2 mediates its genomic actions by binding to their specific nuclear receptors and effecting the expression of target genes. In the female pelvis, estrogen receptors (ERs) are found in the female squamous epithelium of the proximal and distal urethra, vagina, trigone of the bladder, and anal canal. Furthermore, they are expressed in the para-urethral tissues, urethral sphincter, uterosacral ligaments and pelvic floor musculature.[12] Two distinct estrogen receptors are described in the literature - alpha (ERα) and beta (ERβ). Both act as the ligand-activated transcription factors, which are variably expressed in different tissues.[13,14,15,16] Each is coded by its own gene (*ESR1* and *ESR2*, respectively), and requires homodimerization, where two identical proteins are combined, before binding its ligand or to specific DNA sequences called estrogen response elements (EREs). In addition to the full-length ERα isoform (66kDa), several shorter isoforms (36kDa, 46kDa) have been identified as a result of the presence of alternate start codons, or as products of alternative splicing. ERβ also exists in 5 distinct isoforms, ERβ1-5.[17] The shorter isoforms cannot activate transcription. Instead, they form heterodimers with the full-length ERα and inhibit its control of transcriptional activity. Mechanism of genomic signalling is determined by E2 liganding to ERα and ERβ, which regulate expression of specific genes either directly through ERE sites in gene promoters in the nucleus of target cells (**Figure 1**) or indirectly by binding with and modulating the activity of other transcription factors (i.e. NFkB).[18] ERs act through direct binding to EREs to initiate gene expression or to non-EREs by binding other transcription factors, such as Activator Protein-1 (AP1) or Specificity Protein-1 (SP-1). In the absence of ligand, the ER homodimers recruit a complex of factors (co-repressors) that repress transcription and co-activators to promote transcription. Importantly, it has been reported that more than one third of human genes regulated by ERs do not contain ERE sequence elements,[19] with transcriptomic regulation mediated by rapid non-genomic control of gene expression by estrogens. This occurs through a variety of signal-transduction mechanisms with the subsequent production of intracellular second messengers, cAMP regulation, and protein-kinase activation of signalling cascades that result in indirect changes in gene expression[20](**Figure 1**). In addition, there is increasing evidence for the role of extra-nuclear activated ERs, localized either on the cell

membrane (immune cells) or in the cytosol, however nuclear receptors are more abundant. E2 binds to plasma membrane bound ERs that insert into the plasma membrane via post-transcriptional modification of ERα or ERβ or their isoforms [21]. Alternatively, E2 binds membrane G Protein-Coupled Estrogen Receptor (GPER), initiating signal transduction cascades that involve production of cyclic nucleotides, calcium flux, and activation of cytoplasmic kinases capable of phosphorylating substrate proteins and transcription factors that then modulate gene transcription (**Figure 1**). Moreover, it was discovered that traditional ERs activated via E2 could modulate transcriptional changes in mitochondrial genes, influencing mitochondrial function and cell survival (**Figure 1)**. [22]

Estrogen and its receptors play an important role in the pelvic tissues by controlling the synthesis and breakdown of collagen.[23] Various ERs have been identified throughout the uterus, lower urinary tract and vagina[24], with ESR1 being the predominant isoform. [25] These receptors are also expressed in all major pelvic structural components, including uterosacral ligaments, vagina and pelvic floor musculature[26], which respond to the ovarian hormones.[27]

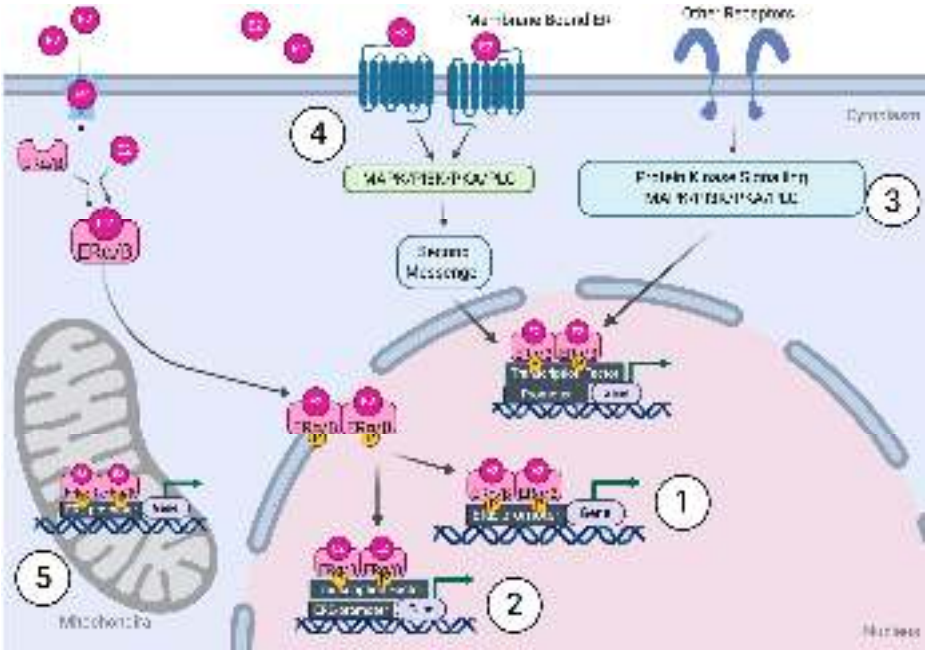

Figure 1. Mechanisms of genomic and non-genomic actions of estrogen via estrogen receptors. (1) Liganding E2 to the estrogen receptor (ERα/β) promotes the formation of homo/hetero dimers, translocation to the nucleus and direct attachment to estrogen response elements (ERE) on DNA, to activate or repress transcription of target genes (genomic pathway); (2) The ligand-activated estrogen receptor binds to other transcription factors (e.g., NFkB), which promote or prevent them from binding to their response elements, thus regulating transcription of its target genes (ERE-independent genomic pathway); (3) E2 exerts its effects in an ERE-independent manner through the activation of intracellular signalling pathways (MAPK/PLC/PI3K/PKA) (non-genomic regulation); (4) The ligand-activated receptor in the plasma membrane (GPER /GPR30) activates cytoplasmic kinases, which in turn cause the phosphorylation of substrate proteins and transcription factors (e.g., Elk-1 and AP-1) that positively or negatively regulate gene transcription;(5) ERs activated via E2 can modulate transcriptional changes in mitochondrial genes influencing cell survival. *The figure was created with BioRender.com*

Molecular mechanism of progesterone action

Progesterone (P4) is the primary sex hormone of pregnancy with essential roles in establishment and maintenance of pregnancy, and the initiation of labor.[28] P4 is produced by the ovaries, placenta and adrenal glands. In these tissues, P4 is synthesized from pregnenolone by the action 3β-hydroxysteroid dehydrogenase (3β-HSD).

P4 mediates it actions via two nuclear receptor isoforms, PRA and PRB, ligand activated transcription factors encoded by a single gene (PGR).[29] Upon binding P4, cytoplasmic PRA and PRB homo- or hetero-dimerize, translocate to the nucleus and bind P4 response elements (PRE) on the promoters of P4-responsive genes to effect gene transcription (**Figure 2.1**) P4 bound PR also acts as a monomer to indirectly initiate gene transcription (**Figure 2.2**). PRA and PRB are expressed in similar concentrations in all human P4 target tissues.[Error! Bookmark not defined.] While PRB often has greater transcriptional activity than PRA and is a positive regulator of P4 effects, both can regulate distinct P4 responsive genes.[30] PRA also has repressor activity over PRB. E2 is an important regulator of PR expression as the promoter of the PGR gene contains several EREs or interacts with other transcription factors that act together with ER. Cells often co-express both ER and PR.[31] P4 also mediates rapid non-genomic effects through binding membrane-coupled progestin receptors (mPR), that inhibit or activate second messenger signalling pathways (**Figure**

2.3) In addition, progesterone membrane components (PGRMC) are single transmembrane spanning receptors that mediate rapid signalling effects of P4 (**Figure 2.4**) These membrane PR may also influence T cell receptor signalling and are important mediators of P4 effects in T lymphocytes during human pregnancy. **Error! Bookmark not defined.** The role of P in the supportive structures of the pelvic floor or LUT remain largely unknown.

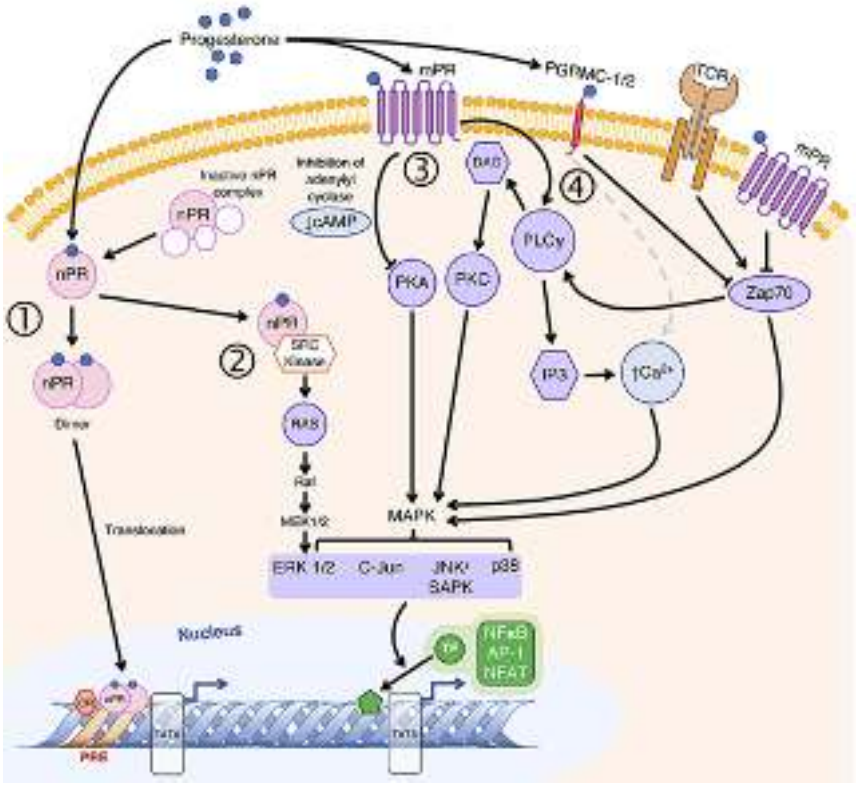

Figure 2 Mechanisms of genomic and non-genomic actions of progesterone via progesterone receptors (PR). (1) Ligand binding of progesterone to inactive, cytoplasmic nuclear PR (nPR) induces dimer formation, translocation to the nucleus and direct attachment to progesterone response elements (PRE) in gene promoters on DNA to activate or repress transcription of target genes. (2) Monomeric progesterone liganded nPR can also act via SRC kinase to activate the MAPK pathway and promote gene transcription. (3) Rapid non-genomic progesterone signaling

via ligand binding to membrane PR (mPR) or (4) progesterone receptor membrane components (PGRMC-1/2) alters gene transcription regulated by second messengers (cyclic AMP or $\uparrow Ca^{2+}$) and their associated protein kinases (PKA, PKC, PLCγ) and modulates MAPK to phosphorylate transcription factors (TF). Reproduced from Shah NM et al 2019 Front Endocrinol 10:198 under Creative Commons Attribution License. **Error! Bookmark not defined.**

Effect of hormonal deprivation on pelvic floor tissues

The prevalence of pelvic floor disorders (PFDs) increases with age[32,33,34], which indicates that age-related modifications of the pelvic floor supportive structures likely plays a crucial role in the pathophysiology of PDFs. Urinary Incontinence (UI) is the most common of all PFDs in women and its prevalence correlates with age and menopause status. Epidemiologic cross-sectional and longitudinal studies suggest an increase in UI around the time of menopause, with 70% of women connecting the onset of UI to their final menstrual period.[35] Up to 40% of post-menopausal women in Italy and Canada reported episodes of incontinence, in particular stress urinary incontinence (SUI).[36,37] Interestingly, Cagnacci et al. noted a higher degree of systemic menopausal symptoms in women with Pelvic Organ Prolapse (POP) as compared to those without POP.[38] The severity of POP/SUI symptoms increases after menopause, possibly due to the loss of protective effects of ovarian hormones.[39,40]Direct causative link between menopause and PFDs is lacking, however the abundance of ERs in the urogenital tract explains why the natural reduction of endogenous E2, the hallmark of menopause, can cause or potentiate PFDs.[22,41]

Hormonal impact on the extracellular matrix of pelvic soft tissues

Collagens and elastin are the two major extracellular matrix (ECM) components of the pelvic connective tissues. The biomechanical properties of pelvic floor connective tissues depend on the total collagen content and ratios of specific collagen isoforms.[42] Cross-linking of precursors, tropoelastin and procollagen, to form mature functional elastin and collagen fibrils, respectively, is performed by one or more members of the lysyl oxidase (LOX) family of enzymes. [43] ECM is degraded by metalloproteinases (MMPs), which are regulated by their tissue inhibitors (TIMPs).[44] The delicate balance between production and degradation of ECM proteins is critical to pelvic floor integrity. Numerous publications have associated POP development in pre- and postmenopausal women with defective ECM synthesis [45] and activated degradation of collagen

and elastin.[46] It is generally accepted that pelvic floor tissues of patients with PFDs have decreased total collagen content, with higher rate of immature collagen that is more susceptible to rupture as compared with age-comparable women without PFDs. [47]

It is likely that the molecular mechanisms underlying POP or SUI in women after menopause are different from those observed in premenopausal women. Vaginal tissue of women with normal pelvic floor support before and after the menopause shows different levels of ECM turnover and stability. Biopsy specimens of the arcus tendineous fasciae pelvis (ATFP, Level II paravaginal supportive tissue) were obtained from 10 premenopausal, 5 postmenopausal, and 12 postmenopausal women on systemic hormone therapy with anterior vaginal wall prolapse who underwent a paravaginal defect repair through a retropubic approach. Scanning confocal and electron microscopy showed that in menopausal women collagen type I in ATFP is significantly reduced compared to premenopausal women, while systemic estrogen therapy is able to reverse this effect.[48] The authors suggest that reduction in collagen I content compromises ATFP tensile strength, increasing susceptibility to anterior prolapse. Importantly, using vaginal biopsies of pre- and post-menopausal women with severe POP (n=13) and women with normal pelvic floor support (n=18), it has been shown that age and menopause influence the expression of genes involved in the ECM biogenesis and remodelling. [49] In premenopausal women, expression of vaginal MMPs varies across the menstrual cycle. Specifically, MMP-1 transcript level is significantly decreased during the proliferative phase compared to the secretory phase in premenopausal women without PFDs. Importantly, active MMP-13 expression by primary fibroblasts derived from human vagina was decreased in the presence of estradiol.[50] A significant increase of MMP-2[51] and MMP-9[52] gelatinase activity was observed in vaginal tissue of women with POP, which may lead to reduced connective tissue strength and progression of disease. Similarly, significantly increased MMP-2 detected in the uterosacral ligaments of women with POP paralleled a dramatic decrease in collagens type I and type III. [53] A possible explanation is that advancing age and ovarian hormone deprivation modulate vaginal ECM components of women affected by PFDs. For instance, it was reported that LOX enzymes and elastin expression diminishes with age.[54] Such correlations may account for the increased incidence of PFDs in the older population. However, the cause and effect relationship between menopause and the development of negative alterations in the pelvic soft tissues' ECM in older women with POP has never been established.

Animal models of human menopause enable studies of chronic ovarian hormone deprivation, clinically relevant hormone therapy and regimens (cyclic vs. continuous), and optimal timing and duration of interventions.[55] One model relevant to pelvic floor research is widely used ovariectomy (OVX) model, in which bilateral ovaries are surgically removed from healthy animals. Experimental interventions can start either at the time of ovariectomy or once systemic E2 level is substantially decreased, which typically occurs within 1–2 weeks post OVX. In rodents and sheep, OVX induces numerous effects: atrophy of vaginal epithelium, upregulation of mature collagen and downregulation of immature collagen, decrease in elastin, upregulation of collagenase MMP13, and downregulation of smooth muscle markers - SM1 and caldesmon.[56] Importantly, most changes caused by OVX in rats, rabbits and sheep can be reversed by exogenous hormones. The studies of systemic and local E2 treatment on collagen assembly and vaginal biomechanical properties in OVX rats[57] demonstrate a modest increase in collagen type I in response to systemic estradiol administration, while low-dose vaginal estrogen treatment resulted in dramatic increases in the content and cross-linking of collagens type I and III. However, the high-dose of vaginal E2 resulted in downregulation of ESR1 and loss of E2-induced increase in vaginal collagen. Another study using KO mice revealed that deposition of type I collagen is regulated by ERS2.[58] These results may have important clinical implications regarding the use of local estrogen therapy (LET) in post-menopausal women with PFDs to reverse the negative effect of menopause on vaginal tissue.

Similarly, OVX rats were used to study the effect of local and systemic E2 on the elastic fiber organization in vaginal wound healing model.[59] Loss of fibulin-5, a key matricellular glycoprotein that promotes elastogenesis and inhibits the matrix degrading MMP-9 in pelvic tissues, was reported after pelvic reconstructive surgery, with no protective effect afforded by E2. In contrast to E2, the general MMP inhibitor, actinonin, decreased excessive ECM degradation after surgical incision of the vaginal wall in rats, potentially enhancing pelvic floor recovery. [60]

Treatment of pelvic floor disorders by systemic and local menopausal hormone therapy

Local Estrogen Therapy (LET) and POP

With the PFD incidence expected to increase further as population ages,[61] it is important to find therapeutic options that will help enhance pelvic support and alleviate symptoms in affected women. To date, there is no definitive evidence for the benefit of LET as a treatment or prevention of POP.[62] To clarify this question, vaginal biopsies from 52 post-menopausal women with severe POP undergoing hysterectomy were collected. Twenty-nine of the 52 women were treated with LET (in the form of vaginal estrogen cream or tablet), while the remaining 23 untreated patients served as controls. Analysis of gene and protein expression showed that LET improves quality of the pelvic connective tissues of post-menopausal women with severe POP.[63] In particular, LET increased the collagen and elastin content, upregulated the expression of biosynthesis enzymes (BMP1), while decreasing the degradation enzymes (MMP1, MMP2 and MMP3) and increasing TIMP1 and TIMP4.[63] In addition, LET was shown to play an important role in the activation of immune system within the local vaginal environment, which was confirmed by the significant increase in gene and protein expression levels of 14 vaginal cytokines involved in leukocyte infiltration, and confirmed by immunohistochemistry.[63] This evidence support the notion that LET treatment offsets menopause-related changes and improves tissue regeneration in post-menopausal patients with POP. However, despite the promising results from some studies, the duration of LET, optimal dosage, long-term effects, and cost-effectiveness remain to be determined

Local Estrogen Therapy (LET) and POP surgery

In a randomized placebo-controlled trial of preoperative LET (Premarin®, Pfizer, New York, NY), LET improved the quality of vaginal tissue by increasing ECM biogenesis and reducing degradation. In particular, preoperative vaginal E2 application for 6 weeks increased synthesis of mature collagen, decreased degradative enzyme activity, and increased thickness of vaginal wall, suggesting that this intervention improves the substrate for suture placement at the time of surgical repair and maintenance of connective tissue integrity of the pelvic floor.[64] In contrast to the reported positive effects of preoperative E2 on the uninjured vagina, study in OVX rats demonstrated that acute administration of postoperative vaginal E2 during an early phase of healing has adverse effects on the fibromuscular layer. On the contrary, postoperative E2 plays a positive role in healing of the vaginal epithelium. [65]

Estrogen and Stress Urinary Incontinence (SUI)

Despite the progress made in determining the molecular role of estrogens in pelvic floor tissue integrity and the development of PFDs, the current knowledge of the role of estrogens in the pathogenesis of SUI remains limited. In particular, the use of MHT as a treatment for SUI is under debate. SUI often appears during the first year after menopause, and women with SUI show lower endogenous serum E2 levels compared to continent women.[66,67] This implies that E2 may play an important role in mediating continence, and hence E2-based hormone therapy might be an important therapeutic modality for SUI in women.

Animal models support the role of E2 in urethral function and continence. In ovariectomized rats, the urethral baseline pressure during sneezing is significantly decreased 6 weeks post-surgery. E2 replacement in these rats increases the urethral baseline pressure, but not urethral response amplitude, indicating partial response. Whereas 63% of the 6-week ovariectomized rats demonstrate SUI with sneezing, E2 replacement reduces this incidence to 25%,.[68] Consistently, in conditional ERα deficient mice, leak point pressure (LPP) and maximum urethral closure pressure (MUCP) values are significantly reduced compared to the wild type controls, and several muscle or cell-matrix adhesion associated proteins are differentially expressed in the ERα deficient urethra (i.e. down-regulation of tropomyosin and up-regulation of myosin) as assessed by mass-spectrometry and confirmed by Western blotting and immunohistochemistry. These ERα mediated imbalances in contractile proteins might cause the observed urethral dysfunction.[69] Likewise, in ERβ deficient mice, LPP and MUCP are decreased compared to wild type mice, and mass spectrometry of urethral tissue also shows differential expression of proteins involved in muscle contraction and development (i.e. up-regulation of myosin and collagen), extracellular matrix proteins (i.e. down-regulation of elastin), metabolism, and other pathways.[70] These studies show that both ERα and ERβ are involved in mediating normal urethral function, and changes in ERα or ERβ signalling likely play a role in the pathogenesis of SUI.

Estrogen Receptor Expression in Urethra and Bladder

Since it is known that ERs have major impact on epithelia, stromal cells, and ECM in many female reproductive tissues (breast, uterus, vagina)[71,72], ER expression has also been investigated in urethral and bladder tissues. Several studies demonstrate ERα and ERβ transcripts in the paraurethral connective tissue, with nuclear receptor proteins for both ERα and ERβ detected in

the interstitial and endothelial cells.[73,74] Earlier studies identified ER protein throughout the urethral epithelial layer, however no discrimination between ERα and ERβ gene expressing cells could be made.[75] Although it is undisputable that both receptors are present in paraurethral connective tissues, the exact cell types that express ER have not been identified. It is likely that most ER-expressing cells in the urethral connective tissue are fibroblasts and endothelial cells in the vessel wall. However, it remains to be determined if ER-positive immune cells, which could mediate estrogen responses and contribute to urethral function, are also present in the female urethra. Additionally, there is no information about ER expression and function in the urethral smooth muscle layers in women. ER expression has been demonstrated in the female rat urethral smooth muscle, where E2 suppresses TGF-β1 signalling by binding to Smad2/3 transcription factors and attenuates elastin gene expression, indicating a role of E2 and ER signalling in urethral ECM remodelling.[76]

Furthermore, there is still uncertainty about the expression of ERα and ERβ in the human bladder, as most studies were performed before the discovery of the ERβ gene in 1996[77], and mainly tissues procured during cystectomy for bladder cancers have been used for analysis. ERβ protein is the predominant isoform detected in the squamous epithelium and in the transitional epithelium of the trigone, whereas ERα is only weekly expressed in squamous epithelial cells.[78,79,80] Variation in the bladder ER expression was neither identified between pre and post-menopausal women, nor in women with E2 supplementation.[81] ERs have been identified in urothelial cells, the bladder trigone and urethra of humans and animals. [81, 82], The role of estrogen receptors is an area of active study and are thought to modulate immune function, detrusor contractility, and neuroinflammation. [82] , [83], [84]

There are some discrepancies on the relative amount of ERα and ERβ expression in urethral tissues of pre- and post-menopausal women with or without SUI. A recent study by Adamiak-Godlewska et al. examined paraurethral connective tissue samples, intraoperatively collected from the external urethral meatus of 49 SUI patients (22 pre- and 27 post-menopausal) and 32 control patients (16 pre- and 16 post-menopausal). [74]One part was used for mRNA quantification, the other part was fixed and paraffin embedded for histology. ERα and ERβ expressing cells were detected in all samples by immunocytochemistry, and quantification of labelled cells revealed no statistically significant difference in ER receptor expression between SUI and control patients. Moreover, there was no difference in ERα and ERβ expressing cells between pre- and post-

menopausal women. Despite the pervasive protein expression, ERα and ERβ transcripts could only be detected in a subgroup of all analyzed tissue samples by quantitative PCR. ERα mRNA was detected in 6/22 (27%) pre-menopausal SUI and 13/16 (80%) control patients and showed a significant reduction of the expression of this gene in SUI patients. ERβ gene expression was unchanged between SUI and control patients, but ERβ transcripts were significantly lower in the postmenopausal versus premenopausal group. The authors conclude that diminished ERα transcript expression in paraurethral tissue could eventually be used to identify women at risk of developing SUI. However, as ERα and ERβ transcripts could not be detected in all samples, possibly due to the low gene expression levels, loss of tissues integrity, or RNA instability that could have biased the results of transcript quantification.[74]

An earlier study by Soderberg et al. found no differences in ERα transcript expression in para-urethral biopsies collected from 12 SUI patients (4 pre- and 8 postmenopausal) and 11 controls (6 pre- and 5 postmenopausal). ERβ mRNA was either undetectable in the samples or too lowly expressed for reliable gene expression analysis by quantitative PCR.[73] Immunocytochemistry for ERα and ERβ revealed a significant increase of ERβ protein in premenopausal women with SUI compared to premenopausal controls, whereas ERα protein expression was not different. Not all analyzed samples had detectable ER transcripts, potentially due to limited amount or quality of tissue samples or low gene expression levels[73,74].

Taken together, these studies suggest that lower urinary tract tissues consistently express ERs independent of age, hormonal status, or the presence of SUI, and are generally responsive to estrogens. The specific spatial expression of ERα and ERβ in the bladder or paraurethral tissues could mediate specific estrogen-related effects on urinary function or the development of incontinence. However, it remains unclear if ER expression levels are directly affected by SUI or hormonal status. Experiments conducted in the ovine model did not show any change in urethral ERα expression of intact versus ovariectomized or E2-suplemented ovariectomized sheep, indicating no effect of the systemic estrogen levels on urethral ERα expression[85]. Larger studies are needed to assess whether ERα/ERβ protein or transcript expression levels, cellular distribution, or hormonal responsiveness of receptor expression can be used to detect, predict or scale the risk of SUI development in women.

Systemic and Local Estrogen Effects on (Para)Urethral Tissues

For decades it has been proposed that estrogens mediate continence by increasing urethral resistance, raising sensory bladder threshold, and detrusor muscle relaxation[86]. However, the mechanisms behind systemic E2-related effects on continence remain to be elucidated. It was shown a few decades ago that E2 deficiency can influence the amount, quality and turnover of collagens in urogenital tissues.[87,88] A biochemical analysis of connective tissue components in punch-biopsies of 34 women (13 premenopausal vs 14 postmenopausal without MHT vs 8 postmenopausal with E2 treatment) showed that in post-menopausal women the paraurethral connective tissue has higher collagen content and crosslinking of fibrils, while proteoglycan/collagen ratio was decreased in post-menopausal compared to pre-menopausal women. Moreover, MHT in post-menopausal women restored properties towards the pre-menopausal state by reducing collagen content, decreasing crosslinking of fibrils, and restoring proteoglycan/collagen ratios.[87] This demonstrates positive molecular effects of systemic E2 on the para-urethral collagen turnover in women without SUI. A comparison of the same ECM parameters in biopsies of 15 women with SUI and 16 control women of reproductive age showed a 30% increase in total collagen content, 30% larger collagen fibril diameters, and a higher cross-linking in the SUI group. [88] In contrast, in a follow up study of post-menopausal women with SUI (12 with SUI without MHT, 17 SUI with MHT, 13 controls without MHT, 11 controls with MHT), no differences in collagen content, fibril structure or proteoglycan/collagen ratios were found in postmenopausal women with SUI compared to controls. Also, ECM of postmenopausal women with SUI reacted differently to MHT as compared to controls, with less fibril cross-linking, and absence of the effect of MHT on reversing the proteoglycan/collagen ratio observed in the control women.[89] *These results show that premenopausal women with SUI already exhibit an altered paraurethral ECM structure, which is characteristic for women after menopause; indicating that the pathogenesis of SUI in pre- vs postmenopausal women has different underlying mechanisms.*

Moreover, as postmenopausal women with SUI react differently to MHT compared to continent women, SUI could be associated with an altered estrogen response in paraurethral tissues that is present prior to menopause. This premise is supported by a study analyzing different markers of collagen turnover in urogenital tissues that found that MHT increases collagen turnover in women without SUI, while having minimal effect in pre-menopausal women with SUI and no effect in post-menopausal SUI patients. This confirms that urogenital tissues in women with SUI generally have different sensitivity to circulating E2 compared to continent women. [86.] Chen et al.

showed that peri-urethral vaginal tissues procured from pre-menopausal women with SUI express less tissue inhibitors of metalloproteinase (TIMP) compared to control women. Fibroblasts, which are integral to the ECM remodeling, derived from the control women showed a dose-dependent increase of TIMPs in response to increasing systemic E2 levels, whereas fibroblasts isolated from incontinent women did not show similar dose response to E2.[90] A change in E2 response of urethral/paraurethral fibroblasts could mediate the onset of SUI. However, the signalling pathways leading to these specific E2-mediated effects on ECM structure, composition, and turnover are still unknown.

Despite the positive effects of MHT reported in some tissue-level studies, the utility of MHT as a treatment of SUI continues to be debated. Several clinical studies challenged the effects of systemic E2-only therapy in restoring continence. A Cochrane database review performed in 2005 and updated in 2012[91] evaluated 34 trials of MHT for incontinence. The meta-analysis of the result from six trials of MHT revealed worsening incontinence in women treated with systemic E2 compared to placebo. Women with uteri receiving E2 +progestogen systemic therapy also showed statistically significant worsening of incontinence. This is supported by the findings from a recent large case-control study that used medical information from the Finnish national databases. The authors demonstrated that all forms of systemic MHT were associated with a two- to three-fold increase in the risk for SUI.[92] In contrast, other studies have reported that MHT increases the maximum urethral closure pressure in women affected by SUI.[91,93]

Most epidemiologic studies are case-control studies and, therefore, cannot establish a causal link between endogenous systemic E2 or estrogen-based therapies and the development of SUI . Also, separation of E2 treatment effects from other SUI risk factors is difficult. Furthemore, most women in these studies were treated with hormones for reasons other than incontinence. Thus, there is a great need for large, placebo controlled, longitudinal cohort studies to establish cause-effect relationships between E-deprivation, MHT and SUI. These studies should include pre-menopausal women with milder forms of SUI to gain knowledge on the effect of endogenous estrogens on the progression of SUI. Such studies should include tissue-level assessmetns and functional outcomes to detect direct effects of MHT on lower urinary tract structures (bladder, urethra), and to discriminate those from the effects on vaginal tissues, which might impact continence. The Cochrane[91] and other reviews[94] support the overall positive effect of LET on mitigating incontinence. [95]

Early human study suggests that a decrease of ER in the pelvic floor tissues might be related to the occurrence of SUI.[96] Recent animal studies suggested an important role for ESR1[97] and ESR2[98] in SUI pathogenesis. Expression and functional analysis show that urethral function was significantly compromised in both, ESR1 KO and ESR2 KO mice. Proteomic analysis of urethral tissue revealed that the majority of the ESR-modified proteins were involved in cell-matrix adhesion, metabolism, immune response, signal transduction, nuclear receptor translational regelation, and muscle contraction and development.[97,98] Altogether, there are significant knowledge gaps in the molecular effects of estrogens on urethral function and the impact on development of SUI. Beside the fact that ERs are expressed in lower urinary tract tissues and that tissues in women with SUI react differently to endogenous or substituted estrogens, the signalling cascades mediating these responses in specific cell types, including immune cells, need to be systematically explored. Modern genomic sequencing and bioinformatics approaches allow the analysis of genome-wide gene expression in tissues and single cells. This will allow the identification of spatial-temporal responses of E2 signaling in the lower urinary tract associated with SUI and of patient-specific variations in responses to MHT.

Modulation of the immune system by steroid hormones in the female pelvic floor and genitourinary tract.

The specific changes in immune cell number and function during the menstrual cycle, pregnancy and menopause signify modulation by E2 and P4. This varies in different regions of the female urogenital tract to meet the challenge of protecting against sexually transmitted infections and enabling the development of an allogeneic fetus.[Error! Bookmark not defined.] How sex steroid hormones interact with the immune system in pelvic support tissues is unclear.

Innate immune system in the female pelvic floor and genitourinary tract

The innate immune system plays a key role in inflammation of the urogenital tract, rapidly initiating a non-specific immune tolerance to sperm. Implanted foreign materials such as vaginal mesh also activate the inflammatory response. Although once regarded sterile, the urinary bladder also hosts a microbiome and the innate immune system has a role in their maintenance, and in the elimination of uropathogens, often derived from the vagina or bowel.[99] Release of cytotoxic granules from natural killer (NK) cells may locally damage pelvic floor and urogenital tissues.

462 Most inflammatory cells express ERα isoforms, but PR isoforms are rarely detected. Both nuclear
463 and plasma membrane ER and PR have also been detected and show functional activity in various
464 innate immune cells (Table 1). E2 generally exerts an anti-inflammatory effect via membrane ERα
465 and ERβ. E2 also promotes the influx of ERα- and ERβ-expressing neutrophils into inflamed
466 tissues.[100] However, it is not known whether E2 has any effect on the resident innate immune cells
467 of the pelvic floor, particularly following vaginal birth injury, and whether these potential effects
468 alter tissue integrity and influence the eventual development of POP or SUI.
469
470 ***Adaptive immune system in the female pelvic floor and urogenital tract***
471 T cells have a critical role in the adaptive immune response. $CD4^+$ T helper cells orchestrate
472 type I (Th1) cell-mediated and type 2 (Th2) humoral immune responses, while $CD8^+$ cytotoxic T
473 cells mediate cytotoxicity. Activated $CD4^+$ Th2 cells produce cytokines that activate B cells of
474 matching antigen specificity to differentiate into plasma cells, which produce specific antibodies.
475 During the adaptive immune response, a small subset of long-lived T and B memory cells are
476 generated to elicit rapid responses on re-encounter with the same antigen. Of note, the majority of
477 immune cells present in the vagina are resident T and B memory cells (Table 1). T regulatory cells
478 (Tregs) downregulate $CD4^+$ and $CD8^+$ T cell responses. Both nuclear and plasma membrane ERα
479 isoforms are expressed in most T and B cells, but PR expression is controversial.
480 E2 promotes proliferation, differentiation and survival of B lymphocytes through complex
481 mechanisms involving ERα and ERβ [101]. These immune cellular responses are mediated by cell-
482 cell contact and production of specific cytokines, lymphokines and chemokines. Each immune cell
483 type has a repertoire of cytokines they respond to through expression of specific receptors, and
484 another that they produce for interaction with other cells, thereby amplifying individual cellular
485 responses to ultimately achieve clearance of foreign invaders. The contribution of these pro- and
486 anti- inflammatory molecules to changes in the pelvic tissues that contribute to POP or SUI is
487 currently unknown.
488 As ovarian function declines with aging, there is an associated marked decline in
489 circulating E2 and P4, together with the development of chronic inflammation and
490 immunosenescence, detailed in *The Role of Aging and Immunity in the Pathogenesis of Pelvic*
491 *Organ Prolapse and Stress Urinary Incontinence* chapter.[102] Thus, postmenopausal changes and

Commented [AM1]: link to aging chapter

aging need consideration together. The aging innate immune system in women is more susceptible to inflammation than in men due to the low levels of circulating E2 (<20 pg/mL). A feature of the aging immune system is "inflammaging", a chronic low-grade inflammation[103] also typical of menopause and characterised by increased inflammatory cytokines IL-1β, IL-6 and TNF.[104] IFNγ rises in early menopause and then decreases over time, while IL-10 continues to increase over the menopausal period.[105,106] The function of innate immune cells also diminishes with age.[107] Importantly, inflammatory responses in younger women serve to remove pathogens and repair tissues, but chronic inflammation in older women contributes to tissue damage. This is particularly important for the female pelvic floor and lower urinary tract.

Much more is known about the effect of aging and menopause on the adaptive immune system due to their pronounced effects on T cell function[108] attributable to the loss of E2. The adaptive immune system also senesces and becomes less functional with aging.[109] Lymphopoiesis is reduced and memory T and B cells accumulate with age, leaving fewer remaining naive lymphocytes to mount immune responses to new pathogens.[110] Aging has specific effects on the various $CD4^+$ T cell subpopulations. $CD4^+$ T cells show reduced responsiveness in older women.[111] T memory cells and antibodies generated early in life persist well into old age, but those arising in older individuals function poorly.

Effect of MHT on the immune system in the pelvic floor and urogenital tissues of post-menopausal women

Menopausal hormone therapy (MHT), particularly the E2 component, partially reverses some of the immunosenescence of menopause.[106] In particular, MHT reduces the inflammatory cytokine levels of IL-1β and TNF, and normalizes IL-10 levels.[105] Older women (30 years post-menopause) taking E2-containing MHT have increased circulating B cells compared to menopausal women not on MHT, pointing to an increased ability to produce an antibody response with hormone therapy.[112] These effects of MHT, particularly EHT, influence these changes by interacting with nuclear and membrane ERs on various innate immune cells (Table 1). However, published studies examining the effect of MHT on innate immune cell number and function are very limited, with small sample sizes and lack the individual cell types assessments. Overall, MHT improves peripheral immune system function, through the actions of E2.

The immune systems of the female lower genital tract are unique in that they require adaptation to specialised physiological processes associated with reproductive function, in addition to the maintenance of commensal microbiota and protection from pathogens.[113] The lower urinary tract has a similar role with respect to its microbial populations, but its close proximity to the vagina and E2 responsiveness also adds to the uniqueness of these tissues. How the bladder immune cells alter in response to menopause or interact with MHT is unknown. It is clear that more experimental and interventional studies on the immune cells and their interaction with MHT in menopausal women with POP and SUI are required.

The vaginal microbiome undergoes major changes during menopause. Lack of E2 alters vaginal cell metabolism resulting in a thinner mucus and lower glycogen production, which reduces *Lactobacilli spp.* abundance and diversity.[114] Local and systemic MHT reverse these changes, increasing *Lactobacilli* and reducing pathogens.[115] The innate immune response in the vagina is largely driven by vaginal bacterial community states.[116] Microbiome gene sequencing analysis showed that postmenopausal women had vaginal communities depleted in *Lactobacilli* and had 10 fold less bacteria than women treated with MHT for at least 12 months.[117] The vaginal community clusters differed between the 2 groups, highlighting their importance in the health of the human vagina. The effects of immunosenescence likely superimpose on the altered hormonal milieu of the menopausal vaginal epithelium, as the immune cells predominantly reside in a thinned epithelium and lamina propria. However more research is required to delineate the effect of the microbiome on immune cell function in the E2-depleted post menopausal vagina and in the MHT/LET treated vagina and whether this influences the structural integrity of the vaginal wall, predisposing to POP and SUI.

Table 1 – Estrogen, estrogen receptors and their effects on immune cell numbers and function in peripheral blood and the lower reproductive and urinary tracts

Immune cell	ER	Function	Reproductive Stage or hormone level	Location	Refs
Neutrophils	ERα, ERβ	↑nNOS ↓adhesion to vessels	cycling, ↑ in ovulatory stage	PB	[118,119]
		↑NET formation, ↑ ROS ↓chemotaxis,	pregnancy	PB	[120]
			cycling	PB	[121]
		↓ROS	unkown	PB	[122]
	GPER1	↑life span, respiratory burst, gene expression	pregnancy	PB	[123,124,125126]
		↓function, ↑ numbers	menopause, aging	PB	
		↓function,			
Monocytes	ERα	E2 induced ERα	cycling	PB	[127]
	ERα46	E2 induces ERα			
	ERα36	↓LPS-induced IL-6, TNF	unknown	PB	[128],
	GPER1	Interacted with ERα36	unknown	PB	
		↑basal IL-1α, IL- β, TNF	luteal vs follicular	PB	[129]
		↓IL-6, ↓TNF, ↓MHC-II	menopause, aging	PB	
Macrophages	ERα	E2 induced ERα	cycling	PB	[127]
	ERα46	E2 induced ERα46			

	ERα36 mERα66 GPER1	↓CXCL8 production BAX translocation ↑survival (BCL-2) phagocytosis, APC Innate immune response	cycling male, pre- and post-menopausal	THP-1 cell line vagina Bladder	[130] [131,132] [133]
pDC	ERα	pDC differentiation ↑TLR7 responses, ↑IFNα Th 1 cytokines ↓numbers, ↓T cell priming ↑basal cytokines	unknown post-menopausal, aging	PB PB	[134] [102 , 107] [135 107]
CD14^{-} DC CD14^{+} DC		Th1 cytokines, induce Th2 cytokines Th1 cytokines	cycling	vagina LP	[132]
Langerhans cells		Th1 cytokines induces Th2 cytokines	cycling	vagina IE	[132]
cNK cells		↑numbers, ↑cytotoxicity ↑numbers cytotoxic phenotype cytotoxicity	luteal vs follicular postmenopausal cycling Pre-and post-menopausal	PB PB vagina bladder	[,136] [106, 108] [137 138] [133]

547

uNK cells	ERβ mERα46	↑motility ↑numbers, ↑cytotoxicity	pregnancy luteal vs follicular	decidua PB	139 136
γδT cells		Cytokine production, cytotoxicity		vagina	140
			mouse	bladder	141
$CD4^+$ T cells	ERα *(ESR2)*		pre- and post-menopausal	PB	142
	ERα	Differentiate to Treg	mouse, pregnancy	spleen	143
	ERα46 mERα46	T cell proliferation	male	PB	**Error! Bookmark not defined.**
	ERβ				
$CD4^+$ Th1 T cells	ERα	↓ IFNγ, TNF	pregnancy, mice	PB	
$CD4^+$ T cells		↓numbers, ↓TCR signalling ↓clonal expansion, ↓differentiation to Th1, Th2 T cells	menopause	PB	144 145 106, 104 109
CD4 Th1		Produce IFNγ, TNF	cycling	vagina	
$CD4^+T_{RM}$		Rapid 2nd response to Ag	cycling	vagina	113 146
$CD8^+$ T cells	mERα46	T cell proliferation	male	PB	
		↑numbers, ↓TCR diversity,	menopause ERT	PB	106
$CD8^+$ T_{RM} cells		↓numbers, ↑IFNγ	cycling	vagina	106 109,123

$CD8^+$ Tcells		Sense own Ag, release IFNγ, rapidly initiate local immune response Innate immune response		bladder	104 147 133
Tregs		↑numbers	pregnancy	PB	148
B cells	ERβ (*ESR2*)		pre- and post-menopausal	PB	142
	GPER1, mERα	BCR signalling ↓numbers ↑numbers, ↑Ab response	 menopause MHT	 PB PB	149 104, 106 112
Plasma cells		Produce specific IgG Abs	cycling	vagina	137

Ab, antibody, Ag, antigen; APC, antigen presenting cell; BAX, BCL-2 like protein; BCL-2, B cell lymphoma; BCR, B cell receptor; cNK, conventional NK cells; CXCL8, C-X-C motif chemokine ligand 8; GPER1, G protein ER 1; mER, membrane ER; MHC-II, major histocompatibility class II; NET, neutrophil extracellular traps; nNOS, neuronal nitric oxide synthase; PB, peripheral blood; pDC, plasmacytoid dendritic cell; ROS, reactive oxygen species; TCR, T cell receptor; Th, T helper; TLR7, Toll-like receptor 7; TNF, tissue necrosis factor; T_{RM}, T resident memory cells; uNK, uterine NK cells; Vagina IE, vagina intraepithelial, VLP, vagina lamina propria.

Table 2 –Progesterone, progesterone receptors and their effects on immune cell numbers and function in peripheral blood and the lower reproductive and urinary tracts

Immune cell	PR	Function	Reproductive stage		Refs
Mouse macrophage	mPRα	↑COX2 (*Ptgs2), Tnf, Il1b* ↓mPRα *(Paqr7*), *Oxtr*		RAW 264.7 Cell line	[150]
Rat mature DC	Cytoplasmic PR	↓LPS-induced Il-1β, Tnf ↓CD80, MHC-II expression ↓LPS-induced	cycling	bone marrow	[151]
pDC		↓T cell activation function	pregnancy	PB	**Error! Bookmark not defined.**
cNK cells	PR	Apoptosis, ↓IFNγ	cycling	PB	[152]
γδT cells	PR	PR induced, ↑ numbers PIBF produced, blocks NK cell function, skews Th1 to Th2 responses	pregnancy	PB	**Error! Bookmark not defined.**
T cells	mPRα, mRPβ	↑intracellular [Ca^{2+}]	bovine	PB	[153]
$CD4^+$ T cells		↓IFNγ	pregnancy	PB	[154]
$CD8^+$ T cells	PR	PR induced, ↑ numbers ↓ granzyme B release	pregnancy	PB	**Error! Bookmark**

					not defined. 154
Tregs	mPRα	↑Tregs	pregnancy	PB	148,155

559 cNK, conventional NK cells; COX2, cyclo-oxygenase 2; DC, dendritic cell; MHC-II, major
560 histocompatibility class II; LPS, lipopolysaccharide; mPR, membrane PR; Oxtr, oxytocin receptor;
561 pDC, plasmacytoid dendritic cell; PIBF, progesterone induced blocking factor; Th, T helper
562
563
564 **Effects of other hormones on the pelvic floor and the association with pelvic floor dysfunction**
565 The conventional "female" hormones such as estrogen and progesterone have been more
566 widely studied for their role in pelvic floor (dys)function; however, other hormones are gaining
567 interest as their roles in pelvic floor pathology have been increasingly investigated. The complex
568 pathophysiology of obesity and the associated conditions, such as metabolic syndrome, diabetes,
569 and alterations in inflammasome and immunity also intersect with imbalances in less studied
570 hormones in women, including androgens, thyroid hormones, and vitamin D. Historically, the
571 impact of obesity on the female pelvic floor has been assumed to be simply mechanical in nature.
572 Here we seek to examine the current literature describing the more intricate role of obesity and
573 associated chronic inflammation and oxidative stress, metabolic syndrome, insulin resistance and
574 diabetes and the overlap of these conditions with hormonal imbalances in androgens, thyroid
575 hormones and vitamin D. Our goal is not only to review the literature, but also integrate the existing
576 body of knowledge in a way conducive to the identification of critical gaps that should fuel novel
577 investigations.
578
579 ***Androgens***
580 Although relatively less studied, androgens also vary in women during their lifetime.
581 Consequently, these hormones may play a putative role in PFDs, the incidence of which increases
582 with menopause. Androgens have established roles in skeletal muscle strength, bone density, and
583 connective tissue integrity in other systems, but their role in the female pelvic floor has not been
584 well characterized.

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Androgens, typically thought of as "male" hormones, are present in women in significant levels throughout the reproductive lifespan. Androgen production is initiated in the ovaries and the adrenal glands in response to luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH). The major androgens produced are testosterone, androstenedione, and dehydroepiandrosterone (DHEA), all of which are cholesterol derivatives. In the ovary, cholesterol is first converted to DHEA and then to androstenedione and testosterone, contributing to plasma testosterone levels directly and indirectly via peripheral conversion of circulating androstenedione. The adrenal glands contribute to circulating levels of DHEA and ultimately DHEA sulfate (DHEA-S) that is derived from DHEA, and testosterone. Circulating testosterone is present in multiple forms: free (1-2%), bound to sex hormone binding globulin (SHBG) (~66%), and loosely bound to albumin (~31%).[216, 217] Loosely bound and free testosterone are bioavailable, in contrast, bound testosterone has diminished biological activity. Consequently, the level of SHBG (produced by the liver) can alter the amount of bioavailable testosterone. Circulating levels of SHBG increase with increased circulating estrogen levels. Circulating testosterone levels can also be diminished by aromatization to estradiol, which occurs in the ovary and peripherally. Circulating testosterone can also be converted by 5α-reductase to the potent androgen - dihydrotestosterone (DHT). More recently identified circulating androgens that are synthesized peripherally include 11-ketoandrostenedione (11KA), 11-ketotestosterone (11KHT), and 11-ketodihydrotestosterone (11KDH).[216] These 11-oxygenated C19 steroids possess similar androgenic potency to testosterone and DHT and can bind and activate androgen receptors in men.[216] The complexity of androgen signaling and difficulty in measuring lower circulating levels of androgens in females have made the characterization and quantification of these hormones in women more challenging.

During the lifespan of a woman, the concentration of circulating androgens changes significantly. A cross-sectional study of 588 premenopausal women ages 18-39 characterized androgen levels across the menstrual cycle. Testosterone and androstenedione are lowest in the early follicular phase and are higher in the midcycle and luteal phases.[216] In contrast, DHEA, 11KA, and 11KT were unchanged during the menstrual cycle. Interestingly the authors also found that overweight women had lower median testosterone, DHEA, and 11KA levels than normal-weight women. All 11-oxygenated C19 steroids were significantly lower in women between 35-39 years old compared to those 18-25 years.[220] As women undergo the menopausal transition, additional androgen alterations are seen. Circulating DHEA and DHEA-S levels decrease with age

as a consequence of ovarian senescence and decreased adrenal activity. [156] Testosterone also decreases with age in women, with the most dramatic decline (up to 50%) occurring between 20 and 40 years of age.[205, 209, 157] SHBG levels remain stable during this time resulting in decreased free testosterone. After menopause, lower estrogen levels reduce SHBG, leading to a minimal increase in free testosterone.

The understanding of the role of androgen signalling in the pathophysiology of PFDs as well as the potential protective effects of androgens in the female pelvic floor is in its nascent stages. Mechanistic studies in murine models have begun to characterize the role of androgens in the pelvic floor. In male rats, pelvic skeletal muscles, including levator ani and external urethral sphincter, are androgen sensitive as evident by levator ani atrophy in response to androgen removal by gonadectomy.[158] Similarly, ovariectomized female rats exhibit levator ani atrophy. Interestingly, this atrophy can be rescued by selective androgen receptor modulators, suggesting that ovarian testosterone rather than estrogen may be needed for preservation of levator ani muscle mass.[159] Functional improvement in PFDs with androgen treatment has also been demonstrated in the rat model. In the sciatic nerve transection female rat model of SUI, leak point pressures return to control values with testosterone treatment.[160]

Despite paucity of studies, these findings suggest that androgen receptors are present in the female pelvic floor and may be needed to maintain muscle mass and function. Additional research is needed to clarify the role of androgens in the pathogenesis and potential treatment of PFDs. Detailed characterization of specific androgen levels using high sensitivity quantitative testing, such as mass spectroscopy-based analysis, is needed to accurately characterize the relationship of androgens with PFDs. In addition, more animal studies are needed to establish a mechanistic link between specific androgens and the function of the integral components of the female pelvic floor. Furthermore, long-term safety and efficacy studies of androgen regimens in women are needed to inform clinical practice and select appropriate patient populations for potential treatments. Topical DHEA vaginal preparations and systemic androgen therapies are clinically utilized to improve sexual health in women. We do not know what impact, if any, these treatments have on the development, progression, or resolution of PFDs long-term.

Vitamin D

Vitamin D is a fat-soluble steroid hormone that is integral for the regulation of calcium homeostasis in bone and skeletal muscle. Vitamin D is predominantly produced in the skin, where provitamin D (7-dehydrocholesterol) is converted to cholecalciferol (D_3) by ultraviolet B rays. Circulating D_3 then binds to vitamin D binding protein (VDBP) and can be metabolized to 25-hydroxyvitamin D_2 [25(OH)D]. In the kidney 25(OH)D_2 is converted to active metabolite calcitriol [1,25 (OH)D_2]. The production of these derivatives is closely coupled to calcium homeostasis and is regulated by calcium, phosphorous and parathyroid hormone.

Vitamin D insufficiency and deficiency are common, affecting approximately 75% of both older (>65) adults and reproductive age women.[161,162,163] Multiple demographic and socioeconomic factors have been associated with vitamin D deficiency, including female sex, obesity, African American race, and low income. [164],[165] Most literature has focused on the adverse impact of vitamin D deficiency on the skeleton and appendicular muscles; however, a body of literature investigating the relationship between vitamin D and PFDs is growing.

Epidemiologic studies examining the relationship between vitamin D and PFDs, such as large cross-sectional analysis [161] of 1881 non-pregnant women enrolled in the National Health and Nutrition Examination Survey, determined that vitamin D levels are significantly lower in women with at least one reported PFD compared to women without self-reported PFDs, regardless of age. In a retrospective study of 394 women with the preponderance of postmenopausal Caucasian participants, mean vitamin D levels were higher in women without compared to with symptomatic PFDs, with worse incontinence impact questionnaire scores associated with vitamin D insufficiency.[166] Overall, *the epidemiologic literature suggests that decreased vitamin D levels coincide with the presence of PFDs independent of age*. Circulating vitamin D levels have primarily been examined, but a small case-control study of 47 women demonstrated an association of vitamin D receptor polymorphisms with the presence of PFDs in women with comparable vitamin D levels, suggesting vitamin D receptor activity may also be relevant for utilization of circulating vitamin D.[167]

It is unclear whether reduced vitamin D levels are directly related to PFDs or alternatively predispose pelvic floor structures to injury during such events as vaginal delivery or subsequent insufficient recovery. A cross-sectional study of 181 postpartum women showed that pelvic floor muscle strength, measured by perineometer, was significantly lower 8 weeks post vaginal delivery in women with vitamin D deficiency compared to women with normal vitamin D levels. No

677 difference in the pelvic floor muscle strength was observed between women with and without
678 vitamin D deficiency 8 weeks post Caesarean section.[168] Overall, this suggests that normal vitamin
679 D levels may offer some protection against the trauma associated with vaginal delivery. On the
680 other hand, vitamin D deficiency may predispose pelvic floor muscles to inadequate recovery after
681 vaginal delivery. Further research is needed to elucidate the pathophysiology of pelvic skeletal
682 muscle damage and recovery in the setting of vitamin D deficiency.
683 Currently, mechanisms by which vitamin D impacts female pelvic floor are poorly
684 understood. Vitamin D receptors are present in human periarticular skeletal muscles supporting
685 direct biological effect. [169] In intraoperative muscle biopsies obtained from women undergoing
686 orthopedic surgery the Vitamin D receptor density decreased with age, as measured
687 histologically.[170] One proposed mechanism of vitamin D related pelvic floor dysfunction is
688 decreased pelvic floor muscle strength in the setting of insufficient vitamin D levels and/or
689 diminished vitamin D signalling. [171] Alternatively, vitamin D may act indirectly by influencing
690 the bioavailability of androgens, which have potent anabolic effect on skeletal muscles. Multiple
691 studies have shown that alterations in vitamin D levels are associated with altered SHBG and free
692 androgen levels. The directionality and magnitude of the relationships varies between studies,
693 suggesting these relationships may be impacted by age and sex.[172,173,174] Overall, additional studies
694 are needed to clearly define the relationship between independent and combined effects of vitamin
695 D and androgen levels and pelvic skeletal muscle function.
696
697 ***Thyroid Hormones***
698 Synthesis and release of the prohormone thyroxine (T_4) and active thyroid hormone,
699 triiodothyronine (T_3) by the thyroid gland are stimulated by thyroid stimulating hormone (TSH).
700 Peripherally, T_4 can be converted to the active T_3 by tissue iodothyronine deiodinases. Thyroid
701 hormone action predominantly relies on binding to thyroid hormone receptors encoded by two
702 genes - TRα and TRβ, but it is also modulated by the co-repressors and activators, as well as
703 thyroid hormone transporters. Thyroid hormone is needed for growth and development, neural
704 differentiation, and metabolic regulation.
705 The role of thyroid hormones in the pathogenesis of PFDs remains largely unknown aside
706 from the epidemiologic association with the dysfunction of the lower urinary tract. In a cohort
707 study of 202 older women (mean age 84yrs), urinary retention, defined as post void residual >200

ml, was independently associated with hypothyroidism, diagnosed by high serum TSH.[175] In a cohort study of 159 postmenopausal women, moderate to high-normal serum TSH was a risk factor for urinary incontinence as measured by the international consultation on incontinence questionnaire short from in women over 65yrs.[176] Overall, these studies indicate that further investigation into the relationship between thyroid hormones and urinary function is warranted.

Animal studies suggest thyroid hormones may modulate both skeletal muscle contractility, as well as sensory and motor nerve conduction. In an induced hypothyroidism rabbit model, fiber cross-sectional area and the number of peripheral myonuclei per fiber were increased in bulbospongiosus and pubococcygeus, with similar expression of TRα and TRβ in these pelvic muscles. The authors speculate that this may result in a polymyositis phenotype seen in other muscles or represent fiber type conversion.[177] Hypothyroidism in this model also increased residual volume and increased the intravesical pressure that triggers the voiding phase while reducing voided volume, maximal pressure and voiding efficiency suggesting impairment of the somatovisceral micturition reflex.[178] These studies suggest that thyroid hormone signaling may play a role in both pelvic muscle morphology and in neurosensory regulation of micturition.

In limb skeletal muscles, thyroid hormones are known to be integral to muscle function through regulation of fiber phenotype, and modulation of muscle regeneration, metabolism and contractility.[179] In response to muscle injury, conversion of the prohormone T_4 to the active T_3 by a specific deiodinase (Type 2, DIO2) is essential for differentiation of muscle stem cells during repair.[180] DIO2 knockout mouse model demonstrates expansion of the limb muscle's stem cell pool after muscle injury without differentiation. This phenotype, similar to that observed in the knockout mouse model of muscle specific Myod gene, can be rescued with T3 administration.[181] [182] Studies are needed to assess whether this phenomenon is also true for pelvic skeletal muscles and if so, what implications this may have for women before and after vaginal deliveries as well as for women who already suffer from PFDs.

Metabolic disorders: effect on pelvic floor function

Despite physiological hormonal fluctuation during a lifespan, hormonal imbalance may intersect with metabolic conditions such as diabetes mellitus, polycystic ovary syndrome (PCOS),

or metabolic syndrome or obesity. These complex conditions share some commonalities: (1) modulation by the common sexual steroid hormones; (2) systemic inflammation; (3) systemic oxidative stress; and (4) systemic vasculopathy. In this section, we examine the current literature describing the role of metabolic disorders in pathophysiology of PFDs, their overlap with hormonal imbalances, and their impact on the pelvic floor structures. do not Specically, we describe molecular/cellular and inflammatory pathways thare are relevant to female genitourinary function and PFDs.[183,184,185, 186, 187].

Hormonal alterations associated with menopause and PCOS are known to impact insulin sensitivity, and adipose distribution, deposition and structure. Menopause increases visceral distribution and accelerates accumulation of white adipose tissue, potentially leading to an increased risk of insulin insensitivity and glucose intolerance.[188],[189] PCOS is a complex endocrine disease that involves hyperandrogenism, ovulatory dysfunction and infertility that is associated with obesity, type 2 diabetes mellitus and non-alcoholic fatty liver disease.[190] Because of the variability in PCOS phenotypes, its association with PFDs is not completely understood.[191] Taghavi et al. found POP symptoms to be significantly higher in women with clinical triad of hyperandrogenism, chronic anovulation and polycystic ovaries compared with non-PCOS women. However, the incidence of POP symptoms in women with PCOS presenting with other phenotypes (only two out of the three above manifestation) were not statistically different from healthy controls. Urinary symptoms also did not differ between the study groups.[192] Interestingly, some studies actually report a lower prevalence of urinary incontinence in hyperandrogenic PCOS women compared to healthy controls regardless of the body mass index.[193],[194] Based on the finding of lower incidence of urinary incontinence in women with hyperandrogenic PCOS, Antônio et al. tested whether androgens could be a protective factor for the pelvic floor by acting directly on the pelvic floor muscles. However, the authors did not demonstrate differences in pelvic floor muscle strength, assessed by manometry, between women with hyperandrogenic PCOS and non-PCOS women.[195] Furthermore, de Melo et al. found that PFM thickness of PCOS patients was not different from the control group.[196] Conversly, Micussi et al. support the beneficial effect of the hyperandrogenic status of PCOS women on the pelvic floor muscles. Comparison of the pelvic floor muscles' function assessed by surface electromyography between PCOS women and premenopausal non-PCOs controls demonstrated that muscle tone, maximum voluntary contraction, and electromyographic activity of the pelvic floor muscles were significantly higher

in PCOS women. In addition, the authors showed a positive correlation between estradiol and testosterone serum levels and pelvic floor muscle tone.[197]

Considering urethral function, Fowler et al. reported in 1998 an apparent association of abnormal electromyographic activity of striated urethral sphincter, characterized by decelerating bursts and complex repetitive muscle discharges with impaired relaxation, with polycystic ovaries in young women with urinary retention.[198] The mechanisms leading to the urethral dysfunction in PCOS women remain unclear. At the time of the above study, the authors speculated that anovulation-related deficiency of progesterone, a cell-membrane stabilizer, might permit transmission of impulses between muscle fibres of the urethral sphincter, giving rise to abnormal electromyographic activity and impairing relaxation of the sphincter. Other theories have been subsequently postulated to explain voiding dysfunction in women with PCOS. Hyperestrogenaemia in PCOS might impair relaxation of the urethral sphincter, resulting in low flow-rates of urine, incomplete emptying of the bladder and, finally, urinary retention.[199] It is also possible that poorly relaxing external urethral sphincter causes increased urethral afferent activity, inhibiting bladder afferent signalling and leading to poor bladder sensation and detrusor under-activity.[200] In addition, hormonal-metabolic disorders such as PCOS lead to a pro-inflammatory environment and oxidative stress that increase the risk of endothelial dysfunction.[285] Thus, it is plausible that endothelial dysfunction is one of the mechanisms by which PCOS predisposes women to PFDs. The above is a fruitful subject for future investigations.

A broader metabolic syndrome (MetS), that includes PCOS, has been identified as significant risk factor for POP , with POP severity increasing with higher glucose levels.[201] Large waist circumference and high triglycerides are also significantly associated with PFDs.[202] Women with MetS have a 2-fold increased risk of symptomatic SUI compared to women without Mets.[202] In 193 Brazilian women, MetS, characterized by high body mass index, waist circumference, triglyceride and glucose levels, was diagnosed in 69.4% of women with SUI compared to 38% in the group without SUI.[203]

The mechanisms by which dyslipidemia contributes to the pathogenesis of PFDs remain unclear and literature is scarce. Peroxisome proliferator-activated receptor (PPARgamma-2) and beta-3-adrenergic receptor (ADRB3) polymorphisms have been associated with co-presence of elevated tryglicerides and connective tissue diseases[204], but only weak assumptions can be made from this. The most accepted mechanistic theories that explain the role of MetS in the development

of PFDs and its effect on the female pelvic floor highlight inflammatory-based vascular, neurogenic, and myogenic tissue damage.

MetS can lead to musculoskeletal diseases through inflammatory pathways such as sarcopenic obesity (muscle loss in obesity), osteoporosis, tendinopathy, and osteoarthritis. Muscle fiber damage happens on a daily basis and is generally considered to be a beneficial stimulus, leading to growth and adaptation through muscle regenerative processes.[205] Chronic inflammation due to obesity and MetS results in unregulated tissue repair and in an imbalance toward negative remodeling of myofibers, resulting in tissue damage. The three most active cells in the regeneration of skeletal muscle are macrophages, resident muscle stem cells, and fibroblasts.[206] The metabolic complications associated with obesity can lead to an inappropriate temporal recruitment of these cells, in turn, impairing angiogenesis and myocyte formation. This process may promote the deposition of fibrotic and adipose tissue, ultimately leading to a reduction in structural integrity and functional capacity of a muscle. These events are driven by increased pro-inflammatory cytokines and chemokines, hyperleptinemia, hyperglycemia, increased oxidative stress products, such as *advanced glycoxidation end products* (AGEs), and reactive oxygen species (ROS). The hallmark of metabolic dysfunction is an impaired muscle integrity, defined as persistent muscle loss, intramuscular lipid accumulation or collagen deposition.[206]

Obesity

Obesity is considered a strong risk factor for urinary incontinence.[207] According to a recent study by Brucker et al., SUI is significantly more prevalent in obese patients than urge incontinence (~25% vs 15%) regardless of women's age.[208] In a prospective study of 30,982 middle-aged women, increasingly higher BMI over time was related to higher odds of developing SUI, with a three-fold higher incidence of SUI in women with BMI ≥40 kg/m2.[209]

POP is also associated with obesity.[210] Kudish et al. studied the relationship between changes in body weight and POP progression/regression in 16,698 postmenopausal women during a 5-year period. The risk of prolapse progression in overweight and obese women compared to women with normal BMI increased by 32% and 48% for anterior prolapse, by 37% and 58% for posterior prolapse, and by 43% and 69% for uterine/apical prolapse, respectively.[211] In a systematic review and metanalysis involving >96,875 participants (17,249 POP cases), Giri et al. showed that obesity, as measured by BMI, was positively associated with POP. Women who were

832 overweight and obese had risk ratios of 1.36 (95% CI, 1.20-1.53) and 1.47 (95% CI, 1.35-1.59),
833 respectively, for having POP.[212]

834 The mechanisms linking obesity and female PFDs are not completely understood. The
835 dominant theory relies on the biomechanics: BMI correlates with intra-abdominal pressure, which
836 increases intravesical pressure, and exerts increased force on the pelvic floor causing chronic stress
837 to the pelvic structures.[213],[214] Some authors have shown that increased sagittal abdominal diameter
838 in obese patients is associated with elevated intra-abdominal pressure compared to normal weight
839 patients.[215,216] Urodynamic findings also demonstrate that increased BMI is associated with
840 increased intra-abdominal pressure [295],[217] and that incontinent obese women have higher intra-
841 abdominal pressure at maximal cystometric capacity.[218] Obesity has also been shown to affect the
842 structure of the urethra. In an animal model of obesity, obese rats with leptin receptor gene
843 mutation demonstrated fibrosis and edema of the periurethral muscularis, marked by collagen
844 infiltration and disruption of striated muscle on histological qualitative analysis of urethral sections
845 stained with Masson`s Trichome. These morphologic changes were accompanied by lower leak-
846 point pressures in obese animals compared to control rats.[219]

847 There is a rising interest in the systemic metabolic changes associated with obesity that
848 may also be involved in the pathophysiology of PFDs. Obesity is metabolically a proinflammatory
849 and nitro-oxidative stress state, characterized by chronic hyperleptinemia and decreased levels of
850 hormone *adiponectin*.[220] In obesity, the visceral adipose tissue (an endocrine organ) undergoes
851 dysregulation of the secreted factors termed *adipokines*, resulting in increased secretion of
852 proinflammatory factors, including leptin hormone and cytokines such as tumor necrosis factor
853 (TNF), interleukin 6 (IL-6), IL-8, and C-reactive protein.[221] Hyperleptinemia has proinflammatory
854 actions, activating NADPH oxidases and inducing the production of reactive intermediates such
855 as hydrogen peroxide that contributes to oxidative stress.[222,223] The low level of adiponectin, which
856 is known to have anti-inflammatory actions, positive input on insulin sensitivity, and is involved
857 in vascular repair, further exacerbates the pro-inflammatory state.[224] Obesity is also associated
858 with an increase in plasma free fatty acids, known to exert negative vascular effects and oxidative
859 stress.[225]

860 Increased dietary AGEs intake, common in high-fat foods, increases the circulating AGEs
861 level.[226] The resultant *lipoxidation* provides excess substrates for endogenous AGE formation and
862 induction of the myeloperoxidase inflammatory pathway[227], leading to more AGE formation,

thereby creating a feed-forward-fueled pathological loop. Taken together, obesity is a systemic chronic inflammation and oxidative stress condition. The pathways of inflammation and pelvic cellular/tissue toxicity due to the accumulation of AGEs and ROS have been underexplored, and, therefore, present many opportunities for researchers in the field of FPMRS.

Diabetes and insulin resistance

Women with glucose metabolism disorders have a higher risk of UI.[228] The National Health and Nutrition Examination Survey (NHANES) that included 7,270 women showed that among women with relatively well-controlled diabetes, each one-unit increase in HbA1c was associated with a 13% (95% CI: 1.03–1.25) increase for *any* UI and a 34% (95% CI 1.06–1.69) increase in risk for SUI.[229] The Action for Health in Diabetes (Look AHEAD) study, a randomized clinical trial with 2,994 overweight/obese women with type 2 diabetes, revealed that 27% had at least weekly incontinence. Of them, 396 (52%) reported predominant SUI, 298 (39%) reported predominant UUI, and 64 (8%) reported an equal number of stress and urgency incontinence episodes. Women with a BMI of ≥35 kg/m^2 had a higher odd of overall UI and stress incontinence (55–85% higher; P=0.03) compared with that for overweight women. With respect to the epidemiology of POP, its association with diabetes is more frequently seen with the coexistence of metabolic syndrome.

Glucose metabolism disorders may contribute to PFDs development and progression through pathways involved in chronic inflammation due to hyperglycemia. This may lead to neurologic and muscular damage that affect pelvic floor structures responsible for urinary continence and pelvic organ support. The findings by Baldassare et al. demonstrate negative vascular and neurological effects of diabetes in pelvic tissues. By analyzing vaginal samples procured from postmenopausal diabetic women, the authors showed morphologically disrupted micro-vessels with increased density in the lamina propria, suggestive of angiogenic compensatory changes and impaired remodeling. In addition, the authors reported that gene and protein endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase isoforms - enzymes that synthetize the nitric oxide (NO) - were significantly reduced in the vagina of women with diabetes.[230] A significant decrease in the expression of nNOS in the anterior vaginal epithelium was observed in women with SUI compared to controls.[231] [232] The exact role of NO and NOS in the pelvic floor function needs to be further investigated. It is known that NO exhibits modulatory effects on

parasympathetic nerves, provoking smooth muscle relaxation. Nerves that utilize NO and neuropeptides as a neurotransmitter in the human vagina may play a role in controlling vaginal blood flow and capillary permeability. The potential role of neuropeptides in pelvic floor tissues has been suggested by some authors.

Diabetes provokes time-dependent changes in urethral morphology, structure and function.[233] Increased urethral pressure during micturition is seen as an early manifestation of the disorder. As the disease progresses, an impaired coordination between bladder and urethra due to dyssynergic activity of external urethra sphincter can occur.[234] In addition, impaired relaxation of the urethral smooth muscle may occur due to a decreased responsiveness to NO[235] and increased urethral smooth muscle responsiveness to α1-adrenergic receptor stimulation.[236] In the late stages, diabetic neuropathy may also play an important role in the lower urinary tract dysfunction. Liu et al. evaluated the urethral structure and function in diabetic rats and observed atrophy of the striated muscle bundles in long-term diabetic animals compared with controls. As a consequence of polyneuropathy seen in diabetic animals, an abnormal pattern of activity in the external urethral sphincter recorded by electromyography partially accounted for the abnormal voiding function..[237] Marini et al. reported that either long-term mild hyperglycemia or short-term severe hyperglycemia have a detrimental impact on urethral muscle health of rats, as evidenced by the reduced striated muscle area in a short-term diabetic model and increased collagen deposition with the resultant severe fibrosis in long-term diabetes. Both diabetic models exhibited similar changes from fast to slow fibers and a decrease in the numbers of fast muscle fibers.[238] Kim et al. reported a more severe SUI, characterized by lower leak point-pressure in female diabetic rats post urethral damage by vaginal distention compared to non-diabetic animals. The authors postulated that diabetes delayed the process of urethral tissue recovery post-trauma.[239]

Considering the impact on pelvic floor muscles (PFMs), Micussi et al. compared PFM tone and maximal voluntary contraction measured by electromyography between 51 nulliparous women with insulin resistance and 35 nulliparous controls. The groups differed significantly with respect to BMI, weight and waist circumference, with all of the above being significantly higher in the insulin resistance group. The authors found an association between high insulin levels and aberrant electromyographic PFMs' signals, marked by lower EMG activity, tone and maximal voluntary contraction in women with insulin resistance.[240]

The elegant research on gestational diabetes and its consequences for the pelvic floor is also worth of attention. Gestation and diabetes seem to provoke more pronounced alterations in the urethra of female rats. Experimental studies showed that mild hyperglycemic state has several effects on extracellular matrix and urethral striated muscle responsible for urinary continence in pregnant rats, marked by a steady decrease in the proportion of fast to slow fibers, fibrotic deposition, and muscle atrophy, compared to only diabetic, only pregnant animals, or controls.[241] With respect to PFMs, electromyography demonstrated a progressive decrease in PFM activity during the rest-and-hold PFM contractions from second to third trimester in women with gestational diabetes. PFM resting activity and active contractions are important for the proper function of the female pelvic floor, as these muscles are involved in postural stability, continence, and mechanical support of the pelvic organs. [242]

Diabetes is a disease marked by biological conditions of low-grade chronic inflammation and oxidative stress, which may lead to systemic tissue damage. In diabetes, oxidative stress mediated damage to neurons has become a popular pathophysiologic mechanism of disease. Oxidative stress not only activates the major pathways namely, polyol pathway flux, AGEs formation, activation of protein kinase C, and overactivity of the hexosamine pathway, but also initiates and amplifies neuroinflammation. The cross talk between oxidative stress and inflammation is due to the activation of NF-κB and AP-1 and inhibition of Nrf2, peroxynitrite mediate endothelial dysfunction, altered NO levels, and macrophage migration. These all culminate in the production of proinflammatory cytokines which are responsible for nerve tissue damage and debilitating neuropathies.[243] Reports from different populations consistently support the mechanistic hypothesis that elevated circulating AGE levels are linked to insulin resistance, metabolic impairment and diabetic complications.[244,245,246,247] In addition, diabetes is related to accumulation of ROS and tissue ischemia, which can interactively or independently contribute to skeletal muscle dysfunctions.[248]

Inflammation / Oxidative Stress and Pelvic Floor Dysfunction

The role of inflammation and oxidative stress processes in the pathophysiology of PFDs has been mostly considered in the context of (1) postpartum tissue remodelling after vaginal birth[249,250]; (2) the use of cell-therapy for PFDs [251,252] (3) the use of synthetic prosthesis in pelvic reconstructive surgeries[253,254]; and (4) age-related changes in the pelvic floor.[255] However, some authors have

studied inflammatory/oxidative stress markers in the context of POP. In vitro studies showed the impact of AGEs on collagen I metabolism. Proliferation of vaginal fibroblasts derived from women with POP is inhibited by AGEs, with decreased expression of collagen I through receptor for advanced glycation endproducts (RAGE) and/or p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-kB-p65 pathways compared to control women.[256] The inverse correlation between AGEs and collagen I levels was confirmed by the analysis of tissue response in an animal model of abdominal defect treated with surgical repair with meshes.[257] The same research group investigated the levels of AGEs and RAGE, and single nucleotide polymorphisms (SNPs) in the vaginal tissues of 44 women with POP and 46 without POP. The authors reported a significantly higher protein expression of AGEs, but lower collagen I levels in the samples from POP compared to the control group. In POP patients, the expression of collagen I decreased in patients ≥ 60 years old, although the AGEs and RAGE expression were not related to age. RAGE gene sequence variance analysis identified two potential SNPs - rs184003 (1806), rs55640627 (2346) - that might be associated with POP. Possible mechanisms of POP development relies on the fact that AGEs are brittle and susceptible to rupture, resulting in tissue with an impaired mechanical strength. AGEs negatively impact the metabolism of collagen through RAGE, similar to the effects of AGEs in other diseases like diabetes. Further studies are needed to determine whether the change of AGEs is the reason or the result of POP.[257]

Vetuschi et al. evaluated the changes of AGEs, ERK, and TGF-β/Smad proteins expression in the muscularis propria of the anterior vaginal wall in 20 patients affected by POP compared with 10 women without POP. They observed that AGE, ERK1/2, Smad-2/3, MMP-3, and collagen III were upregulated in the POP group, whereas in controls, Smad-7 and collagen I were increased.[258] This study suggests that AGE plays a role in the ECM homeostasis and remodeling and could influence the pathogenesis and progression of POP. Possible interactions between MAPK, stimulated by AGEs, and Smads could lead to increased MMPs' synthesis and collagen III deposition.[259]

Weli et al. support the idea that glycation is a cause rather than an effect of prolapse based on the finding that age-related estrogen decline is a key player in glycation accumulation in prolapsed vaginal tissues.[259], [260], ER and Glyoxalase I (GLO-I), an antioxidant enzyme, were reduced in association with higher glycation in non-pregnant female Sprague-Dawley rat vaginal tissues.[257] Similar evidence was observed by analyzing full-thickness vaginal samples from a

group of 49 POP and 16 control women.[260, 258] The authors observed lower expressions of ER-α and GLO-I, and significantly higher pentosidine content (an AGE´s marker as product of sugar fragmentation) in the POP tissues in the comparison to the control. Prolapsed tissue had more notable age-dependent increase in pentosidine with significant differences between the 6th and 7th decade. Hypertension and smoking were also associated with higher amounts of pentosidine in the vaginal tissues and are cofounding variables that should be considered. [260]

An extensive body of literature links hormonal imbalance, glucose and lipids metabolism disorders with systemic inflammatory and oxidative stress markers and changes. Epidemiological data also strongly support the association between those conditions and PFDs. Even though the aforementioned reports do not clearly define how hormone-metabolic disorders affect PFDs, the findings suggest that inflammation/oxidative stress are the mechanisms that likely play a significant role in POP development. It is also not known if the cellular or molecular changes observed in the pelvic tissues are the cause or the consequence of POP. Moreover, the interaction of multiple risk factors (endocrino-metabolic disorders, ageing, menopause status, chronic mechanical stress, among others) makes it more challenging to study, necessitating preclinical models to perturb each factor.

Knowledge gaps and future research directions

The severity of POP/SUI symptoms increases after menopause, which is at least partially due to the loss of protective effects of ovarian hormones.[261],[262]While the direct causative link between menopause and PFDs is still lacking, the abundance of ERs in the urogenital tract explains why the natural reduction of endogenous E2, the hallmark of menopause, can cause or potentiate PFDs.[22,263] Mechanistic studies devoted to the above are urgently needed. Furthermore, the mechanisms governing the differential effects of systemic MHT vs vaginal LET in the lower urinary tract and pelvic floor tissues and organs needs to be determined to optimize exogenous hormonal therapies.

The role of hormonal signaling via less conventional “female” hormones such as androgens, vitamin D and thyroid hormones and its impact on PFDs is in its incipient stages. The role of these hormones in the function of skeletal muscle of the limb and early studies of the pelvic floor portend significant roles for these potent hormones in modulation, modification and

regulation of PFM function. Future research is needed to understand and capitalize on the putative role of these hormones in PFM function.

Metabolic conditions such as diabetes mellitus, polycystic ovary syndrome (PCOS), metabolic syndrome or obesity are usually intricated with hormonal imbalance. Epidemiological data support the association between those conditions and PFD. There is some evidence exploring potential mechanisms by which those conditions affect the pelvic floor leading to stress urinary incontinence and POP. However, there is still an open venue for future investigation. One difficulty in understanding the contribution of those conditions for pelvic floor dysfunction relies on their complexity, and the fact that women commonly present a combination of them.

The literature links hormonal imbalance, glucose and lipids metabolism disorders with systemic chronic inflammation and oxidative stress. Thus, it is highly plausible that these mechanisms play a role in the development of SUI and POP. The role of the isolated or combined risk factors (endocrino-metabolic disorders, ageing, menopause status, chronic mechanical stress, among others) needs to be studied, exploring the inflammation and oxidative stress pathways.

The immunobiology of skin, intestinal and respiratory mucosa is well studied. In contrast, the lower female urogenital system has received scant attention and the existing studies are mostly focused on sexually transmitted and urinary tract infections. While there is emerging knowledge on the role of sex steroid hormonal milieu on innate and adaptive immune function in the vagina and lower urinary tract, there is much to be learned, in particular related to their effects on the structure and function of these tissues. More studies on how E2 and P4 interact with nuclear ERs and various membrane ERs of the resident immune cells across the female lifespan are needed. The concept that the bladder is not sterile but hosts microbiota also needs further research, with focus on the role of E2, ER and resident immune cells. Research on the interplay between sex steroid hormones and urogenital tissue structure and function is almost non-existent. It is, therefore, important that such investigations are fostered to assess the effects of inflammation and its resolution on tissue micro- and macro-structure and function.

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Chapter 4: The Role of Aging and Immunity in the Pathogenesis of Pelvic Organ Prolapse and Stress Urinary Incontinence

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Pelvic organ prolapse (POP) and stress urinary incontinence (SUI) are complex, multifactorial conditions resulting from a weakening of supportive structures and deterioration in structure and function of the vagina and urethra, respectively. Aging, a complex multifactorial process involving the gradual decline of physiologic functions designed to maintain homeostasis of the tissues and organs, is a significant risk factor for both POP and SUI. Thus, understanding the mechanisms by which aging affects these conditions remains of great importance. The identification of critical molecular pathways involved in the attenuation of the pelvic organs, like the vagina and urethra, and their supportive tissues is essential for developing effective prevention and treatment strategies.

There is growing evidence that age dependent changes in cells, termed *cellular senescence mechanisms*, play a role in the compromise of the urethra and vagina, and their support systems. In this review, we highlight what is known about the mechanistic influences of aging on the cells and tissues of the urethra, vagina and their supportive tissues; the relationship between these changes and the pathogenesis of POP and SUI; and their impact upon the success of surgical interventions. Further, the present review highlights significant knowledge gaps in immune underpinnings which remain unstudied in the context of aging and these conditions. It is noteworthy that, to date, the impact of age on the urethra has largely been studied in men related to prostatic hypertrophy contributing to the knowledge gap on the pathophysiologic basis of worsening incontinence in women with age. The rapid decline of ovarian function after age 50, or menopause, undoubtedly contributes to aging in women, and it is difficult to disentangle tissue structural and functional decline attributable to the absence of ovarian hormones, mainly estrogen, described in XXXXXX and those solely due to aging. In this chapter, however, we focus on aging appreciating that these processes are highly interrelated.

Here, we highlight the mechanistic influences of aging on the cells and tissues, the relationship between these changes and the pathogenesis of POP and SUI, and their impact upon the success of surgical interventions. Further, the present review highlights significant knowledge gaps in immunological underpinnings characterized by elevated systemic cytokine milieu, infiltration of proinflammatory macrophages, and adaptive immune cells which remain unstudied in the context of aging and POP and SUI. Addressing these gaps may open novel avenues of therapeutic interventions in management of POP and SUI with the ultimate goal of improving women's health.

Introduction

POP and SUI, while two distinct conditions, share risk factors and hence, their pathophysiologic bases are likely similar. Both substantially negatively impact quality of life and mental health of affected individuals and thus, incur substantial cost to the livelihood of women world-wide. These conditions are common, impacting roughly 30-50% of the female population over the age of 50 years and incur a lifetime risk of undergoing a single surgical procedure to repair either POP or SUI of 20% by age 80 (1). In the United States, the separate cumulative risk for POP surgery is 12.6% and for SUI surgery 13.6%, with societal costs estimated to exceed $10 billion annually (2-5). A significantly higher number of women seek non-surgical or conservative treatment options, also with significant associated costs. Despite the high prevalence of POP and SUI, the frequent need for surgical intervention, associated costs, and the substantial impact upon quality of life, the natural history and underlying mechanisms of these pelvic floor disorders (PFDs), and especially POP, remain poorly understood.

The predominant risk factor for both SUI and POP is vaginal delivery. Additional risks include age, menopause, obesity, and race (6-11). Among these additional risks, age is perhaps the most impactful. Women above the age of 50 represent the majority of patients presenting for management of clinically significant POP (12). The prevalence of SUI peaks in the 5th decade and the prevalence of mixed incontinence, which includes SUI and urge urinary incontinence, dramatically increases with age thereafter, resulting in a disproportionate number of women seeking care for their incontinence after age 60 (8, 13). As the population over 65 is forecasted to increase to 88.5 million by 2050, and as 55% of those 65 and older will be female (11), the human and economic impact of POP and SUI is expected to rise substantially. Thus, improving our understanding of aging as a risk factor for POP and SUI is imperative if preventative measures are to be developed.

Aging is a multifaceted process associated with an overall functional decline spanning nearly all tissue and organ systems including those in the pelvis, and is a significant risk factor for many diseases and conditions (14). Aging has been well investigated in the context of major conditions including cancer, and cardiovascular and neurodegenerative diseases. Mechanistic investigations of the impacts of aging at the cellular level in these conditions have led to the identification of nine suggested *"hallmarks" of aging* (14). These hallmarks manifest during the

degenerative processes of aging and include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (14). These hallmarks provide a framework for the study of the impact of mechanisms of aging on degenerative processes. Investigations of aging at the cellular level have led to significant improvements in the understanding and treatment of many prevalent age-related disease processes. However, despite the known increases in the incidence of POP and incontinence with aging, there have been few mechanistic studies into the role of aging in the processes which underlie POP and SUI. Furthermore, a key aspect of aging, namely the immune changes that occur with age, termed *inflammaging* or *immunosenescence*, have not been investigated in depth in the pathogenesis of POP or SUI and will be explored here.

The pathophysiology of POP and SUI can be broadly attributed to deterioration in the structure and function of the vagina and urethra, respectively, and the soft tissues that support them. While initial injury may be damage to nerves, connective tisues, and muscles at childbirth, additional decompensation occurs mostly from aging and age-related diseases over time. The primary structures involved in support of the pelvic organs are the pelvic floor muscles (PFMs) and supportive connective tissues (reviewed in (15)). The vagina is comprised of a stratified squamous epithelium followed by a dense connective tissue enriched with collagen and elastin and a bilayer of smooth muscle. The urethra is supported by the periurethral ligaments, the pelvic diaphragm, and the levator ani muscles. It is comprised of a stratified squamous epithelium, a spongy submucosa, a middle smooth muscle layer, and an outer fibroelastic layer. Urethral closure is achieved via active and passive mechanisms (13, 16, 17). At rest, the urethra is closed by submucosal vessels, elastin and connective tissue, smooth muscle and neural networks. A voluntary striated sphincter muscle forms the outermost layer that is highly interactive with the levator ani muscles. During increases in intra-abdominal pressure, levator ani and the supportive connective tissues interact in a highly orchestrated fashion to maintain the pelvic organs, such as the vagina and the urethra, in their normal anatomic position.

Changes in Cellular Function with POP and SUI in Aging

While each of the hallmarks of aging described above have been shown to have distinct impact upon cellular behavior, the overall result of these changes is generally referred to as *cellular*

senescence (14). Cellular senescence is characterized by cell cycle arrest and a *senescence associated secretory phenotype* (SASP) (14, 18, 19). SASP positive senescent cells accumulate over the life span in rodents, primates, and humans, and are more often found in renewable tissues and sites of chronic inflammation (19, 20). SASP cells have also been shown to be present in settings characterized by trauma or excess loading such as posttraumatic osteoarthritis (21, 22). The most common cell-intrinsic change resulting in a senescent phenotype is the accumulation of genetic changes within cells over the course of a lifetime. Such changes are continually occurring due to exogenous physical and chemical challenges, DNA replication errors, and exposure to oxidative stress resulting in a diverse range of point mutations, translocations, chromosomal gains and losses, and telomere shortening. The ensuing changes can occur in the nuclear DNA as well as mitochondrial DNA, resulting in a range of dysfunctional outcomes, including alteration of essential gene and transcriptional mechanisms as well as functional phenotypes. Certain regions of the DNA are more susceptible to age-related modifications (23). For example, it is well established that telomere shortening occurs with aging (20, 23), eventually leading to the progressive loss of proliferative capacity of cells known as the *Hayflick limit* (24). Each of these changes can lead to dysregulation of tissue homeostasis and SASP phenotype. A recent review by Huang, et al, examined multiple aspects of cellular senescence and their relationship to POP, suggesting a role for senescence of fibroblasts in the pathogenesis of POP (25). However, POP-specific studies of cellular senescence are few and the exact implications of how the observed cellular changes contribute to POP and SUI pathogenesis remains an important avenue for investigation. Only a handful of studies investigating genetic instability in the context of POP have been performed. Microarray studies of the pubococcygeus muscle as well as uterosacral and round ligaments, have suggested that there are differences in gene expression associated with SUI and POP (26, 27). These studies, as well as a recent systematic review examining both specific gene and whole genome/proteome level data sets demonstrate that genes associated with extracellular matrix (ECM) production and maintenance, estrogen receptors, inflammatory mediators, as well as structural proteins related to actin and myosin are altered in patients with POP (26-30). However, the degree to which these expression profiles are induced by age-related changes versus hereditary- or injury-induced mechanisms remains an open question.

Cellular Dysfunction in POP and Aging

Age-related mitochondrial dysfunction is a well-known component of the aging process (14, 31). The *mitochondrial free radical theory of aging* suggests that progressive mitochondrial dysfunction leads to increased production of reactive oxygen species (32), resulting in further cellular, mitochondrial, and tissue level damage as well as dysfunction in cellular bioenergetics (energy production, expenditure); however others suggest that oxidative stress is only one of many mechanisms which underlie aging and cellular dysfunction (14, 33). Studies of mitochondrial dysfunction in postmenopausal women with POP have shown that fibroblasts isolated from the uterosacral ligaments of patients with POP were associated with slower proliferation, reduced viability and increased expression of mitofusin 2 (Mfn2), an outer mitochondrial membrane GTPase critical for mitochondrial fusion, as well as downregulated expression of procollagen. These data suggest a link between mitochondrial function and collagen production as the increased expression level of Mfn2 could inhibit the proliferation and cell cycle of fibroblasts by mediating Ras/Raf/ERK pathway, leading to the decrease in collagen synthesis and eventually tissue degradation (34). Another study examined the prevalence of mitochondrial DNA (mtDNA) changes in the uterosacral ligaments, showing that depletion of mtDNA, including rearrangements of mtDNA4977, were accumulated in the uterosacral ligaments of women with POP, especially those with stages III and IV (35). mtDNA4977 is among the most common mtDNA variations found in aging human tissues (36). This deletion is also associated with increased oxidative stress within aging tissues' environment, suggesting POP may be linked to oxidative damage. The negative impact of aging on the function of mitochondria in the urethra and its supportive tissues is less clear. Elucidation of the mechanisms underpinning this process might provide novel avenues for therapeutic interventions to manage POP.

Oxidative stress has been suggested as a primary driver of the genetic changes observed with aging. Increases in oxidative stress are known to occur over the lifespan, potentially due to concurrent decreases in the production and function of oxidative stress regulating molecules such as superoxide dismutase. In the context of POP, Kim, et al., demonstrated that indicators of oxidative stress were increased in the uterosacral ligaments of women with prolapse (mean±SD age 55.5±9.0 years) compared to age matched controls (mean±SD age 54.9±8.4 years), as evidenced by the increased expression of 8-OHdG and 4-hrdroxy-2-nonenal identified by immunohistochemistry (37). Additional analysis demonstrated that women with stage III and IV prolapse also demonstrate evidence of mitochondrial apoptosis (37). In vitro studies have

demonstrated that mechanical strain can induce cellular oxidative stress via the PI3K/Akt pathway, leading to apoptosis and senescence of human uterosacral ligament fibroblasts (38, 39). Further investigation showed that oxidative stress also induced a shift towards collagen catabolism, reduced collagen I production, and increased matrix metalloproteinase (MMP) expression in human uterosacral ligament fibroblasts (38, 39). While these studies did not specifically address the effects of aging upon the production of reactive oxygen species or the susceptibility to oxidative stress, the observed changes in collagen metabolism and regulation of MMPs in uterosacrarl ligament fibroblasts have been shown in multiple studies examining pelvic tissues procured from women with POP, as well as in numerous studies of aging appendicular muscles and ligaments. Together, these studies clearly highlight the potential for age-related oxidative stress and changes to cellular machinery in the pathogenesis of POP; however, whether there is a link between oxidative stress and other age-related cellular changes in POP remains to be elucidated.

By comparison, fewer studies have been performed examining oxidative stress in the setting of SUI specifically; however, as oxidative stress can lead to muscle wasting, and as relationship exists in POP, it has been hypothesized as a potential mechanism underlying SUI (40, 41). Limited evidence from a study conducted in a rodent model suggests that mechanical-trauma-induced oxidative damage and ECM remodeling contribute to the pathogenesis of SUI. The above is largely based on the protective effect of the potent anti-oxidant punicalagin (42, 43). The ability of this compound to mitigate the reduction in leak point pressures after vaginal distension injury was via activation of TGF-β1/Smad3 and the nuclear factor erythroid 2–related factor 2 (Nrf2)-regulated antioxidant response element signaling (44). Low intensity extracorporeal energy shock wave therapy (2000 to 3000 pulses in 0.20–0.25 mJ/mm^2) which has been shown to reduce oxidative stress, enhance wound healing, promote angiogenesis, induce vascular endothelial growth factor (VEGF), stimulate proliferation and differentiation of stem cells, and promote tissue regeneration was shown to decrease SUI symptoms in prospective studies, including one small randomized trial (45-48).

Epigenetic alterations in Aging and POP

While genetic mechanisms such as point mutations in DNA nucleotide sequences, chromosomal mutations and gene copy number variations can predispose individuals to various

diseases and accelerated aging, the epigenome is responsible for the stability and plasticity of the function of our genes in response to our environment, which also influences the fate of all cells and tissues (49, 50). The epigenome includes alterations that are defined as reversible heritable changes to the genome that do not involve changes in the DNA sequence. The mechanisms responsible for these changes include alterations in DNA methylation patterns, posttranslational modification of histones, chromatin remodeling, and non-coding microRNAs (miRNA) (50). These epigenetic modifications have been identified as one of the hallmark mechanisms of aging, and are thought to potentially explain the diverse patterns of physical decline within the population (14, 49, 51-53). Epigenetic mechanisms may also play a role in the compromise of the pelvic support system. Since aging is a risk factor for POP and urinary incontinence, epigenetic mechanisms could explain how aging affects the deterioration of the tissues providing support to the pelvic organs including the vagina and urethra.

Longevity studies have demonstrated that genetic factors explain 20-30% of the differences observed in the lifespans of monozygotic twins, and that epigenetic drift accumulated during their lifetime was responsible for the remaining differences (54-57). Similarly, in a twin study conducted by Altman et al., 3,376 mono- and 5,067 di-zygotic female twin pairs were identified from the Swedish Twin Registry and their records were cross-linked to the Swedish Inpatient Registry that contains data on individual hospital discharges to determine the genetic and environmental influence on the incidence of SUI and POP surgery. Using statistical modeling, it was determined that genetic and non-shared environmental factors equally contribute ~40% of the variation in risk for the development of POP and SUI, however, the interrelationship between genetics and the environment remain unknown (58). Investigating the influence of environmental factors and epigenetic regulation of genes is promising to close this gap in knowledge. To date, there are a limited number of studies investigating DNA methylation and microRNA as epigenetic mechanisms involved in POP and SUI pathogenesis, which could elucidate signals of early or advanced aging processes. We highlight these studies here.

DNA methylation regulation in POP and Aging

Methylation of cytosines in CpG (cytosine nucleotide followed by guanine nucleotide) dinucleotides is an epigenetic modification that alters gene expression and is a well known mechanism for gene silencing. DNA methylation can result in alterations in transcription factor

binding sites, control of gene expression at important regulatory sites such as enhancer regions, change in chromatin structure, and gene imprinting (59). To date, there are 2 studies evaluating DNA methylation in tissues procured from women with POP, however, there are no studies evaluating DNA methylation as a potential mechanism in SUI, though it is reasonable to suggest that similar mechanisms may affect tissues in SUI.

Lysyl oxidase is an enzyme involved in cross-linking of collagen and elastin fibrils in the extracellular matrix of tissues (60). Transgenic mice deficient in lysyl oxidase (LOX) and its family member, lysyl oxidase-like 1 (LOXL1), have been shown to develop POP spontaneously and after vaginal birth, and also demonstrate lower urinary tract dysfunction similarly to that observed in women with prolapse (61-63). Alterations in biomechanical properties of the vaginal wall and its supportive connective tissues in LOXL1 deficient mice demonstrate a causal link between LOXL deficiency and POP in these transgenic mouse models (64). A few studies have shown this enzyme to be deficient in the vagina and uterosacral ligaments in women with POP compared to women with normal support (65-68). Based on findings in animal models and in women with POP, Klutke et al., investigated if DNA methylation was a potential mechanism for altered LOXL1 expression in women with POP. The authors treated genomic DNA isolated from paracervical uterosacral ligament biopsies with sodium bisulfite modification, and then sequenced amplified plasmid DNA samples containing the LOX gene promoter region from each woman to idenfy methylated CpG islands by sequence comparison. They found increased DNA methylation in the promoter of LOX in paracervical biopsies of the uterosacral ligaments in 31 women with stage III or greater POP compared to 29 women with minimal to no POP, who were of similar age (mean (range) 45.4 years (38–49) and 47.6 years (36–59), respectively) and parity (3.4 (2–5) and 3.1 (0–7), respectively) (69). These data suggest that DNA methylation may down regulate the expression of LOX enzyme leading to abnormal collagen and elastin in pelvic supportive structures.

A recent genome-wide DNA methylation analysis of uterosacral ligament biopsies taken at the the time of hysterectomy in postmenopausal women with and without POP (n= 5 and 4, respectively) further revealed thousands of differentially methylated CpG sites including hypermethylated and hypomethylated sites in patients with POP compared to normal controls (70). Overall, there were more hypermethylated CpG sites, but a high ratio of hypomethylation

between CpG islands and the northern shelf region, (a 2 kilobase pair region adjacent to and upstream of the 2 kilobase shore that is directly upstream of the CpG island), was also seen. Gene ontology analysis demonstrated that these differentially methylated genes were associated with mechanisms involving 'cell morphogenesis', 'extracellular matrix', 'cell junction', 'protein binding' and 'guanosine triphosphatase activity'. Analysis using the Kyoto Encyclopedia of Genes and Genomes identified several significant pathways including 'focal adhesion' and 'extracellular matrix-receptor interaction pathway'.

Together, these two studies suggest that epigenetic mechanisms are involved in the regulation of the transcription of genes responsible for differentiation of tissues, extracellular matrix proteins and key intra- and intercellular functions are altered in women with POP. Targeting these pathways with epigenetic markers that accumulate with aging could lead to novel treatment options for tissue engineering through modulation of these identified proteins. However, robust studies that define POP and control groups using a standardized staging system such as the Pelvic Organ Quantification System (POP-Q) that control for risk factors for POP (i.e. menopausal status and BMI) as well as for methylation status (i.e aging, smoking) are needed. In addition, standardization of the procurement of specimens and confirmation of the histology using a standardized histologic quantification system should be employed. This is important since the histology of the proximal, middle and distal portions of uterosacral ligaments are distinctly different and since histologic pheontypes of prolapsed tissues exist. Thus, cellular content will dictate methylation patterns (71-73). Lastly, confirmatory studies demonstrating that hyper- and hypomethylated status of genes correlate to their expression are necessary in order to determine if the changes in methylation affect transcription. Future studies looking at DNA methylation as a contributor in developing SUI in women are also needed.

MicroRNA regulation in POP and Aging

MicroRNAs (miRNA) are small endogenous RNA molecules that play important regulatory roles by targeting mRNAs for cleavage or translational repression (74). Remarkably, although miRNA genes represent only 1% of the genome, it is estimated that approximately 30% of the protein-encoding genes are regulated by at least one miRNA (75, 76). MicroRNAs play key roles in diverse regulatory pathways including developmental processes, cell growth, differentiation and apoptosis. Alterations in miRNA expression are associated with various human

316 diseases (77, 78) and are emerging as a regulatory mechanism in development and maintenance of
317 the pelvic supportive structures requiring collagen.

318 For example, the homeobox gene, HOXA11, which is necessary for the development of
319 uterosacral ligaments and is an important upstream regulator of collagens and matrix
320 metalloproteinases, has been shown to be deficient in the uterosacral ligaments in women with
321 POP compared to women with normal pelvic support (79-82). Jeon et al. performed miRNA
322 microarray expression profiling of the uterosacral ligaments of 38 POP patients and 38 controls as
323 staged by the POP-Q system. There were no differences between the two groups in terms of age,
324 parity, BMI or menopausal status. Notably, both the POP and control groups were largely
325 comprised of postmenopausal women (68% and 55%, respectively). They identified 10 miRNAs
326 which target HOXA11 mRNA and found them to be overexpressed in POP patients. Notably,
327 miR-30-d and 181a expression was inversely correlated with HOXA11 mRNA levels and forced
328 overexpression of miR-30d or 181a suppressed HOXA11 mRNA and protein levels in human
329 embryonic kidney cells, whereas the knockdown of these miRNAs enhanced HOXA11 levels and
330 collagen production (81).

331 In expeirments to determine potential differential expression of miRNA in women with
332 SUI and to predict putative target genes, Liu et al. evaluated periurethral vaginal tissues of
333 postmenopausal women with stage 0-II POP, as determined by the POP-Q system, who were not
334 using exogenous estrogen with and without SUI (n = 13/group). Groups were matched by age,
335 parity, BMI and duration of menopausal status. Using miRNA mircroarray analysis, they identified
336 12 miRNAs that were differentially expressed, and by integrating data from a previous gene
337 microarray study and computational algorithms, they predicted 3 miRNA pairs. Furthermore, the
338 expression of predicated miRNAs , mRNAs, and corresponding proteins was determined using
339 PCR and western blot, respectively, and an inverse association between miRNAs, paired mRNAs,
340 and proteins was detected, confirming that the identified miRNAs regulated transcription and
341 downstream translation. Interestingly, all three predicted target genes were associated with
342 neurodegenerative conditions, indicating the potential significance of neurodegenerative
343 mechanisms in the etiology of SUI in this well designed study of older women (83).

344 Yang et al. demonstrated that vaginal biopsies from women with SUI and little-to-no POP
345 (SUI group: n=18; average age 49.56±6.41years; number of vaginal deliveries 1.56±0.70; BMI
346 22.16±2.40 kg/m^2) demonstrated significantly lower expression levels of miR-93 compared to

vaginal tissues from women without SUI (control group: n=20; average age 52.90±8.09 years; number of vaginal deliveries 1.70±0.80; BMI 23.10±2.23 kg/m^2). In order to further investigate the function of miR-93 in the development of SUI, a luciferase reporter assay was performed to investigate its association with calpain-2 (CAPN2) - a neutral protease that degrades ECM components, including matrix metalloproteinase-1 (MMP1) that has been shown to be upregulated in vaginal tissue in women with POP (84). They used a primary fibroblast cell line and an established SUI cell line to perform confirmatory validating studies. They demonstrated that miR-93 was able to bind to CAPN2, and that overexpression of miR-93 decreased the expression of calpain-2, indicating that calpain-2 may be a direct target of miR-93. Furthermore, they observed that MMP1 was upregulated in the periurethral vaginal tissue of patients with SUI and in SUI primary fibroblasts in cell culture, suggesting that MMP1 may be negatively regulated by miR-93, which was consistent with a previous report (85). Unfortunately, the menopausal status of these middle age women in two groups was not reported.

Together, these studies have identified miRNAs that modulate the ECM of the pelvic support structures as important regulators of genes expressed in the uterosacral ligaments, periurethral connective tissue, and vagina in women with POP and SUI. Future studies should evaluate pre and postmenopausal women separately to determine the effects of estrogen status on the expression of miRNA. Similar to the techniques in determining methylation status of complex tissues with several cell types, characterizing the histology of the specimens is also important in determining miRNA expression as it may vary with difrerences in the proportions of cellular content between specimens. As the science emerges on the use of miRNA therapeutics in gene therapy, advances to overcome issues with stability, delivery and toxicity are being developed with the use of directed vectors and nanoparticles. Since miRNA are generally cell-type specific, understanding how they function in other cell types will be critical to determine specificity and undesired effects of surrounding tissues. It is therefore plausible that directed miRNA therapeutics could be used in the future to specifically target the genes that modulate the expression of ECM proteins (86).

As mentioned above, hormones can also be a key player in epigenetic regulation. For example, the sex hormone estrogen, mediates its biological effects through estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (87) which are expressed in the vagina, uterosacral ligaments, and the uretha of pre- and postmenopausal women, with and without POP/SUI (28, 88,

89). ERβ expression declines with age regardless of postmenopausal estrogen use in women with POP (88). Based on these findings, a few studies have evaluated regulation of estrogen receptors via miRNA since menopause is associated with aging and the development of POP and SUI. MiR-92 is a small non-coding RNA that has been shown to regulate ERβ1 in breast cancer cells (75). It was later found that miR-92 expression is increased in the paracervical biopsies of the uterosacral ligaments of women with POP (n=56) compared to matched counterparts without POP (n=48). These groups were matched by age and had similar parity and BMI. Both groups consisted of pre- and postmenopausal women, however postmenopausal women in each group predominated. They found that women with more advanced stage of POP had higher expression levels of miR-92 as determined by qRT-PCR, and that an inverse correlation between ERβ1 protein and miR-92 expression existed, suggesting that ERβ1 may be a target of miR-92 (90). ERα is also a target gene for two other miRNAs, miR-221 and miR-222, which inhibit ERα protein expression. In a study comparing paracervical biopsies of the uterosacral ligaments in 40 POP and 40 controls matched for age, menopausal status, parity and BMi, Zhi et al. found an increased expression of miRNA-221 and miRNA222 via RT-PCR in women with POP. Conversely, they found decreased protein expression of ERα in the uterosacral ligaments of women with POP compared to controls (91). Future studies with lager cohorts separating pre and postmenopausal groups, and experiments to eluciate direct cause and effect of miRNA on ER expression are needed. These studies warrant further investigation as they provide insight on epigenetic regulation of estrogen signaling as a potential mechanism impacting tissue integrity which is important since both hypoestrogenism and epigenetic changes occur with aging.

Immune-Matrix interactions in POP and SUI in Aging

Aging affects the integrity of the tissues at the level of intercellular communication with the ECM and immune cells. These vital pathways of cross-talk can be disrupted due to several mechanisms (92-94). Inflammation has been identified as a prominent mechanism of altered intracellular communication that is associated with decline of the integrity and function of tissues and progression of disease states (95, 96). Mechanisms include the secretion of pro-inflammatory cytokines by senescent cells, activation of inflammatory pathways involving NF-κB transcription factor signaling, or deficiencies in the autophagy response, which identifies and clears defective macromolecules and damaged mitochondria. Reduced recognition and clearance of abnormal

409 cells, such as senescent and premalignant cells, can lead to deterioration in the functional and
410 mechanical properties of tissues in a spectrum of conditions, as reviewed by Lopez-Otin (14).

411 Studies are now emerging investigating the role of the immune system and the ECM
412 components, and how these affect the function and structural integrity of the pelvic floor support
413 system. Transforming growth factor-□ (TGF-β), a multifunctional cytokine produced by white
414 blood cells, has been shown to be a key mediator of signaling in cellular senescence and age-
415 related pathologies. Aging or senescent fibroblasts in skin demonstrates reduced or dysregulated
416 collagen production, increased protease activity, elastin metabolism, and decreased expression of
417 TGF-β. (10) TGF-β isoforms are known to regulate the balance in synthesis and degradation of
418 collagen matrix via MMPs and their inhibitors [tissue inhibitors of metalloproteinases (TIMPs)]
419 (97). Thus, in the context of POP, a reduction of TGF-β could reasonably lead to dysregulation
420 and weakening of the tissue of the pelvic floor. Indeed, a recent study demonstrated that TGF-β
421 is expressed at significantly lower levels in the uterosacral ligaments of 40 postmenopausal women
422 with POP compared to 40 controls matched by age, parity, BMI and length of postmenopausal
423 status, and was negatively correlated with severity of POP (98). The modulation of the immune
424 system by steroid hormones in the female pelvic floor and genitourinary tract is discussed in detail
425 in the *The Impact of Hormonal Milieu on the Female Pelvic Floor Structure and Function* chapter.
426 Further investigation on how the immune system regulates ECM and how aging and menopause
427 modulate the immune system are needed to develop preventative strategies and to help develop
428 new strategies in tissue engineering for more favorable host response reactions (99).

429 Thus far, studies of the immune system and POP and SUI have focused on the
430 inflammatory reaction that occurs with mesh implantation in animals and with exposure/erosion
431 of mesh in women, and not necessarily on the role of the immune system in the general
432 maintenance and/or dysregulation as seen with aging (100-102). Polypropylene mesh can elicit a
433 foreign body response causing aggregates of inflammatory cells, such as lymphocytes, plasma
434 cells, macrophages and giant cells (103). Puerarin is a plant-based isoflavone compound with anti-
435 inflamatory properties that is used in traditional Chinese medicine (104-106). Using a Puerarin
436 based drug-loaded matrix, Qin et al attempted to modulate the immune response of cultured
437 vaginal fibroblasts obtained from women with POP in an attempt to improve the biocompatibility
438 of implants used in POP repairs. This anti-inflammatory biomaterial reduced the aggregation of

inflammatory cells, inhibited inflammatory cytokines, and promoted matrix remodeling which provided a stable immune environment around implants (107). Future studies like this promise new approaches for application in tissue regeneration, and may provide insight if there are any age related differences in foreign body responses between older and younger women. This is important since age-related changes in the immune system may play a significant role in postmenopausal women with POP.

Other studies evaluating POP and intercellular communications are focused on the expression of *fibulin-5*, an integrin binding matricellular protein that is essential for elastic fiber assembly, and *integrins*, which are transmembrane receptors that facilitate the binding between cells and ECM in pelvic support structures. The majority of these studies have shown that there is decreased expression of fibulin-5, and altered expression of integrins including ECM protein-1, integrin β-1 and integrin β-3 in pelvic floor support tissues in women with POP (65-68). Budatha et al. elegantly defined the mechanism of the interactions of fibulin-5, integrin binding, and regulation of MMPs in vaginal tissue, and importantly, how disruption of this pathway can lead to POP (108). Fibulin-5 has a unique motif that contains an evolutionally conserved arginine-glycine-aspartic acid (RGD) sequence known to mediate binding to cell surface integrin receptors (109). Fibulin-5 controls assembly of elastic fibers in an RGD-independent manner and MMP-9 activity in an RGD-dependent manner in the vaginal wall of wild type mice and prevents development of POP. In fibulin-5 knock out mice, the structure of elastic fibers is altered, and MMP-9 activity is upregulated through increased fibronectin-integrin interactions and generation of reactive oxygen species in vaginal stromal cells, ultimately leading to POP. Importantly, creating a double knock out mouse, where mice are deficient in fibulin-5 and MMP-9 , resulted in significant rescue of the prolapse phenotype where thick and continuous bundles were observed in the vaginal wall compared to the thin, irregular, and disrupted collagen fibers seen in the *Fbln5*$^{-/-}$ mice. When the RGD motif was mutated, upregulation of MMP-9 alone was not sufficient to cause POP due to the presence of intact elastic fibers. However, inhibition of the activity of lysyl oxidase, an enzyme responsible for cross linking elastin fiber during assembly, in combination with increased MMP-9 led to milder POP. This suggests that an imbalance between synthesis and degradation pathways in elastin production could also contribute to development of POP, and that MMP-9 upregulation plays a significant role in the pelvic floor dysfunction in *Fbln5*$^{-/-}$ mice.

In another study of adhesion molecules and POP, Kufaishi et al. investigated the effects of static mechanical loading on the expression of ECM and cellular adhesion proteins in vaginal cells derived from premenopausal women with stage II - IV POP (n=8) and compared them to women with stage 0 POP (n=7, controls). The demographic data including age, parity, BMI were not reported. They found that stretched POP cells demonstrated differential expression of transcript levels of collagens, MMPs and integrins compared to controls (n=7). In a second study, they found that resting (non-stretched) cultured primary vaginal cells procured from women with POP respond differently when placed on varying matrigels. These cells demonstrated altered cellular adhesion, expression of integrins, collagens and MMPs compared to cells from controls. This demonstrates that risk factors that induce stretch may alter ECM composition and the critical cell-ECM interactions that are necessary to maintain the pelvic floor tissues (110-112). Since POP is a multifactorial disease, disruptions at the level of cellular communication from mechanical stretch and decline from aging processes may have an additive effect on the quality of the pelvic support structures. Future expansive studies are needed evaluating mechanical stretch in postmenopausal tissues and cells form older women, and animal models could be useful in determining these interactions.

Aging and Immunity in POP and SUI pathogenesis

The above hallmarks of aging are known to be present in many age-related diseases and tissue degradation. Clear links have been established between cellular dysfunction and tissue degradation, including the progressive changes in ECM content and structure seen in POP and SUI. However, tissue resident fibroblasts and muscle cells are not the only populations which participate in the pathogenesis of these conditions. Aging is also well known to affect the immune cells of both the innate and adaptive immune systems. These changes are likely to have significant impact both on the pathogenesis of POP/SUI as well as the ability to treat using surgical interventions.

Immunosenescence is a phenomenon occurring with advanced age and has been associated with increase susceptibility to infection, autoimmune disorders, and cancer related mortality (113-115). While the definition of senescence usually includes the arrest of cell division, such as that discussed above for fibroblasts (Hayflick limit), immunosenescence is better characterized by a reduction in circulating cells, delayed migration to sites of injury, and dysregulated immune

responses (115, 116). Immunosenescence, much like cellular senescence, is characterized by increased systemic levels of inflammatory cytokines and oxidative species (117, 118). This process has been referred to as "inflammaging" as the effects of this elevated systemic cytokine milieu and resultant oxidative stress can cause tissue damage and senescence of stromal cells, resulting in their acquisition of the SASP (19, 118).

The role of innate immune system in the female pelvic floor and genitourinary tract is detailed in the *The Impact of Hormonal Milieu on the Female Pelvic Floor Structure and Function.* The innate immune system includes neutrophils, eosinophils, basophil, mast cells, innate lymphoid cells, and antigen presenting cells. Each of these cells provides a coordinated response which ideally results in the restoration of tissue homeostasis (119). Multiple defects in this response have been observed in aging. For example, dendritic cell activation is impaired in aging, resulting in impaired ability to cross tissue barriers, and to elicit responses from the adaptive immune system (120, 121). Similarly, monocytes and macrophages, which have recently been shown to be key mediators of tissue homeostasis, wound healing, and the response to implanted materials, are altered over the lifespan (122, 123). Macrophages have been shown to accumulate in multiple tissues including the liver, heart and adipose tissues with age (115, 124, 125). Accumulation has also recently been observed in the reproductive system with aging, corresponding with fibrosis in the stroma of the ovary (126); and in aging bladders (127, 128). Of note, though multiple studies have shown increases in inflammatory mediators in patients with prolapse, studies of uterosacral and round ligaments of women undergoing hysterectomy for POP were not found to have increased inflammatory infiltrates as compared to controls (26).

Macrophage recruitment is delayed in aging, and a reduction in major histocompatibility complex II has been observed, leading to reduced antigen presentation and cross talk with the adaptive immune system (129, 130). Further, a reduction in the ability of macrophages to polarize towards M1, pro-inflammatory, and M2 anti-inflammatory, homeostatic phenotypes has been observed (131). Reductions in macrophage function have been correlated with increased incidence of infections with age, particularly in postmenopausal women. This dysregulation in the innate immune response is associated with slow and incomplete wound healing as well as the loss of tissue homeostasis with aging and has been implicated in a wide range of age-related diseases. Recent studies of 12 women (mean age 57.21±12.11) have taken bioinformatics approaches to identify immune changes in POP and identified mast cells and neutrophils infiltration to be higher

in POP tissues (132). In addition, inflammatory cytokines and genes associated with cytokine-cytokine-receptor interactions and chemokine signaling pathways have been implicated in POP. Few studies have identified immunologic changes in women with SUI. However, one recent study utilized genome-wide association to study 8,979 European women with stress incontinence, urgency incontinence, and all incontinence phenotpyes (133). The results identified and confirmed two signle nucleotide polymorphisms, endothelin 1 (EDN1) and macrophage receptor with collagenous structure (MARCO) to be associated with urgency incontinence and stress incontinence, respectively. These findings suggest a potential role for the innate immune response in stress urinary incontinence, possibly due to persistent bacterial coonization in lower urinary tract system. However, significant work is needed to demonstrate and elucidate the role of the innate immune system in SUI.

Adaptive immune cells, which include T cell and B cell types could also play a role in POP and SUI. However, little is known regarding these populations in the aging pelvic floor. Mysorekar and colleagues have recently shown that there are distinct age dependent changes in the immune landscape of the genitourinary tract with enrichment of adaptive immune cells and formation of tertiary lymphoid tissues in the bladder mucosa (127). Persistence of the recruited adaptive immune cells in the bladder tissue were not conducive to repair and associated with increased risk of UTIs. Vaginal estrogen has shown efficacy in improving age associated adaptive immune changes in reconstructive surgery for pelvic organ prolapse (134). Taken together, the evidence for alterations of both innate and adaptive immunity seen with aging, changed immune cell gene expression in POP, and paucity of understanding of adaptive immunity contributions in POP and SUI warrants significant further study.

Aging and Immunity in Surgical interventions in POP

Nowhere is the importance of understanding immune infiltration and inflammation more clear than in the response to surgical reconstruction or the outcome of prosthetic mesh implantation and other surgical interventions to treat POP or SUI (135). Surgical interventions naturally elicit a wound healing response. Wound healing is a highly dynamic process characterized by a series of overlapping events orchestrated by numerous resident and recruited immune cells, soluble factors, and matrix assembly (136, 137). Immediately following injury, a rapid inflammatory response ensues with the initiation of the innate immune response (138, 139). Resident dendritic cells and

monocytes release cytokines to facilitate migration and differentiation of macrophages, at the site of injury. Macrophages are a key mediator of the wound healing process, and dysregulation of their phenotypes results in delayed or deficient wound healing (138, 139) . This is followed by the deposition of extracellular matrix and subsequent remodeling with resolution of the wound healing process or scarring as an outcome. This series of events occurs following tissue injury, regardless of the cause, including those caused by vaginal delivery or surgical intervention, for example.

Prosthetic mesh materials are commonly used in the repair of POP to provide mechanical support to the pelvic floor and to reduce recurrence rates (140-142). While mesh implantation has been shown to improve anatomical outcomes in the anterior and apical compartments, complications associated with mesh usage are observed (143-146). These include mesh exposure through the vaginal wall, shrinkage, erosion and pain. Recent work has shown that these complications may be attributable, at least in part, to the host immune response following implantation (102, 103). Chronic activation of the M1, pro-inflammatory macrophage response has been associated with erosion, while chronic activation of a more M2, pro-healing, phenotype has been associated with fibrosis and pain (102). Thus, careful orchestration of the macrophage response to implantable mesh materials is required for integration and long-term success. Multiple studies have now demonstrated that mesh materials which elicit stronger M1 type responses are associated with increased vaginal tissue degradation following implantation in primate studies (103, 135, 147-149). Others have shown that methods which shift the early host response towards an M2 phenotype are associated with improved integration and remodeling outcomes (150-152).

While age-related defects in the wound healing process have not been clearly tied to the pathogenesis of POP, they may play an important role in the success or failure of surgical reconstruction or the outcome of prosthetic mesh implantation. A recent study investigated age-associated differences in macrophage response in a rodent model of vaginal wound healing (153). Histological analysis showed clear differences in wound healing, with more rapid wound closure in young rats (12 weeks old) as compared to aged rats (12 months old). The aged rats also exhibited a more robust and sustained macrophage response characterized by increased TNF-α (Tumor Necrosis Factor alpha) and iNOS (inducible Nitric Oxide Synthase) expression and an increased ratio of M1:M2 immunolabeled macrophages within the wound site than was observed in young animals. Additional studies in this rat model demonstrated that these changes in the host response

were correlated with expression of macrophage-migration inhibitory growth factor within the site of tissue remodeling and resulted in lower tensile strength of tissues at 30 days post injury (154).

Recent studies have further examined variations in the host response to implanted materials with increasing age (150, 155). For example, one study examined the host response to polypropylene mesh in an aged rodent model (155). These studies showed a delayed macrophage response as well as dysregulation of macrophage polarization with a chronic increase in the M1 phenotype in aged (18 months) animals. The study further examined the circulating macrophage population, showing that the ability of the cells to migrate and switch polarization states was largely intact, suggesting that the local tissue environment may have been responsible for the observed delay and dysregulation of the macrophage response. A follow up study demonstrated that delivery of IL-4, an M2 polarizing cytokine, was effective in modulating the immune response in young (2 months) animals, but not in aged (18 months) animals (150). A sequential delivery strategy utilizing dual macrophage chemoattractant protein-1 and IL-4 to first recruit circulating macrophages and then polarize them to an anti-inflammatory phenotype was required to achieve immune modulation and improvements of outcomes in aged animals. While this phenomenon has not been examined in human patients, it suggests that a more robust understanding of the impacts of aging upon immune senescence and wound healing can lead to strategies which produce better tissue remodeling outcomes following surgical intervention with or without a mesh prosthesis.

Summary

In summary, there is significant evidence for age-related changes in the cells of the pelvic floor during the pathogenesis of POP and SUI. As each of these hallmarks of aging is interrelated, significant mechanistic work is needed to demonstrate that the observed changes are related to cell intrinsic dysfunction, cell cell communication, epigenetic changes, and how the observed changes affect both cellular behavior, proteostasis, nutrient sensingas well as overall tissue structure and function leading to pelvic floor dysfunction. Impact of recruitment of stromal cells and immune cells, stem cell exhaustion and other changes to the tissue environment upon the pathogenesis of POP and SUI needs to be determined. Immunological underpinnings driving pathogenesis of POP/SUI and implicated in mesh repair remain an important and highly understudied aspect of understanding of POP and SUI mechanisms and clinical management. Role for innate immunity and adaptive immunity are beginning to be identified along with immune signatures of POP and

SUI. Better understanding of types of immune cell infiltrates, timing and dynamics of infiltration, and impact on pelvic floor homeostasis, injury, and repair in this tissue is needed for limiting adverse conditions with mesh replacement and other interventions. Thus, these are areas of research that has many opportunities for advancement of the field of Female Pelvic Medicine and Reconstructive Pelvic Surgery since a better understanding of the role of aging in POP and SUI will rationally lead to an improved ability to prevent or treat these conditions..

Knowledge Gaps

Aging is a clear risk factor for POP and SUI. However, the mechanisms which lead to the pathogenesis of these conditions are not well elucidated. Evidence exists to suggest that the pathogenesis of POP and SUI is tied to age-related changes in cellular function, leading to imbalanced proteostasis, and resulting in degradative changes at the tissue level. However, while such mechanisms have been well studied in other tissues and organs as well as in many age-related disease processes, they remain under-investigated in the pelvic floor and in the setting of POP and SUI. Understanding the mechanism of age-related changes in the pelvic floor will logically lead to improved ability to manage or prevent POP. We consider the following to be significant knowledge gaps which need to be filled:

- Elucidating impact of aging on specific tissues and cell types within the supportive connective tissues and muscles of the pelvic floor as well as the vagina, urethra and bladder.
- Investigation of immune cell responses following surgical intervention is key for the development of treatments and therapies which lead to successful outcomes and reduced complication rates.
- Given challenges of using small animal models prevalent in aging research for clinically relevant surgical studies of the pelvic floor, new animal models are needed.
- Development of multicellular in vitro systems such as “pelvic floor-on-chip” type to address the complex biomechanical environment of pelvic floor tissues.
- Advances in the development of tools and models for the study of aging in the context of POP and SUI will significantly enhance the ability to derive actionable mechanistic insights and improve overall care for women with POP and SUI.

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POP	**Pelvic Organ Prolapse**
SUI	**Stress Urinary Incontenence**
SASP	**Senescence Associated Secretory Phenotype**
ECM	**Extracellular Matrix**
Mfn2	**Mitofusin 2**

MMP	**Matrix Metalloproteinase**
miRNA	**MicroRNA**
LOX	**Lysyl Oxidase**
ERα	**Estrogen receptor alpha**
ERβ	**Estrogen receptor beta**
TGF-β	**Transforming growth factor-beta**
RGD	**Arginine-glycine-aspartic**
TNF-α	**Tumor Necrosis Factor-alpha**
iNOS	**Inducible nitric oxide synthase**

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