after the first vaginal delivery, being symptomatic. By contrast, only 2% of symptomatic POP is found in nulliparous women. A prospective cross-sectional study including 548 nulliparous and 892 multiparous women showed that vaginal delivery is not associated with severe POP (POPQ stages III and IV), but is associated with a 30% increase in POPQ stage II.

Second Stage of Labour
Greater levator trauma and significant effects on muscle function were seen in women that had a longer second stage of labour. Caesarean section in the second stage does not protect against these changes, considering neurogenic and myogenic effects on the pelvic floor. Less pelvic floor damage seems to occur after elective, but not necessarily emergency, Caesarean section.

Assisted Vaginal Delivery
Although the evidence is weak, operative vaginal delivery and episiotomy are potential risk factors for POP. They are known to cause direct injury to the anal sphincter and pelvic floor musculature, which would logically predispose to the development of prolapse. The use of forceps and a vacuum extractor was shown to cause marked increase in bladder mobility in theValsalva manoeuvre. The risk of POP surgery was more than double with forceps deliveries when compared with both non-instrumental normal deliveries and the use of a vacuum extractor. MRI measurements detected that after vacuum extraction, descent and mobility of the bladder base, bladder neck and anorectal junction on straining increased considerably when compared with nulliparous women, the posterior wall defect being more prominent. More studies need to be carried out but it is postulated that suction by the vacuum extractor in a posterior–inferior direction possibly protects the anterior vaginal wall, while increasing the risk of damage to the posterior vaginal wall.

Caesarean Section
Overall, preventative Caesarean delivery in order to protect the pelvic floor remains controversial, although some large studies showed that Caesarean section is associated with a lower risk of POP than vaginal delivery. In a case-control study involving 15,007 women, Larsson et al. observed that the hazard ratio for POP (Caesarean only versus vaginal births only), controlling for age and parity, was 0.20 (0.18–0.22). Recently, Leijonhufvud et al. in a large sample cohort study involving 33,167 women only delivering by Caesarean and 63,229 only having vaginal deliveries, estimated the risk of POP in relation to the mode of childbirth. With a mean follow-up time around 26–27 years for both groups, the results showed that women that only had vaginal deliveries had an increased overall risk of prolapse surgery compared with women having Caesareans. The rate of POP surgery in the Caesarean group was 0.7 per 10,000 person-years, while in women having vaginal deliveries it was 8.1. Compared with Caesarean delivery, the risk of POP surgery increased nine-fold after both normal vaginal delivery and vacuum extraction delivery, while a 20-fold increased risk of POP surgery was found among women having forceps deliveries. Although highly suggestive of the protective effect of Caesarean section, the results of the studies were not controlled for potential behavioural and lifestyle factors possibly associated with POP development. Faced with the current imperfect evidence, a randomised controlled trial comparing modes of delivery is necessary to determine the role of Caesarean section in the prevention of POP.

Other Factors
Women with limited pelvic organ mobility antepartum will have a greater risk of developing postpartum vaginal prolapse. Increased maternal age is another risk factor for intrapartum pelvic floor trauma. An increase in the risk of levator trauma of approximately 10% for every year of delay in childbearing was observed. Logistic regression analysis derived from a retrospective evaluation of 983 women assessed by 3D ultrasound showed that levator avulsion was strongly associated with age at first delivery, vaginal operative delivery, prolapse symptoms and minimal muscle strength by Oxford grading. Puborectalis defects were more likely in women who had their first child by vaginal operative delivery over the age of 30.

Pelvic Organ Prolapse – Inherited or Acquired?
Vaginal childbirth, despite being the major causal factor, fails to provide a total explanation of the origin and progress of pelvic floor dysfunction. POP has been observed in nulliparous women, and the absence of the condition has been confirmed in many multiparous women. These findings raise the possibility of significant individual variability in predisposition to pelvic floor dysfunction.

Epidemiological studies suggest that there is a genetic component in POP. Familial incidence of genital prolapse is described as being about 60%. The risk of POP among siblings of young women (less than 55 years old) with severe prolapse was found to be five times higher than in the general population, and the prevalence of symptomatic prolapse was higher in women whose mothers or sisters had undergone surgery for prolapse compared with women without such family history (odds ratio 3.1, 95% confidence interval 1.4–3.0). Some authors believe that POP is part of a generalised connective tissue disease affecting joints, skin and pelvic floor support. Histories of varicose veins, hernias, haemorrhoids, joint hypermobility and abdominal striae were found to show significant positive associations with symptomatic POP. Moreover, women with Marfan syndrome or Ehlers–Danlos syndrome have higher rates of POP. Dietz et al. showed that bladder mobility is a heritable trait, 50% of the variability being due to genetic factors in nulligravid women.

On the other hand, it is known that pregnancy per se and childbirth trauma due to vaginal distention and stretching of the structures of the pelvic floor may cause dysregulation in components of the pelvic floor connective tissue. Several studies have identified alterations in gene and protein expression in women with POP without being able to draw conclusions about the causes of these alterations. Women with POP may have inherited ‘weak tissues’ that predispose them to develop the dysfunction, either spontaneously or initiated by events such as pregnancy and vaginal childbirth, or the childbirth trauma may directly affect the tissues influencing gene expression and protein activities. Therefore more studies are necessary to provide a comprehensive understanding of the biological basis of prolapse. Identifying women predisposed to develop POP would be extremely useful in counselling and assisting these patients.

Conclusions
Vaginal delivery is the main aetiological agent of POP. Although operative vaginal delivery is associated with other pelvic floor dysfunctions, the current literature is weak regarding its role in POP development. Use of Caesarean section to prevent damage to the pelvic floor is still open to wide discussion. Despite the lack of controlled
studies, recent observational epidemiological studies with large sample sizes and long follow-up times suggest a protective effect against POP. There is little evidence demonstrating the exact importance of each risk factor in the aetiology of pelvic floor dysfunctions, which limits our ability to develop effective prevention strategies. However, researchers now face the challenge of establishing the role of genetic predisposition in the genesis of POP and the molecular alterations secondary to pregnancy and childbirth trauma. A full understanding of the biological basis of these disorders would help in devising individualised management plans in obstetric patients. 6*
Vaginal delivery and pelvic floor dysfunction: current evidence and implications for future research

M. A. T. Bortolini · H. P. Drutz · D. Lovatsis · M. Alarab

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Abstract Vaginal delivery is the major risk factor for the development of pelvic organ prolapse and urinary and fecal incontinence, resulting from damage to the pelvic floor muscles, nerves and connective tissue. This article reviews the perineal trauma mechanism during vaginal delivery and discusses implications of current and future research projects.

Keywords Vaginal delivery · Incontinence · Pelvic organ prolapse

Introduction

Female pelvic floor dysfunction (PFD) is a term applied to a wide variety of clinical conditions, including urinary incontinence (UI), anal and fecal incontinence (FI), pelvic organ prolapse (POP), sensory and emptying abnormalities of the lower urinary tract, defecatory dysfunction, sexual dysfunction, and several chronic pain syndromes [1]. Because of its high prevalence, deleterious effects on quality of life and its impact on the health care system, PFD is an important public health issue [2]. This article considers aspects of the three most common conditions: urinary incontinence, fecal incontinence and pelvic organ prolapse. There is an 11.1% chance that a woman will need surgery to correct pelvic organ prolapse or incontinence during her lifetime [3]. Estimates show 135,000 women undergo surgery for UI and 225,000 have POP repair each year in the United States [4, 5]. Statistics about procedures for FI have not been reported [6].

Risk factors associated with PFD include multiparity, age, chronic increase of abdominal pressure (cough, overweight, physical effort), previous pelvic surgeries, spinal cord conditions and injury, genetic conditions, ethnicity, and family history. However, most cases are related to vaginal childbirth trauma [7, 8].

Mant et al. [8] described relative risk of developing pelvic floor dysfunction of 10.8 during pregnancy and puerperium. It is estimated that 4–25% of primiparous women suffer from fecal incontinence in the postpartum period [9], while 26% develop stress urinary incontinence [10] and 52% have some degree of anterior vaginal wall prolapse after the first vaginal birth [11].

Even under normal circumstances, vaginal delivery causes mechanical trauma to the pelvic floor that is sometimes irreversible. The integrity of muscle and ligaments composing the endopelvic fascia is key for maintaining support, suspension, and anatomic position of the pelvic organs in physiological conditions. In addition, innervation has an important role in maintaining the support structure function and continence mechanism integrity [12–17].

This article reviews the mechanism of childbirth trauma and consequences on pelvic floor functions and then discusses current and future research related to this common and debilitating condition.

Mechanism of childbirth trauma and PFD: evidence

Pelvic floor dysfunction results from loss of support and suspension provided by the endopelvic fascia and pelvic floor muscles, especially the components of the levator ani muscle. Many researchers had claimed that the vaginal delivery would
cause direct damage to these structures as well as indirect damage to the nerves of the pelvic floor [18, 19]. With nerve injuries, pelvic muscular tonus decreases, and the weakness of levator ani muscle overwhelms the uterosacral and parametrial ligaments and endopelvic fascia implicating in secondary injuries of these structures [20, 21].

**Pelvic floor muscle injury and PFD**

During vaginal delivery, the passage of the fetal head causes significant deformations in the pelvic floor muscles and other tissues [22, 23]. The decrease of pelvic muscle resistance after vaginal birth can be observed using techniques of measurement of muscle strength, such as standardized physical examination, use of vaginal cones, measurements of intra-vaginal pressure and use of translabial ultrasound [12, 24, 25]. Nielsen et al. [26] found that patients did not recuperate muscle strength until 8 months postpartum, while 34% of them were not able to contract the pelvic muscles voluntarily after six weeks of puerperium [27].

The puborectalis muscle fibers and the medial portion of the pubococcygeus muscle stretch 3.26 more times than other pelvic muscles during the expulsion of the fetus, exceeding by 217% the maximum point of stretching muscle without any disruption of fibers in muscles of non-pregnant mammals [28]. Avulsion of the puborectalis muscle has a marked effect on pelvic floor muscle strength [25].

Magnetic resonance imaging studies have shown that women with injury to the puborectalis muscle at 6 weeks postpartum tended to have normal images at 6 months. A 60% increase in puborectalis muscle thickness was observed in 6 months compared with that seen in 6 weeks postpartum, indicating the extent of the injury. However, the total recovery was not observed in injuries to both the puborectalis and ileococcygeus muscles [29]. Image studies have shown that up to 20% of women who have delivered vaginally have visible levator ani defects, of which the majority is in the pubovisceral portion of the levator ani muscle [30, 31]. These defects can be unilateral or bilateral and are associated with symptomatic stress urinary incontinence (SUI).

Women with levator avulsion defects postpartum detected by 3D ultrasound were about twice as likely to show POP of stage II or higher than those with an intact levator muscle, especially cystocele and uterine prolapse [32].

Women with limited pelvic organ mobility antepartum have a greater risk of developing postpartum vaginal wall prolapse [33]. Echography studies evaluated the elevation of the bladder neck in the postpartum period during voluntary contraction of the levator ani muscle at rest and during effort. In the period from 3 to 8 days before birth there was a decrease in elevation of the bladder neck, loss of bladder neck support at rest, with increased mobility in stress maneuvers. However, elevation of the bladder neck returned to antenatal levels after 6 to 10 weeks [34, 35].

Functional magnetic resonance imaging also detected significant differences in almost all the structures evaluated, such as in the pelvic position of the bladder, bladder neck, posterior vaginal fornices, anorectal junction, genital hiatus and depth of rectocele after vaginal childbirth. Urethral and bladder mobility contributed to SUI after vaginal childbirth [36–38].

An increase in the size of the genital hiatus was observed after vaginal childbirth and related to development and recurrence of POP [39, 40]. A levator hiatus area of greater than 25 cm² is considered abnormal and associated with symptomatic prolapse [41].

**Pelvic floor nerve injuries and PFD**

Pudendal nerve lesions usually result in demyelination of the fibers; axonal break may occur in severe cases without recovery of the tissues [13]. c-Fos expression (an early reactive nerve injury marker) in the L6 to S1 spinal cord segments was observed in rats after simulated birth trauma, indicating acute nerve injury or irritation in spinal neurons. Also, histological studies have revealed a marked decrease of ganglion cells in the neural plexuses posterolateral to the vagina in experimental rats after simulated birth [42].

Neuromuscular abnormal pelvic floor activation patterns may also contribute to PFD [43–45]. Electroneuromyography studies have shown that 80% of primigravidae developed evidence of partial denervation with signs of reinnervation and increase in the density of nerve fibers in the postpartum period after vaginal delivery [46, 47]. The latency time of pudendal nerve motor fibers increased after 2 to 3 days of vaginal delivery, but values normalized after 6 months in 66% [47]. Most nerve lesions spontaneously recover within a year by regenerative processes [13]. However, pudendal nerve damage, even with partial reinnervation of the external anal sphincter muscle, may persist and become more marked in the long term [48].

Neurophysiological tests revealed nerve damage in 36% of women with persistent SUI at 3 months postpartum. Compared with nulliparous control subjects, patients with SUI and POP had changes in the levator ani and external anal sphincter consistent with either motor unit loss or failure of central activation, or both [49].

**Urethral lesion and PFD**

Structural and functional alterations in the components of the urethra and anus after vaginal delivery will be specifically and separately addressed for didactic purposes.
Some experimental studies have described the effects of vaginal delivery in the urethra. Rocha et al. [50, 51] observed that vaginal delivery and simulation of childbirth trauma significantly decreased the number of nerve and muscular fibers; increased the amount of collagen and elastic fibers and the ratio collagen-muscular fibers in the mid-urethra of adult female rats.

A rat model of simulated birth trauma assessed the effects on lower urinary tract function of prolonged dilation and compression of pelvic floor tissues during vaginal delivery. Leak point pressure was significantly decreased in both 1- and 4-h distention groups 4 days after distension, compared to the sham-distended rats group, indicating a short-term decrease in urethral resistance. Six weeks after vaginal delivery, urethral resistance returned to normal values. However, the voided volume in sham-distended animals was still significantly increased at 6 weeks compared to the distended animals. In conclusion, this model shows that prolonged vaginal delivery results in reduced bladder capacity and long-term urinary dysfunction [52]. A similar experimental model found that, four weeks after simulated birth injury, SUI was noted in 40% of experimental rats. A significant decline in urethral wall musculature (both smooth and striated) and a wider genital hiatus were noted in incontinent rats [42].

A prospective study in nulliparous women found a remarkable decrease of urethral closure pressure and the functional length of the urethra 8 weeks after vaginal delivery compared with the antenatal period [53]. Lower maximum urethral closure pressure was shown to be the factor most associated with de novo SUI [54]. A study collating imaging and urodynamic data showed bladder neck descent also to be a strong predictor of stress urinary incontinence [37].

**Anal lesion and PFD**

Lesions of the external anal sphincter are significantly related to lacerations and/or episiotomy, while internal anal sphincter lesions may occur with an intact perineum [55]. Wai et al. [56], in a rat model, observed that anal sphincter transection, with or without antecedent, prolonged vaginal distention results in severe changes of external anal sphincter function immediately after injury. However, the contractile function of the external anal sphincter was fully recovered by 3 months and was sustained at 6 months. Over 35% of primiparous women suffer some degree of damage to the external anal sphincter, persisting until six months post vaginal delivery [55]. Women with recognized third and fourth grade perineal lacerations during vaginal delivery have a higher rate of fecal incontinence compared to those not injured (7.8% and 2.9%, respectively) [57].

Endoanal ultrasound findings have shown that 35% of primiparous women who delivered vaginally and required repair of clinically evident anal sphincter third- or fourth-degree tears, still exhibited internal sphincter gaps compared with 3% of women with no anal sphincter tear (OR 2.3, 95% CI 1.3–4.0). This condition is associated with fecal incontinence severity [58].

Damage to the innervation of the external anal sphincter often co-exists with direct injury to the muscle [59, 60]. A computer model of vaginal delivery simulation showed that during the second stage of labor, the nerves innervating the anal sphincter are stretched beyond the 15% strain threshold known to cause permanent damage in the peripheral nerve [28]. In a cohort study, 83 women with fecal incontinence underwent neurophysiologic assessment, together with anal endosonography and anal manometry, as part of the investigative evaluation of their incontinence symptoms postpartum. 26 women (31%) were found to have abnormal pudendal nerve function directly attributable to past obstetric trauma [61].

**Genetics, heredity, and pelvic floor dysfunction**

Vaginal childbirth, despite being the major causal factor, fails to explain completely the origin and progression of pelvic floor dysfunction. Pelvic organ prolapse and urinary incontinence have been observed in nulliparous women; the absence of these conditions has been confirmed in many multiparous women [8, 55]. These findings raise the possibility of an important individual variability in the predisposition to pelvic floor dysfunction [62, 63].

Epidemiological studies have shown higher prevalence of POP and SUI in patients with a positive familial history [63–69]. Family history appears to be a strong predictor of urinary incontinence, indicated by the high degree of concordance in the rate of incontinence between parous women and their nulliparous sisters [32]. This was also evident in a study of identical twins who had identical continence status and relaxation of pelvic support, despite parity status [69]. Mushkat et al. [68] found a threefold increased prevalence of SUI in first-degree relatives of women with this condition.

Familial incidence of POP is described to be about 60% [70]. The risk of POP among siblings of young women (fewer than 55 years) with severe prolapse is found to be five times higher than in the general population [38] and the prevalence of symptomatic prolapse was higher in women whose mothers or sisters had undergone surgery for prolapse compared with women without such family history (OR 3.1, CI 95% 1.4–3.0) [63].

The evidence suggests that there is a genetic predisposition to PFD [62, 71–73], as history of conditions suggestive of deficient connective tissue, such as varicose
veins, hernias, and hemorrhoids, was found significantly and positively associated to symptomatic POP [63]. Women with Marfan or Ehlers–Danlos syndrome have higher rates of SUI and POP. Studies have shown that women with POP and SUI have an increased incidence of joint hypermobility [71] and abdominal striae [73]. Dietz et al. [74, 75] showed that the mobility of the bladder, bladder neck and urethra, determinants of PFD, is a heritable trait, composing 50% of the variability due to genetic factors in nulligravid women. In addition, bladder mobility appears to correlate to joint hyperextensibility, mostly at the elbow, composing 14% of variation in bladder neck mobility accounted for by genes influencing elbow mobility [76].

Recent animal studies have shown that female reproductive organs are rich in elastic fibers that undergo massive remodeling in the reproductive organs throughout pregnancy and birth [77–79]. Deficiency in lysyl oxidase-like-1 (LOXL1), a protein essential for the postnatal deposition of elastic fibers, led to severe POP in mice 1 to 2 days after vaginal birth, weakness of the vaginal wall, paraurethral pathology, and lower urinary tract dysfunction [77–79]. Fibulin-5 (FIB-5) is an ECM protein that acts to stabilize and organize elastic fibers [80]. FIB-5 deficiency in vaginal tissues also resulted in prolapse in mice [81]. Vaginas of FIB-5 knockout mice that developed prolapse exhibited decreased maximal stress, increased distensibility and strain, and decreased stiffness [82], phenotypes that resemble pelvic floor dysfunction in humans.

Based on those observations, some authors have explored the field of genetic basis for pelvic floor dysfunction in humans [83–86]. Most research concentrates on the analysis of extracellular matrix proteins and genes involved in the function of muscular and connective systems. Candidate genes have been identified that may result in alteration of normal metabolism of various structural proteins, which may predispose some women to PFD, but certainly more studies are necessary to provide a comprehensive understanding of the biological basis of these disorders.

**Conclusion**

Despite the absence of controlled studies, current literature reaffirms vaginal delivery as the main etiological agent of pelvic floor dysfunction. However, sparse evidence supports the exact participation of each element in the etiology of pelvic floor dysfunction; this limits our ability to develop effective prevention strategies. The challenge for researchers, therefore, is to establish the role of genetic predisposition in the genesis of pelvic organ prolapse and urinary and fecal incontinence. A full understanding of the biological basis of these disorders would help in planning individualized management for the obstetric patient.

**Conflicts of interest** None.

**References**


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3. PROPOSIÇÃO
Propusemo-nos a analisar a expressão gênica e protéica, bem como a localização in situ de importantes enzimas envolvidas na biossíntese da matriz extracelular em tecidos vaginais de grupos de mulheres com e sem prolapse de órgãos pélvicos controlados pelo estado hormonal, por meio das tecnologias de RT-PCR em tempo real, imunobloting e imuno-histoquímica.

Objetivos:

Estudo 1
(1) Comparar as expressões dos genes e proteínas da família lisil oxidase (LOX e LOXL1-4), fibulina-5 e fibrilinas-1 e -2 em tecidos vaginais de pacientes na fase proliferativa do ciclo menstrual (pré-menopausa) com POP acentuado e controles assintomáticas;

Estudo 2
(2) Analisar as expressões diferenciais do gene e da proteína pró-colágeno C proteinase (PCP/BMP1) em tecidos vaginais de pacientes com e sem POP acentuado na pré-menopausa (fase proliferativa) e na pós-menopausa;

(3) Determinar e comparar a expressão da proteína pró-colágeno C proteinase (PCP/BMP1) no tecido vaginal de mulheres saudáveis não-a cometidas por POP consoante o estado menopausal.
4. CASUÍSTICA E MÉTODOS
Todas as etapas deste trabalho seguiram as normas de boas práticas em estudos clínicos envolvendo seres humanos e foram aprovadas pelo Research Ethics Board (REB) do Mount Sinai Hospital, University of Toronto, Canada (Anexo 2) e Comitê de Ética em Pesquisa (CEP) da Universidade Federal de São Paulo, Escola Paulista de Medicina (Anexo 3).

Trata-se de estudo transversal, caso-controle, no qual foram selecionadas mulheres atendidas ambulatorialmente nas clínicas de Uroginecologia e Ginecologia Geral do Mount Sinai Hospital. As participantes do estudo foram instruídas e assinaram o Termo de Consentimento aprovado pelo REB (Anexo 4).

4.1 Casuística

4.1.1 Grupo Controle

Formado por mulheres sem prolapso dos órgãos pélvicos, com ou sem incontinência urinária, que se submeteram a histerectomia total abdominal por doenças benignas: 70% miomas uterinos, 20% algia pélvica e 10% por lesões cervicais ou tumores anexiais suspeitos de malignidade, porém não confirmados.

Critérios de inclusão

- Caucasianas
- Diagnóstico, por exame físico, de ausência de POP (POPQ Estadio 0)
- Ausência de história clínica e/ou tratamentos prévios para POP
- Ausência de uso de terapia hormonal (estrogênio, progestogênio e androgênio) e corticoterapia, tópica e/ou sistêmica
- Mulheres na pré-menopausa na fase proliferativa do ciclo menstrual sem uso de pílulas contraceptivas
- Mulheres na pós-menopausa sem terapia hormonal há pelo menos 12 meses
Critérios de não inclusão

- Pacientes que se submeteram a cirurgias de assoalho pélvico prévias
- Pacientes na fase secretória do ciclo menstrual
- Mulheres com história prévia de doenças do tecido conjuntivo
- Mulheres portadoras de endometriose
- Mulheres portadoras de enfisema pulmonar
- Diagnóstico de doenças malignas do trato genito-urinário
- Não consentimento da paciente com a participação no estudo

4.1.2 Grupo Caso - POP

Foram incluídas mulheres com prolapso dos órgãos pélvicos acentuado, com ou sem incontinência urinária associada, que se submeteram à hysterectomia vaginal para tratamento do POP.

Critérios de inclusão

- Caucasianas

Pacientes com prolapso uterino acentuado (POPQ: ponto C estádios III e IV)
- Ausência de história clínica e/ou tratamentos prévios para POP
- Ausência de uso de terapia hormonal (estrogênio, progestogênio e androgênio) e corticoterapia, tópica e/ou sistêmica
- Mulheres na pré-menopausa na fase proliferativa do ciclo menstrual sem uso de pílulas contraceptivas
- Mulheres na pós-menopausa sem terapia hormonal há pelo menos 12 meses

Critérios de não inclusão

- Pacientes que se submeteram previamente a cirurgias de assoalho pélvico
- Pacientes na fase secretória do ciclo menstrual
- Mulheres com história prévia de doenças do tecido conjuntivo
• Mulheres portadoras de endometriose
• Mulheres portadoras de enfisema pulmonar
• Diagnóstico de doenças malignas do trato genito-urinário
• Não consentimento da paciente com a participação no estudo

Sabendo que a etnia e o estado hormonal são potenciais fatores de risco para desenvolvimento do POP, optamos pela homogenização dos nossos grupos de estudo quanto a estas variáveis a fim de reduzir vieses. Assim, incluímos somente mulheres caucasianas, e dividimos os grupos de acordo com o estado hormonal. Para estudos de grupos de pacientes na pré-menopausa, somente analisamos os tecidos das mulheres que se encontravam na fase proliferativa do ciclo menstrual na ocasião da coleta das amostras.

O estado menopausal foi definido com base no relato dos ciclos menstruais das pacientes. Classificamos como estando na pós-menopausa, as mulheres que informaram que sua menstruação havia cessado há mais de um ano, e na fase pré-menopausa, se elas relataram apresentar ciclos menstruais regulares nos últimos 12 meses. O estado hormonal foi posteriormente confirmado por exame histopatológico endometrial do material proveniente da histerectomia.

4.2 Métodos

4.2.1 Coleta de Dados Clínicos

Na semana anterior ao procedimento cirúrgico, as pacientes submeteram-se à anamnese dirigida e exame ginecológico. A anamnese continha dados como: nome, número do registro hospitalar, dados demográficos, história familiar de POP, antecedentes obstétricos, data da última menstruação, uso de medicações ou terapia hormonal, história de constipação intestinal e incontinências fecal e urinária, história patológica prévia, dentre outros (Anexo 5).
Todas as participantes foram pesadas e medidas para o cálculo do índice de massa corpórea e posteriormente submetidas ao exame ginecológico, no qual o prolapso fora graduado e estadiado pela classificação POPQ.

O exame para estadiamento por POPQ seguiu as instruções preconizadas pela Sociedade Internacional de Continência (ICS) (Bump et al, 1996). As pacientes foram examinadas em decúbito dorsal com bexiga confortavelmente cheia, e foram instruídas a realizar a manobra de Valsalva. A descida dos compartimentos vaginais foi aferida no máximo esforço realizado, com o uso de uma régua de escala em cm. O comprimento vaginal total foi medido em repouso com a redução do prolapso com auxílio de um espéculo vaginal. Depois disso, a manobra de Valsalva foi repetida com a paciente na posição em pé para confirmação da extensão do POP.

4.2.2 Coleta de Material Biológico

Padronizamos o local e tamanho das biópsias de forma a homogenizar as coletas uma vez que é sabido haver variações de espessura e composição da parede vaginal ao longo de seu comprimento e, com isto, diminuir os vieses do estudo (Weber, Walters, 1997; Weng et al, 2009). Após a remoção do útero, a amostra de tecido vaginal (pelo menos 1cm²) foi obtida pela dissecção cortante do espaço avascular de tecido conjuntivo frouxo da vagina com tesoura de Metzenbaum. As amostras foram obtidas da porção central da parede vaginal anterior (cúpula), região menos exposta a estiramentos causados pelo próprio prolapso ou inerentes aos procedimentos cirúrgico e de coleta (Boreham et al, 2002) (Figuras 12 e 13). Os tecidos foram coletados logo após a sua excisão no centro cirúrgico. Para a extração de RNA e proteínas, as amostras vaginais foram imediatamente congeladas em nitrogênio líquido e armazenadas a -80˚C. Para os estudos de imuno-histoquímica, os espécimes foram seccionados longitudinalmente e fixados em paraformaldeído a 4%.
4.2.3 Processamento e Análise do Material Biológico

RNAm e proteínas totais foram extraídos usando Trizol e RIPA Buffer, e os genes e proteínas de interesse quantificados utilizando-se as tecnologias de RT-PCR em tempo real e Imunobloting, respectivamente. Com técnicas histológicas e imuno-histoquímicas, observamos a morfologia tecidual e a localização in situ das proteínas de interesse. A descrição detalhada dos protocolos de técnicas e condições de reações utilizados, bem como seqüências de primers, anticorpos e concentrações dos mesmos estão detalhadas nos manuscritos que se seguem.
5. RESULTADOS E DISCUSSÃO
Os resultados das análises, bem como a discussão dos mesmos estão descritos nos manuscritos que se seguem.
LOX family enzymes expression in vaginal tissue of premenopausal women with severe pelvic organ prolapse

May Alarab · Maria AT Bortolini · Harold Drutz · Stephen Lye · Oksana Shynlova

Introduction

Pelvic floor dysfunctions (PFDs), including pelvic organ prolapse (POP) and urinary incontinence, are major health issues affecting millions of women worldwide [1]. With the aging population, it has been projected that over the next 30 years, the rate of women seeking care for PFD will double [2]. Despite the apparent importance of this medical condition, the etiology of POP remains obscure. Numerous risk factors have been attributed to this condition, including vaginal parity, advancing age, obesity, previous pelvic surgery, spinal cord conditions, and ethnicity with vaginal birth considered the most important [3–5]. However, not all women who undergo vaginal delivery have severe POP, and it is equally puzzling that severe POP and urinary incontinence have been observed in nulliparous women [6]. Recent studies suggest that pregnancy itself, distinct from vaginal delivery, augments the risk of POP [7]. Multiple reports have shown an association between POP and generalized connective tissue disorders [8, 9].

Structurally, the pelvic organs are supported by ligaments, muscles, and the bony pelvis. The spaces within are filled with connective tissue made of a network of tough extracellular matrix (ECM) protein fibers embedded in a polysaccharide gel. The two main proteins composing...
ECM fibers are collagen and elastin. Elastin provides elasticity and resilience to tissue, while collagen is responsible for the tensile strength and integrity of the pelvic floor [10]. The production of mature functional collagen and elastin fibers is a complex process, where monomers (tropoelastin and procollagen) are cross-linked in the extracellular space by one or more members of the lysyl oxidase (LOX) family of enzymes to form polymers. Recent animal studies have shown that a failure to maintain elastic fiber homeostasis in LOX like-1 (LOXL1)-deficient mice caused an inability of reproductive tissues to replenish elastic fibers after parturition, leading to POP, weakening of the vaginal wall, pararectal pathology, and lower urinary tract dysfunction [11]. Similarly, human studies have shown a significant reduction in LOX protein levels in the uterus, and vasculature [16].

Fibrillins guide elastogenesis by forming extracellular microfibril suprastructures [15] fibulins stabilize elastic fibers and anchor them to cells in the skin, lung, aorta, uterus, and vasculature [16].

We hypothesized that the deficiency of the LOX enzymes and/or related proteins expression could cause a failure of ECM fibers formation in reproductive tissues and could predispose the development of POP in women. We chose vaginal tissue biopsies as earlier data suggest that collagen content and metabolism of vaginal tissue closely resembles that of the endopelvic fascia and uterosacral ligaments [17]. As ovarian hormones are known to modulate collagen metabolism during menstrual cycle [18], only patients in the proliferative phase of menstrual cycle were included in this study. We aimed to examine the expression and localization of LOX, LOXL1-4, fibrillin-1, fibrillin-2, and fibulin-5 in the anterior vaginal tissue of premenopausal women with advanced POP and asymptomatic control patients using real-time reverse transcriptase polymerase chain reaction (RT-PCR), Western immunoblotting, and immunohistochemical analysis.

Materials and methods

Patient's selection and tissue collection

The Institutional Review Board of Mount Sinai Hospital, University of Toronto, Canada, approved this study. Premenopausal women undergoing vaginal hysterectomy for POP equal or greater than stage 3 by POP-Q [19] were identified as cases, while patients with POP-Q of stage 0 undergoing abdominal hysterectomy for reasons other than prolapse (menorrhagia, fibroids) were identified as controls. All cases and controls were Caucasians. The principle author obtained written informed consent, performed the examination for POP staging, and collected clinical data (patients' demographics) and tissue samples. Women with history of gynecologic malignancy, endometriosis, connective tissue disorders, emphysema, previous pelvic surgery, and steroid therapy were excluded. Premenopausal status was defined as regular menstrual cycle with no hormonal supplement. To minimize the effect of hormonal modulations on protein expression, we used only tissue samples from patients in the proliferative phase of the menstrual cycle; the phase was confirmed by endometrial histology report of uterine specimens. After removal of the uterus, full-thickness tissue specimen (approximately 1 cm²) was obtained by sharp dissection down to the avascular space of loose areolar tissue of the vagina using Metzenbaum scissors. To account for variations in stretch conditions and muscularis (MUS) thickness throughout the vaginal length [20], we standardized the site of tissue collection at the anterior middle portion of the vaginal vault for all POP cases and controls. For RNA and protein extraction, the vaginal biopsies were washed in ice-cold phosphate buffered saline (PBS), flash frozen in liquid nitrogen, and stored at −70°C. For immunohistochemical studies, the vaginal tissue specimens were sectioned longitudinally and fixed in 4% paraformaldehyde for 48 h.

Real-time PCR analysis

Reverse transcription

RNA was extracted from the frozen tissues using TRIZOL (Gibco, Burlington, ON), column purified using RNeasy Mini Kit (Qiagen, Mississauga, Canada), and treated with 2.5 μL of DNase I (2.73 Kunitz unit/μL, Qiagen). Two micrograms of RNA was reverse transcribed into cDNA in a total reaction volume of 100 μL using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). To assess for genomic DNA contamination in the RNA samples, we used an “RT (−) control”.

Real-time PCR protocol

The primer sequences for LOX, LOXL1-4, fibrillin-5, fibrillin-1, and fibrillin-2 were generated through Primer Express 2.1 ABI (Applied Biosystems, Foster City, CA, USA), verified for specificity by BLAST analyses and designed to span from two adjacent exons (Table 1). Twenty nanograms of cDNA from the previous step was subjected to real-time PCR in a total reaction volume of 20 μL containing SYBR Green Master Mix (BioRad, Hercules, CA) using Eppendorf realplex Mastercycler (Eppendorf, Hamburg, Germany). After PCR, a dissociation curve was

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constructed by increasing temperature from 65°C to 95°C to verify the specificity of PCR products. A cycle threshold (CT) mean value was recorded for each sample. Values obtained for each gene were normalized to the geometric mean of three housekeeping genes: TBP, SDHA, and YWHAZ [21, 22]. Relative quantitation (ΔΔCT method) [23] was used to compare the gene expression. mRNA levels for POP patients were expressed as fold changes relative to the control mRNA levels. Validation experiments were performed to ensure that the PCR efficiencies between the target genes and the housekeeping genes were approximately equal.

Western immunoblot analysis

Tissues were crushed under liquid nitrogen and homogenized, and proteins were purified in RIPA lysis buffer supplemented with protease inhibitor cocktail tablets (Complete™ Mini; Roche, Canada). Protein concentrations were determined using the BioRad protein assay buffer (BioRad). Protein samples (40–60 μg) were solubilised in Laemmli buffer, denatured by heating for 5 min. Lysates were resolved by electrophoresis on a 12% to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Blots were blocked with 5% skimmed milk, incubated with the appropriate primary antibody (Table 2a), washed three times in PBS-T (PBS with 0.1% Tween 20), and incubated with the required secondary antibody, followed by three washes, and the detection was performed using ECL kit (Amersham, Little Chalfont, Buckinghamshire, UK). Individual PVDF membranes were used for each protein studied to avoid cross-reactivity between the antibodies; membranes were stripped and reprobed with housekeeping protein (β actin) to correct variations in protein content among samples. Probed membranes were exposed to X-ray film (HyBlot CL, Denville, NJ) and analyzed by densitometry using ImageJ software (National Institutes of Health, USA). The density of each band was measured, and the values of each sample were expressed as a ratio of the protein of interest to the housekeeping protein.

Immunohistochemistry

The fixed vaginal biopsy samples were sectioned at 5 μm thickness and collected on Superfrost Plus slides (Fisher, ON, Canada). Antigen retrieval was performed by treatment with 0.125% trypsin, followed by blocking with 5% normal horse serum, and overnight incubation with primary antibody at 4°C followed by appropriate secondary antibodies (Table 2b). For the negative controls, ChromPure nonspecific mouse IgG (Jackson Laboratories, West Grove, PA), nonspecific rabbit IgG, and nonspecific goat IgG (both Santa Cruz) were used at the same concentration as primary antibody (Table 2b). Counterstaining with Harris’ hematoxylin (Sigma, St. Louis, MO) was carried out before slides were mounted with Cytoseal XYL (Richard–Allan Scientific, Kalamazoo, MI).

### Table 1 Real-time PCR primer sequences of a panel of genes involved in elastogenesis and housekeeping genes

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sequences</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOX</td>
<td>Forward 5′-AGGCCACAAAGCAAGTTTCTG-3′ Reverse 5′-AACAGCCAGGACTCAATCCT-3′</td>
<td>NM_002317</td>
</tr>
<tr>
<td>LOXL1</td>
<td>Forward 5′-CTGTGACTTCGCCAACCCTCAA-3′ Reverse 5′-TGCACTGGTTATGTCGAT-3′</td>
<td>NM_005576</td>
</tr>
<tr>
<td>LOXL2</td>
<td>Forward 5′-TCGAGGTTGCAAAATCCGATT-3′ Reverse 5′-TTCCTGCTCTCAGCTGAAG-3′</td>
<td>NM_002318</td>
</tr>
<tr>
<td>LOXL3</td>
<td>Forward 5′-CGGATGTGAAAGCCAGGAAACT-3′ Reverse 5′-AGGCAATCAAATGTGGCA-3′</td>
<td>NM_032603</td>
</tr>
<tr>
<td>LOXL4</td>
<td>Forward 5′-ACGGGCATGACATTGGATGC-3′ Reverse 5′-CATTACTTGCACGGGACT-3′</td>
<td>NM_03211</td>
</tr>
<tr>
<td>Fibulin-5</td>
<td>Forward 5′-ATGCCGCTTTTGGATAACCAGAT-3′ Reverse 5′-CCGCTCTTCAGTATGCAG-3′</td>
<td>NM_006329</td>
</tr>
<tr>
<td>Fibrinin-1</td>
<td>Forward 5′-TGACTGGCCACACGTCATAG-3′ Reverse 5′-TGACATCGACCCCCCTTGGACAGGA-3′</td>
<td>NM_000138</td>
</tr>
<tr>
<td>Fibrinin-2</td>
<td>Forward 5′-CACGACGGTAATGACTGCTGCTC-3′ Reverse 5′-TGCCCTTTGTAAATCCCACATCGG-3′</td>
<td>NM_001999</td>
</tr>
<tr>
<td>SDHA</td>
<td>Forward 5′-TGGGAACAGAGGACATCGT-3′ Reverse 5′-CCACACTGACATCAAATTCATG-3′</td>
<td>NM_004168</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>Forward 5′-ACTTTTGTACTTGGTGGCTCA-3′ Reverse 5′-CCGCCAGGAGCAAAACCAGAT-3′</td>
<td>NM_003406</td>
</tr>
<tr>
<td>TBP</td>
<td>Forward 5′-TGCAACAGGGCAAGATCGA-3′ Reverse 5′-CACATCAGAGCTCCCCACCA-3′</td>
<td>NM_003194</td>
</tr>
</tbody>
</table>
Vaginal tissue sections were observed on a Leica DMRXE microscope (Leica Microsystems, Canada). A minimum of three fields were examined for each specimen, and representative tissue sections were photographed with Sony DXC-970 MD (Sony, ON, Canada) 3CCD color video camera.

Double immunostaining

Immunohistochemical staining was performed as above. Antigen retrieval was performed by treatment with proteinase K, followed by blocking with serum-free protein block for 30 min (DAKO Corporation, Carpinteria, CA), and incubation with primary anti-LOXL1 and anti-CD68 antibodies overnight at 4°C (Table 2). Secondary antibodies used for detection were rabbit anti-mouse IgG, fluorescein isothiocyanate (FITC) conjugated, and donkey anti-goat IgG, Cy3 conjugated (Table 2b). Slides were then mounted with Vestashield mounting medium (Vector, Burlingame, CA), observed on a Leica DMRXE microscope (Leica), and representative tissue sections were photographed with a Sony DXC-970 MD (Sony) 3CCD color video camera.

Statistical analysis

Unpaired comparison between the expression of LOX family genes and proteins in POP patients and controls was performed using Wilcoxon signed-rank test (Prism version 4.02). Fisher's exact test was used to compare differences in the family history of POP and the incidence of stress urinary incontinence (SUI) between the two groups. The level of significance was set at \( P<0.05 \). Experimental error was reported as SEM.

Results

Vaginal tissue biopsy samples were obtained from 15 POP patients and 11 control patients matched for age (42.9 vs. 44.9 years), race (Caucasian), body mass index (BMI) (27.5 vs. 29.2), and phase of the menstrual cycle (proliferative). The mean parity, the family history of POP, and the incidence of SUI were significantly higher in POP patients compared to controls (\( P=0.001, 0.01, \) and 0.0001 respectively). No significant difference was noted regarding smoking habit between the two groups. LOX, LOXL1–4, fibrillin-1, fibrillin-2, and fibulin-5 mRNA transcripts were expressed in all tissue samples. LOX, LOXL1, and LOXL3 mRNA levels were significantly reduced in patients with prolapse compared to control patients (\( P=0.005, 0.0008 \) and 0.001, respectively). No significant difference between the two groups was observed for all other genes (Fig. 1). To confirm these changes, we performed Western immunoblot analysis using antibodies that specifically

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Summary of antibodies used in immunoblot and immunohistochemical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Primary or secondary</td>
</tr>
<tr>
<td>(a) Immunoblot</td>
<td></td>
</tr>
<tr>
<td>LOX</td>
<td>Primary polyclonal</td>
</tr>
<tr>
<td>LOXL1</td>
<td>Primary polyclonal</td>
</tr>
<tr>
<td>LOXL3</td>
<td>Primary polyclonal</td>
</tr>
<tr>
<td>b-actin</td>
<td>Primary polyclonal</td>
</tr>
<tr>
<td>HRP-conjugated anti-rabbit IgG</td>
<td>Secondary Polyclonal</td>
</tr>
<tr>
<td>HRP-conjugated anti-goat IgG</td>
<td>Secondary Polyclonal</td>
</tr>
<tr>
<td>HRP-conjugated anti-mouse IgG</td>
<td>Secondary Polyclonal</td>
</tr>
<tr>
<td>(b) Immunohistochemical</td>
<td></td>
</tr>
<tr>
<td>LOX</td>
<td>Primary polyclonal</td>
</tr>
<tr>
<td>LOXL1</td>
<td>Primary polyclonal</td>
</tr>
<tr>
<td>LOXL3</td>
<td>Primary monoclonal</td>
</tr>
<tr>
<td>8-Smooth muscle actin</td>
<td>Primary polyclonal</td>
</tr>
<tr>
<td>CD68</td>
<td>Primary monoclonal</td>
</tr>
<tr>
<td>Biotin-conjugated anti-rabbit IgG</td>
<td>Secondary Polyclonal</td>
</tr>
<tr>
<td>Biotin-conjugated anti-goat IgG</td>
<td>Secondary Polyclonal</td>
</tr>
<tr>
<td>Biotin-conjugated anti-mouse IgG</td>
<td>Secondary Polyclonal</td>
</tr>
<tr>
<td>Cy3-conjugated anti-goat IgG</td>
<td>Secondary Polyclonal</td>
</tr>
<tr>
<td>FITC-conjugated anti-mouse IgG</td>
<td>Secondary Polyclonal</td>
</tr>
</tbody>
</table>

**HRP** horseradish peroxidase
recognize LOX, LOXL1, and LOXL3 proteins. Bands of the appropriate molecular weight were observed for all proteins studied in both groups (Fig. 2). LOX was detected as a double band at 47 kDa (proform) and 35 kDa (active form) (Fig. 2). We found a significant reduction of 47 kDa form of LOX in vaginal tissue of POP patients compared to controls ($P=0.005$), as well as a similar trend in the expression of the 35 kDa LOX protein ($P=0.05$). While the mean expression levels of LOXL1 proteins was lower in POP patients than in controls, this did not reach the level of significance, in part due to the considerable variability in the expression levels of control subjects. The mean expression levels of LOXL3 protein was significantly reduced ($P=0.01$), indicating a direct correlation with transcript changes.

Prior to characterization of the localization of LOX family proteins in the vaginal tissue of patients with POP ($n=3$) and controls ($n=3$), we assessed the morphology of these tissues by hematoxylin and eosin staining and α-smooth muscle actin immunostaining and confirmed that all vaginal samples include the three layers: the stratified squamous epithelium (SSE), lamina propria (LP), and MUS (data not shown).

The immunohistochemical staining shows that LOX, LOXL1, and LOXL3 proteins were expressed in the vaginal specimens from both groups (Fig. 3). The immunolabeling with anti-LOX antibody was detected in all three layers of the vaginal tissue: in the nuclei of (1) basal part of SSE, (2) fibroblasts, and vascular SM cells in the LP, and...
LOXL1 and LOXL3 immunostaining did not show the same distribution as LOX enzyme: (1) the nuclei in SSE were negative for the enzyme; (2) SM bundles in the MUS layer were stained weakly (Fig. 3). LOXL3 immunostaining was observed at the vascular smooth muscle cells (SMCs) in the LP and MUS layer. Immunolocalization was similar in POP patients and controls.

Interestingly, we detected a large number of macrophage-like cells scattered in the LP and MUS around blood vessels that displayed a strong LOX1 and LOXL3 immunostaining (Fig. 3). To identify the origin of these cells, we used CD68, a macrophage-specific marker, and confirmed the presence of CD68-positive macrophages in vaginal tissue (Fig. 3). We also performed double immunostaining for LOX1 and CD68, discovering that CD68-positive macrophages also expressed LOX1, a finding that may have new implications for the enzyme function (supplemental Fig. 1).

Discussion

We hypothesized that deficiency of LOX enzymes represents one of the key factors responsible for the development of POP. This deficiency may result in anomalous ECM synthesis and/or inadequate repair in response to mechanical strain and/or hormonal stimulation. Therefore, we set a goal to compare the expression of LOX enzymes and
related proteins between POP patients and asymptomatic controls. We restricted our analysis to premenopausal Caucasian women in the proliferative phase of the menstrual cycle to rule out the influence of major cofounders of age, ethnic background, and hormonal status that play a role in the pathophysiology of POP [24]. However, in our patients group, we found that women affected by POP showed a strong familial history for this disease and significantly higher incidence of SUI. This finding provides an additional support to the idea of genetic predisposition for the development of POP. Our data confirm that all members of LOX family of enzymes were expressed in the vaginal tissue of patients with severe POP and asymptomatic controls. We detected a significant reduction of LOX, LOXL1, and LOXL3 mRNA levels in vaginal samples of patients with POP compared to controls. Both LOXL3 and LOXL4 transcripts were found in many tissues; however, these enzymes have not been studied in relation to POP before. Their biological function is to stabilize elastin and collagen fibers by oxidative deamination of the peptidyl lysine residues, thus, contributing to the development and maintenance of ECM [25, 26].

Our immunoblot analysis confirmed the expression of LOX, LOXL1 and LOXL3 proteins in all vaginal samples studied. LOX and LOXL1 both are secreted as 47-kDa proproteins (proLOX and proLOXL1, respectively) and are proteolytically cleaved by procollagen C-protease enzyme to form active LOX and LOXL1 with a molecular weight of 32 kDa [27]. Though only the mature form of LOX and LOXL1 are catalytically active against collagen and elastin substrates, it has been shown that the proforms initially target the enzymes to the appropriate matrix substrate to be acted upon [28]. We detected a significant reduction in the proform of LOX (47 kDa) protein expression in patients affected by POP compared to the control group; LOXL1 protein showed a similar trend. Evidence exists to support our finding as LOX mRNA levels were reduced in the uterosacral and cardinal ligaments of patients with POP compared to controls, suggesting that generalized deficiency of LOX enzymes in pelvic floor tissues may cause the development of POP. We speculate that the reduction of LOX and LOXL3 proteins in vaginal tissue of patients with POP may compromise ECM protein synthesis or assembly, leading to impaired cell–ECM interactions and result in defective connective tissue formation.

We examined the spatial distribution of LOX proteins in human vaginal tissue biopsies. Previous studies have shown that both LOX and LOXL1 are expressed in different tissues by the same cell types [29]. Unlike other reports, our study did not show similar cellular distribution of LOX and LOXL1 proteins in the vaginal biopsy samples. We have observed the nuclear localization of LOX in vaginal tissue fibroblasts, epithelial cells, and SMCs. This result agrees with reports of Noblesse and colleagues showing that LOX was expressed in the nuclei of cultured SMCs, foreskin fibroblasts, keratinocytes, and vascular endothelium [30]. In contrast to LOX, prominent expression of LOXL1 was found in macrophages as defined by CD68 immunostaining. To our knowledge, this is the first report showing the expression of LOXL1 by resident leukocytes in human tissue. The importance of this finding is still to be elucidated.

We acknowledge that with our strict selection criteria and over 3 years’ period, we were able to recruit 15 patients and 11 controls. This did not meet our target of n = 20; however, the advantage was the strictly homogenous study group regarding age, race, BMI, and menstrual status. We also acknowledge that our study groups were not matched for parity; this may validate even further the importance of this parameter in the etiology of PFDs.

In summary, we have confirmed that LOX enzymes controlling mechanical properties of the fibrillar ECM are all expressed in human vaginal tissue, and certain enzymes are reduced in patient with severe POP compared to asymptomatic controls. We suggest that women with deficiency in proteins responsible for ECM biogenesis may have anomalous ECM fibers, which render them unable to maintain the structural and functional integrity of the pelvic floor connective tissue. Furthermore, we speculate that exposure to repeated mechanical stresses during pregnancy and vaginal delivery in this group may result in permanent or progressive pelvic floor damage. Further research is required to directly address the potential alteration of LOX enzymes activity in response to mechanical stretch and hormonal perturbation.

Acknowledgements The study funding was provided by the Research Fund, Department of Obstetrics and Gynecology, Mount Sinai Hospital, Toronto. There is no conflict of interest to be disclosed. We thank Dr. V. Miranda for assisting us in the statistical analysis.

Conflicts of interest None.

References

Pelvic organ prolapse (POP) is a major health issue for women of all ages.\(^1\) Risk factors such as multiparity and vaginal birth, ageing, menopause, obesity, neuropathies, ethnicity, family history, and genetic predisposition,\(^2,3\) separately or superposed, lead to failure of the structures that support the pelvic floor: connective tissue in the form of ligaments and endopelvic fascia, and levator ani muscles.\(^4\) Microscopic anatomy of the vaginal wall indicates that endopelvic fascia represents the fibromuscular layer of the vagina.\(^5\) Therefore, abnormal vaginal connective tissue may play a role in the cause of POP.\(^6\)

Collagen and elastin are 2 main proteins composing the connective tissues. Collagen is responsible for the tensile strength and integrity, whereas elastin provides elasticity and resilience to the pelvic floor tissues. Bone Morphogenetic Protein-1 (BMP1), also known as procollagen C proteinase (PCP), is a matrix metalloproteinase that cleaves the C-terminal propeptide from procollagen chains, originating the mature collagen. Furthermore, BMP1 plays an important role in the collagen cross-linking necessary for tissue stability, by activating lysyl oxidases (LOX) family of proteins.\(^7,8\) Therefore, BMP1 is considered a biologic control point for the regulation of collagen deposition. The National Center for Biotechnology Information has described 7 different BMP1 isoforms (1-7).\(^9\)

Despite contradictions in the literature, it seems that patients with POP present decrease in the total collagen content, with higher rate of immature collagen more susceptible to rupture.\(^10,11\) We have previously reported that premenopausal women with POP show differential expression of LOXs enzymes in vaginal tissue compared with asymptomatic women.\(^12\) BMP1 is crucial for extracellular matrix (ECM) biogenesis, being involved in the maturation of collagen and LOXs. Because of its importance, we have now investigated BMP1 expression and possible association with POP. As reproductive hormones substantially modulate the turnover of the...
pelvic floor connective tissue, we have controlled our study groups according to the menopausal status. We have hypothesized that BMP1 gene and protein expressions are (1) altered in women with POP and (2) affected by menopausal status. We aimed to analyze the expression and localization of BMP1 in the anterior vaginal wall tissue of white women with advanced POP and asymptomatic controls according to the menopausal status. We used real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), immunoblotting, and immunohistochemical analysis.

The goal of this cross-sectional case-control study is to describe the changes in the vaginal tissue observed in POP and healthy women before and after the menopause. We believe that this study will point a new target for future researches that may elucidate the mechanism underlying this dysfunction.

**Materials and Methods**

**Patient’s selection and tissue collection**

The study was reviewed and approved by the Research Ethics Board of Mount Sinai Hospital, University of Toronto. We recruited white adult women undergoing vaginal hysterectomy for cervical prolapse equal or greater than stage III by POP-Q classification as “patients,” and women with stage 0 undergoing total abdominal hysterectomy for benign conditions other than POP as “controls.” We rationalized that stage 0 is the “gold standard” for normal pelvic support. Women with history of urogenital malignancy, endometriosis, connective tissue disorders, emphysema, previous pelvic surgery, and on estrogen and/or progestogen or steroid therapy were excluded. The initial gynecologic examinations were performed by the Urogynecology staff (H.P.D. and M.A.), and by the Gynecology team at Mount Sinai Hospital during regular activities. The first author obtained written informed consent, confirmed the POP staging of all participants, and collected clinical data a week before the surgical procedure. The patients were examined in the lying position with a referred full bladder, and asked to perform the Valsalva maneuver. The descensus of the vaginal compartments were measured at the maximum straining point using a centimeter scale ruler. Total vaginal length was measured at rest under POP reduction with a vaginal speculum. Afterward, straining examination in the standing position confirmed the full extent of the POP. We divided patients and controls in groups according to the menopausal status. We considered women in the postmenopausal phase if they reported that their menstrual periods had stopped for more than a year, and as premenopausal if they were having regular periods over the preceding 12 months. Only tissue samples from premenopausal women in the proliferative phase of the menstrual cycle were analyzed. The hormonal status was confirmed by endometrial histology report of uterine specimens. After removal of the uterus, vaginal tissue specimen (at least 1 cm²) was obtained by sharp dissection down to the avascular space of loose areolar connective tissue of the vagina using Metzenbaum scissors. The dissected structure corresponds to the adventitia layer that separates the vaginal from the bladder muscularis. As easily torn during dissection, adventitia was excluded from our analysis. To account for variations in stretch conditions and muscularis thickness throughout the vaginal length, the site of tissue collection was standardized at the anterior middle portion of the vaginal vault. The first author (M.B.), not blinded for the samples status, immediately received the tissue biopsies from the surgeon in the operative room and further performed the biochemical assays under direct supervision of the senior author (O.S.). For RNA and protein extraction, the vaginal samples were washed in ice-cold phosphate buffered saline solution (PBS), flash-frozen in liquid nitrogen and stored at −80°C. For immunohistochemical studies the specimens were sec-

### TABLE

**Demographic data of the study groups**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Group 1 Premenopausal patients</th>
<th>Group 2 Premenopausal Control</th>
<th>Group 3 Postmenopausal patients</th>
<th>Group 4 Postmenopausal control</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>23</td>
<td>16</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Mean age</td>
<td>43 (± 8)</td>
<td>44.9 (± 6.1)</td>
<td>67.5 (± 9.1)</td>
<td>57 (± 5.4)</td>
</tr>
<tr>
<td>Mean BMI</td>
<td>27.18 (± 6.2)</td>
<td>26.65 (± 4.3)</td>
<td>26.21 (± 4)</td>
<td>27.6 (± 5.1)</td>
</tr>
<tr>
<td>Mean parity</td>
<td>2.65 (1-4)</td>
<td>1.67 (0-3)</td>
<td>3.56 (1-6)</td>
<td>1.6 (0-2)</td>
</tr>
<tr>
<td>SUI</td>
<td>69.6%</td>
<td>6.25%</td>
<td>54%</td>
<td>0%</td>
</tr>
<tr>
<td>Family history POP (%)</td>
<td>56.5%</td>
<td>28%</td>
<td>54%</td>
<td>0%</td>
</tr>
<tr>
<td>Stage of POP (n, %)</td>
<td>III C (20, 87%)</td>
<td>Stage 0 (16)</td>
<td>III C (8, 61.5%)</td>
<td>Stage 0 (5)</td>
</tr>
<tr>
<td></td>
<td>IV C (3, 13%)</td>
<td></td>
<td>IV C (5, 38.5%)</td>
<td></td>
</tr>
</tbody>
</table>

Fisher’s exact test; level of significance: P value < .05. BMI, body mass index; POP, pelvic organ prolapse; SUI, stress urinary incontinence.

* Indicates statistical difference between groups 2 and 4; † Indicates statistical difference between groups 3 and 4; ‡ Indicates statistical difference between groups 1 and 2.

Bortolini. BMP1 in POP. Am J Obstet Gynecol 2011.
tioned longitudinally and fixed in 4% paraformaldehyde for 48 hours.

**Real time-PCR analysis**

*RT.* RNA was extracted using TRIZOL (Gibco, Burlington, Ontario, Canada), column purified using RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) and treated with 2.5 μL DNase I (2.73 Kunitz U/μL, Qiagen), according to the manufacturer’s instructions. The mRNA quality of each sample was checked through electrophoregram (Bioanalyzer, Agilent Technologies, Santa Clara, CA). The 2 μg RNA was reverse transcribed into complementary DNA (cDNA) in a total reaction volume of 100 μL using the TaqMan Reverse Transcription Kit (ABI, Carlsbad, CA). To assess for genomic DNA contamination in the RNA samples, a “RT (−)” control was used.

**Real-time PCR protocol.** The primers sequences were generated through Primer Express 2.1 (ABI), verified for specificity by BLAST analyses and designed to span from 2 adjacent exons. The BMP1 primers sequences were designed to amplify part of the region common to all RNA spliced variants (Gene Bank: NM_006132; forward: 5’-GCCACATTCA-ATCGCCCAA-3’; reverse: 5’-TGGCCGCTCAATCTCAAAAGGAC-3’). The primer sequences of the housekeeping genes are: ACTB (forward: 5’-ACCTTCAACACCCAGCCATGTACG-3’; reverse: 5’-CTGATCCACATCTGCTGGAAGGTGG-3’), TBP (forward: 5’-TGCAAGAGGGAAGAGTGGA-3’; reverse: 5’-CACATCACAGCTCCACCAAC-3’), and SDHA (forward: 5’-TGCTGAAGAGGGAAGAGTGGA-3’; reverse: 5’-CCACACCTCAGCTCCACCAAC-3’). The 20 ng cDNA was subjected to real-time PCR in a total reaction volume of 20 μL containing SYBR Green Master Mix (BioRaD, Hercules, CA) using Realplex Mastercycler (Eppendorf, Hamburg, Germany). After PCR, a dissociation curve was constructed by increasing temperature from 65°C to 95°C to verify the specificity of PCR products. A cycle threshold (CT) mean value was recorded for each sample. Values obtained for each gene were normalized to the geometric mean of 3 housekeeping genes. Relative quantitation (ΔΔCT method) was used to compare the gene expression. The mRNA levels for POP patients were expressed as fold changes relative to the control mRNA levels, and postmenopausal controls expressed as fold change relative to

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**FIGURE 1**

**BMP1 gene expression**

Real-time quantitative RT-PCR analysis were performed to compare the level of BMP1 mRNA in vaginal wall biopsy samples from premenopausal POP patients (black bars, n = 23) and controls (white bars, n = 16); and postmenopausal POP patients (black bars, n = 13) and controls (white bars, n = 5). Values represent mean ± standard error of measurement. A significant difference is indicated by * (P < .05).

BMP1, Bone Morphogenetic Protein-1; POP, pelvic organ prolapse.

Bortolini. BMP1 in POP. Am J Obstet Gynecol 2011.

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**FIGURE 2**

**BMP1 protein expression in premenopausal women**

A, Representative immunoblotting analysis of vaginal BMP1 protein isoforms of premenopausal POP patients and controls. B, Densitometric analysis of the BMP1 isoforms (130 kDa, 92.5 kDa, 82.5 kDa, and 70 kDa) expression levels in vaginal tissue of premenopausal POP patients (black bars, n = 14) and asymptomatic controls (white bars, n = 7) normalized vs ACTB. Values represent mean ± standard error of measurement. A significant difference is indicated by * (P < .05).

BMP1, Bone Morphogenetic Protein-1; POP, pelvic organ prolapse.

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the premenopausal controls. Validation experiments were performed to ensure that the PCR efficiencies between the target genes and the housekeeping genes were approximately equal.

Western immunoblot analysis

Tissues were crushed under liquid nitrogen, homogenized, and proteins were purified in RIPA lysis buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (vol/vol) Triton X-100, 1% (vol/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, supplemented with 100 μM sodium orthovanadate and protease inhibitor cocktail tablets (Complete Mini; Roche, Mississauga, Ontario, Canada). Protein concentrations were determined using the protein assay buffer (BioRad). Protein samples (40-60 μg) were solubilized in LDS sample buffer (Invitrogen, Carlsbad, CA) and denatured by heating for 5 minutes. Lysates were resolved by electrophoresis on a gradient 4-12% Novex Tris-Glycine Pre-Cast Gels using Novex SDS Running Buffer (both Invitrogen). Protein extracted from human placenta was used as positive control, and brain tissue lysate (Abcam, Cambridge, MA) as negative control.21,22 We used Page-Ruler Plus Prestained Protein Ladder (SM1811; Fermentas, Burlington, CA) as molecular weight marker. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using Novex Tris-Glycine Transfer Buffer 25× (Invitrogen). Blots were blocked with 5% skimmed milk/1% BSA, incubated with rabbit antihuman BMP1 primary antibody (1:3000; Abcam), washed in PBS-T and incubated with HRP-conjugated antirabbit IgG secondary antibody (1:3000; GE Healthcare, Chalfont, UK). Detection was performed using Immuno-Star Western C kit (Biorad). Membranes were stripped with Restore Plus Western Blot Stripping Buffer (ThermoScientific, Rockford, IL) and reprobed with housekeeping protein (ACTB) to correct variations in protein content among samples (rabbit antihuman ACTB primary antibody; 1:3000; BioVision, Mountain View, CA, and HRP-conjugated sheep antirabbit IgG secondary antibody; 1:3000; GE Healthcare). Probed membranes were exposed to Versadoc Imaging System 5000 MPs and quantified by densitometry using Quantity One Analysis Software (both Biorad). The values of relative optical density for POP patients were expressed in fold change relative to the corresponding controls.

Immunohistochemistry

The formalin fixed vaginal tissues were gradually dehydrated in ethanol and embedded in paraffin. Sections of 5 μm thickness were collected on Superfrost Plus slides (Fisher, Ontario, Canada). Paraffin sections were deparaffinized and rehydrated. After immersion in 3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ), antigen retrieval was performed by treatment with 0.125% trypsin, followed by blocking with 5% normal horse serum and overnight incubation with primary antibody at 4°C, followed by appropriate secondary antibodies. To verify the morphology of the vaginal samples, we used alpha-smooth muscle actin immunostaining (rabbit antihuman ACTC1 primary antibody; 1:50; Dako, Glostrup, Denmark) as positive control, and brain tissue lysate (Abcam, Cambridge, MA) as negative control.21,22 We used Page-Ruler Plus Prestained Protein Ladder (SM1811; Fermentas, Burlington, CA) as molecular weight marker. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using Novex Tris-Glycine Transfer Buffer 25× (Invitrogen). Blots were blocked with 5% skimmed milk/1% BSA, incubated with rabbit antihuman BMP1 primary antibody (1:3000; Abcam), washed in PBS-T and incubated with HRP-conjugated antirabbit IgG secondary antibody (1:3000; GE Healthcare, Chalfont, UK). Detection was performed using Immuno-Star Western C kit (Biorad). Membranes were stripped with Restore Plus Western Blot Stripping Buffer (ThermoScientific, Rockford, IL) and reprobed with housekeeping protein (ACTB) to correct variations in protein content among samples (rabbit antihuman ACTB primary antibody; 1:3000; BioVision, Mountain View, CA, and HRP-conjugated sheep antirabbit IgG secondary antibody; 1:3000; GE Healthcare). Probed membranes were exposed to Versadoc Imaging System 5000 MPs and quantified by densitometry using Quantity One Analysis Software (both Biorad). The values of relative optical density for POP patients were expressed in fold change relative to the corresponding controls.
Denmark). For the BMP1 tissue localization, we used rabbit antihuman BMP1 primary antibody (1:1000; Abcam). BMP1 immunostaining of human umbilical cord tissue served as positive control. For the negative controls, ChromPure nonspecific rabbit IgG (Jackson Laboratories, West Grove, PA) was used in vaginal tissue at the same concentration as primary antibody. Biotin-conjugated goat antirabbit IgG (1:200; Vector Laboratories, Burlingame, CA) was used as secondary antibody. Counterstaining with Harris’ Hematoxylin (Sigma-Aldrich, St. Louis, MO) was carried out before slides were mounted with Cytoseal XYL (Ricard-Allan Scientific, Kalama-zoo, MI). Vaginal tissue sections were observed on a DMRXE microscope (Leica Microsystems, Thornhill, Ontario, Canada). A minimum of 3 fields were examined for each specimen, and representative tissue sections were photographed with Sony DXC-970 MD 3CCD color video camera.

Statistical analysis

Pilot studies for quantification of BMP1 gene expression in premenopausal women indicated that 10 samples in each study group would be required to achieve a difference of at least 2-fold between women with and without POP at the $P \leq .05$ for a power of 80%.

Unpaired comparisons between the expression of BMP1 gene and protein in POP patients and asymptomatic controls as well as premenopausal vs postmenopausal healthy women were performed using Wilcoxon signed-rank test (Prism version 4.02). Fisher’s exact test was used to compare demographic variables between the groups. The level of significance was set at $P < .05$. Experimental error was reported as SEM.

RESULTS

Vaginal tissue biopsy samples were obtained from 23 premenopausal POP patients (group 1) and 16 premenopausal controls (group 2), 13 postmenopausal POP patients (group 3) and 5 postmenopausal controls (group 4) (Table). The groups were matched for race and body mass index (BMI); however, postmenopausal POP patients were significantly older than the controls (67.5 vs 57 years, respectively; $P < .05$). Mean parity, family history of POP, and incidence of SUI were significantly higher in patients when compared with controls in premenopausal (groups 1 and 2) and postmenopausal phases (groups 3 and 4) ($P < .05$ for both). Among the POP groups, the majority of the women had stage III cervical prolapse. In addition, 15 of premenopausal POP patients and 9 of postmenopausal POP patients had stage III anterior vaginal wall prolapse.

BMP1 transcripts were expressed in all tissue samples collected. BMP1 mRNA levels were reduced in patients with POP compared with controls in both premenopausal (groups 1 vs 2) and postmenopausal phases (groups 3 vs 4) ($P = .01$, 3-fold for both) (Figure 1).

Immunoblotting analysis was performed using antibody that specifically recognizes BMP1 protein in all study groups. Assays results consistently showed a pattern of 4 different bands of appropriate molecular weight (130 kDa, 92.5 kDa, 82.5 kDa, and 70 kDa) expression levels in vaginal tissue of premenopausal women (white bars, $n = 7$) and in postmenopausal women (black bars, $n = 4$) normalized vs ACTB. Values represent mean ± standard error of measurement. A significant difference is indicated by *(P < .05). BMP1, Bone Morphogenetic Protein-1.

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82.5 kDa, and 70 kDa) in vaginal tissue samples, corresponding to different BMP1 isoforms derived from the same gene. The tissue-specificity of the BMP1 variants may explain the absence of the 82.5 kDa isoform in our positive control assays. A significant 2.5-fold up-regulation in the expression of 130 kDa isoform was found in vaginal tissue of premenopausal POP patients compared with asymptomatic controls (groups 1 vs 2, $P = .009$), which does not mirror the gene expression pattern (Figure 2). The other isoforms were not statistically different between the premenopausal groups. In contrast, a direct correlation between mRNA changes and the protein expression was observed in the postmenopausal groups. The expression of BMP1 isoforms 130 kDa, 92.5 kDa, and 82.5 kDa were significantly down-regulated in POP patients compared with postmenopausal controls (groups 3 vs 4) ($P = .01$ for all; 3-, 3-, and 6-fold change, respectively; Figure 3). Comparing vaginal tissues from healthy women (groups 2 vs 4), we observed highly significant decrease in the expression of BMP1 130 kDa and 92.5 kDa proteins after menopause ($P = .04$, 18-fold and $P = .02$, 6-fold, respectively, Figure 4).

ACTC1 immunostaining confirmed that all collected vaginal samples included the stratified squamous epithelium, lamina propria, and muscularis layers. Our preliminary results using tissues of premenopausal patients ($n = 3$) and ovariectomized mice, and that physiologic estrogen replacement reversed those changes, 13 provided us evidence that reproductive hormones may influence BMP1 expression in vaginal tissue of women, and guided us in our study design.

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**COMMENTS**

BMP1 is considered a key factor for ECM proteins regulation. 7,8 The literature describes that PCP-null mice are perinatally lethal, with the most obvious gross abnormality being failure of ventral body wall closure, and persistent herniation of the gut, condition that resembles the human gastroschisis. 23 This phenotype likely reflects the defective, weakened nature of the ECM and particularly aberrant collagen fibrils. This finding together with the well-known association between POP and herniation, 24 gave an additional support to our hypothesis that PCP expression deficiency is associated with POP. The fact that BMP1 protein expression was decreased in vaginal tissue of ovariectomized mice, and that physiologic estrogen replacement reversed those changes, 13 provided us evidence that reproductive hormones may influence BMP1 expression in vaginal tissue of women, and guided us in our study design.

The experimental findings confirmed our original hypothesis as we detected a significant reduction of BMP1 mRNA
levels in vaginal samples of patients with POP compared with controls, before and after the menopause. The cause of this alteration, inherited or acquired, may be a deficiency in one of the numerous transcription regulators. Our immunoblotting analysis detected the 2 main and most studied isoforms: mammalian toloid BMP1/mTld or BPM1-3 (MW = 130 kDa) and its spliced-variant BMP1/PCP or BMP1-1 (MW = 70 kDa). In addition, we detected BMP1-5 (MW = 82.5 kDa) and BMP1-7 (MW = 92.5 kDa) in vaginal tissue. Although BMP1-5 and BMP1-7 proteins are less described in the literature, they are expressed in other reproductive tissues including uterus and ovaries. The structural, molecular, and functional properties of the BMP1 variants are not completely understood.

We observed different patterns of BMP1 protein regulation according to the menopausal status. Surprisingly, premenopausal POP patients showed up-regulation in the BMP1 protein expression, which does not correspond to the gene expression profile, whereas postmenopausal patients showed BMP1 protein down-regulation. These findings led us to speculate that the ovarian hormones are involved in the BMP1 translation process. To check this proposal, we analyzed vaginal samples of healthy women before and after the menopause. We observed significant decrease in the expression of BMP1 in women after the menopause, which agreed with previous animal study that described down-regulation in BMP1 protein in vaginal tissue of castrated mice. Therefore, we proposed that reproductive hormones modulate BMP1 in human vaginal tissue.

The analysis of spatial distribution of BMP1 proteins in the vagina showed positive intracellular and extracellular staining. This result agreed with previous reports demonstrating that this protein is synthesized inside the cells; however, the procollagen C-proteinase activity of BMP1 takes place in the extracellular space.

Our study, however, has few limitations. The patients groups have higher parity than the controls. This finding was expected once parity and vaginal delivery are considered the major risk factors for POP development. We acknowledge that some of the control women may not have presented vaginal descensus up to the surgery because of an enlarged uterus caused by fibroids, preventing the pelvic organs to prolapse. However, we avoided including women with early stages of POP as controls because their tissues had already been exposed to some degree of stretch and damage, and this would influence the results of the study. The sample size of the postmenopausal asymptomatic group was small, which reflects the difficulty in obtaining vaginal biopsy from elderly women as only few benign conditions justify the hysterectomy in this population. We were not able to analyze the protein level of all collected samples because of the biopsies sizes, sometimes just sufficient for mRNA quantification. Moreover, our immunoblotting quantified the total amount of BMP1 protein, but functional assays are needed to evaluate the expression of the protein that became active. Similarly, we were not able to perform BMP1 immunostaining in the postmenopausal

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**FIGURE 6**

BMP1 immunolocalization in healthy women

A, Alpha-smooth muscle actin immunostaining in vaginal biopsy of premenopausal healthy woman. ACTC1 immunolabeling is indicated by a brown deposition. B-E, In situ localization of BMP1 protein in vaginal biopsy of premenopausal control woman. F and G, Immunolocalization of BMP1 protein in human umbilical cord tissue (positive control). The BMP1 immunolabeling is indicated by a brown deposition. A, B, F, magnification is ×50, scale bar = 200 μm; C, D, E, G, magnification is ×400, scale bar = 25 μm.

BMP1, Bone Morphogenetic Protein-1; LP, lamina propria; MUS, muscularis layer; SSE, stratified squamous epithelium.

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groups because of scarce samples. IHC in
the premenopausal group was per-
formed to localize the proteins in the
vaginal tissue. We are currently refining
our methodology to study the different
layers of the vaginal tissues separately
and add the principles of stereology to
compare BMP1 distribution and expres-
sion between the 4 study groups. We be-
lieve that the new model will give us
more precise results considering the role
of BMP1 in the vaginal support.

There is a general agreement that the
dysregulation in the components of the
pelvic floor connective tissue in POP
may be the cause or effect of this dys-
function. Women with POP may have
inherited “BMP1-deficient tissues” that
predispose them to develop the dysfunc-
tion spontaneously or initiated by events
such as pregnancy, vaginal childbirth, or
menopause. In contrast, BMP1 dysregu-
lation may be secondary to the POP pro-
gression per se. BMP1 expression is
modulated by mechanical loading in
dermal fibroblasts and skeletal mus-
cle. It is possible that the different
traction applied to the tissue during
the vaginal vs abdominal hysterectomy
may have influenced our results. In
fact, standardize pelvic tissue collect-
ion is a challenge for surgeons that
study POP. Biomechanical principles
considering viscoelastic properties of
the tissues would help us to unify tech-
niques for sample collection to be ap-
plied in clinical studies.

Therefore, our study model does not allow us
to make conclusions regarding cause/ef-
fect of POP, but show that BMP1 is in-
volved in the molecular mechanisms
underlying the dysfunction.

In summary, this study analyzes BMP1
in human vaginal tissue and its possible
association with POP. The strengths of
our study are the strictly homogeneous
population regarding ethnicity and hor-
monal status, the inclusion of only stage
0 POP women as controls, and the inclu-
sion of a postmenopausal control group,
not commonly seen in POP studies. We
speculate that the alteration in the ex-
pression of BMP1 in vaginal tissue of pa-
ients with POP may contribute to defi-
cient ECM synthesis, assembly, and/or
inadequate repair in physio-
logic or pathologic conditions. There-
fore, anomalous ECM fibers are unable
to maintain the structural and functional
integrity of the pelvic floor connective
tissue. We also suggest that the BMP1
molecular pathway is influenced by
ovarian hormones in vaginal tissue and
should not be neglected as important co-
founder in POP studies. Functional con-
trolled studies are necessary to answer
the numerous shortcomings in the liter-
ature. We hope that our findings bring
attention to this important regulatory
protein and may encourage future BMP1
studies.

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6. CONCLUSÕES
Conclusões

A partir dos resultados obtidos, concluimos que:

**Estudo 1**

1- Os genes LOX, LOXL1 e LOXL3 acompanhados das proteínas LOX e LOXL3 estão significativamente hipoexpressos no tecido vaginal de mulheres na pré-menopausa com POP acentuado comparadas com controles assintomáticas;

**Estudo 2**

2- O gene PCP/BMP1 é significativamente hipoexpresso nos tecidos vaginais de mulheres com POP acentuado comparadas com controles, independente do estado hormonal (pré ou pós-menopausa); os níveis das isoformas 130 kDa, 92,5 kDa e 82,5 kDa da proteína PCP/BMP1 estão significativamente reduzidos no tecido vaginal de pacientes na pós-menopausa comparadas com controles; na pré-menopausa, o nível da isoforma 130kDa da PCP/BMP1 é significativamente aumentado nas pacientes com POP acentuado comparadas com mulheres assintomáticas;

3- As isoenzimas 130 kDa e 92,5 kDa da PCP/BMP1 encontram-se diminuídas no tecido vaginal de mulheres saudáveis após a menopausa.
7. CONSIDERAÇÕES FINAIS E PERSPECTIVAS
A quantidade e a qualidade das fibras de colágeno e elastina no organismo são reguladas por meio de um equilíbrio preciso entre síntese, maturação e degradação, resultando num processo dinâmico e constante de remodelamento tecidual. Estudos mecanísticos sobre o papel da remodelação do tecido conjuntivo no desenvolvimento e progressão do POP são de extrema importância para melhorar a nossa compreensão sobre a fisiopatologia desta disfunção. Até o momento, as pesquisas nesta área são limitadas, principalmente pela complexidade inerente ao estudo funcional de proteínas estruturais. Além disto, a maioria dos tecidos do assoalho pélvico são difíceis de serem obtidos sem que se aumente a morbidade cirúrgica e eventual risco de desenvolvimento de incontinência urinária ou POP com a coleta, especialmente nos grupos controle.

Os resultados das pesquisas nesta área são caracterizados muitas vezes por dados discrepantes e inconsistentes provavelmente pela heterogeneidade da população dos grupos de estudo em relação ao estado menstrual, idade, etnia, severidade do POP e estilo de vida, os quais desempenham papel importante na fisiopatologia desta condição.

Em nossos estudos, tentamos minimizar estes vieses de estudo incluindo grupos homogêneos de mulheres caucasianas controladas pelo estado hormonal. Ao analisar as enzimas relacionadas à biossíntese da matriz extracelular, pioneirizamos a análise da expressão gênica e protéica de todos os membros da família LOX e PCP/BMP1 na vagina humana, e observamos correlação entre suas expressões e POP. Além disso, observamos também que os hormônios ovarianos podem modular o perfil de expressão das proteínas PCP/BMP1 em mulheres saudáveis.

No entanto, nosso modelo de estudo (caso-controle transversal) não nos permite concluir sobre possíveis associações entre os demais fatores de risco, POP e as alterações biomoleculares observados nos tecidos de mulheres com a disfunção. De fato, a investigação de qualquer condição de etiologia multifatorial em seres humanos como o POP, que nunca é perfeitamente controlada, é agravada pelo fato de que as expressões do RNAm e das proteínas são reguladas e influenciadas por variações genéticas individuais, diferenças de estilo de vida e fatores ambientais externos.
Há um consenso comum de que a desregulação dos componentes do tecido conjuntivo do assoalho pélvico em POP pode ser a causa ou o efeito dessa disfunção. Mulheres com POP podem ter "tecidos deficientes" herdados, que as predispõem a desenvolver a disfunção espontaneamente, ou incitada por eventos tais como a gravidez, o parto vaginal, a menopausa ou envelhecimento. Por outro lado, o trauma do parto pode influenciar diretamente a expressão de genes e proteínas, ou mesmo as alterações moleculares podem resultar da progressão do POP por si só, pelo estiramento e tensão pelos quais os tecidos do assoalho pélvico são submetidos.

Atualmente, o principal objetivo da comunidade uroginecológica que se dedica ao estudo da etiopatogenia do POP é prever quais mulheres desenvolverão a disfunção, para fins de aconselhamento e assistência, especialmente obstétrica, destas pacientes. É evidente, portanto, a necessidade de compreender melhor as bases moleculares do POP, reconhecer os potenciais marcadores moleculares da disfunção e seus moduladores nos tecidos de suporte do assoalho pélvico e identificar as mulheres que são geneticamente predispostas a desenvolver o POP.

Nossas perspectivas para as pesquisas em POP incluem:

1- A avaliação de outras importantes enzimas para a síntese de colágeno e elastina, tais como pró-colágeno N proteinase e TGF-β-like nos tecidos do assoalho pélvico, que podem estar envolvidas no desenvolvimento e/ou progressão do POP.

2 - O estudo do efeito e modulação dos fatores mecânicos na expressão dos vários componentes da MEC com o intuito de elucidar a associação de causa/efeito entre estes genes e proteínas e POP. Para tal, utilizaremos modelos funcionais in vitro, onde fibroblastos humanos derivados de tecidos pélvicos serão submetidos a estiramento por meio de sistema computadorizado de vácuo.
Considerações Finais e Perspectivas

3 - Estudos *in vitro* utilizando células primárias derivadas de tecidos humanos e animais, a fim de investigar as respostas celulares e moleculares a terapias tais como com hormônios, agentes inibidores de MMPs e terapia celular.

4- Estudos *in vitro* utilizando células primárias derivadas de tecidos humanos e animais, com o intuito de elucidar os mecanismos epigenéticos (por ex. padrão de metilação) de controle da expressão dos genes previamente identificados na literatura e associados com POP.

5- A pesquisa de novos polimorfismos isolados de DNA na população de pacientes afetadas por POP, tais como mutações nas seqüências que codificam as LOXs, PCP/BMP1, fibulina-3 e fibulina-5.

6- Estabelecer colaboração com outros centros de excelência em uroginecologia tanto nacionais como internacionais, a fim de viabilizar estudos populacionais de seqüenciamento genômico em larga-escala.

Assim, esperamos compreender melhor os mecanismos que mantêm a integridade do assoalho pélvico e que estão subjacentes ao POP, e talvez determinar potenciais alvos de intervenção terapêutica. Além disto, esperamos contribuir para a identificação de pacientes predispostas a desenvolver a disfunção, possibilitando futuras estratégias e ações preventivas.