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ORIGINAL ARTICLE

Collagen XVIII and LOXL-4 polymorphisms in women with and without advanced pelvic organ prolapse

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Abstract

Introduction and hypothesis We verified the presence of single nucleotide polymorphisms (SNP) rs2236479 of the collagen 18 (*COL18A1*) and rs2862296 of the lysyl oxidase-like 4 (*LOXL-4*) genes and the association with pelvic organ prolapse (POP) in Brazilian women and determined risk factors for POP development.

Methods We assessed 532 postmenopausal women divided into POP (stages III and IV) and control (stages 0 and I) groups by examination and peripheral blood sample collection. DNA sequences of interest were analyzed by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). We used logistic regression models for the analyses, with $p < 0.005$ for significance.

Results The frequency of homozygous polymorphic alleles (AA) in *COL18A1* and (GG) in *LOXL-4* were similar in both groups (17.5% and 15.4% for *COL18A1* and 18.9% and 20.6% for *LOXL-4*, respectively). There were no associations between those polymorphisms or other genotypes and POP. Multiple logistic regression analysis identified age [odds ratio (OR) = 1.10, confidence interval (CI) 95% = 1.07; 1.14), number of vaginal births (OR = 1.66, CI 95% = 1.36; 2.03), and family history (OR = 2.55 CI 95% = 1.43; 4.55) as independent risk factors for POP.

Conclusion Our study suggests lack of association between DNA polymorphisms rs2236479 of *COL18A1* and rs2862296 of *LOXL-4* with advanced POP in this population.

Keywords Pelvic organ prolapse · Polymorphism · Collagen · Lysyl oxidase · Extracellular matrix

Introduction

Pelvic organ prolapse (POP) is defined as the descent of one or more vaginal walls toward or through the vaginal introitus [1]. It is estimated that 11% of women will require surgery to repair prolapse and/or urinary incontinence (UI) by the age of 79 years, with a 29% reoperation rate [2]. Epidemiological studies have described numerous risk factors for POP, such as aging, estrogen deficiency, parity, vaginal delivery, pelvic surgery, bowel dysfunction, connective tissue diseases, and lifestyle (obesity, smoking/chronic obstructive pulmonary disease, heavy lifting). Among them, vaginal delivery is the main risk factor for POP [3]. However, not all women undergoing vaginal delivery develop severe POP, and it is equally intriguing that POP is seen in nulliparous women [4]. It has been reported that sisters of women <55 years with advanced POP have a five times increased risk for POP development [5]. With that, the interest in investigating the potential genetic predisposition to POP has increased [6, 7].

Genetic studies have mainly focused on extracellular matrix (ECM) of the connective tissues that compose pelvic fascia and ligaments [7]. It is believed that disorders of ECM play a role in POP development [8], and its components may be potential markers for POP. Collagen and elastin are among the main proteins in the human body, and the mechanical and physiological properties of both fibers are highly dependent on the lysyl oxidases (LOX), enzymes that act in collagen and elastin fiber maturation and are essential for ECM remodeling [8, 9].

Allen–Brady et al. reported the results of a genome-wide association study (GWAS) that assessed Caucasian women

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with POP and a strong family history of POP (n = 115) and matched controls (n = 2976) that identified six DNA single nucleotide polymorphisms (SNPs) significantly associated with POP [10]. One was rs2236479, an intronic A/G variant of the collagen XVIII gene (*COL18A1*), in which the allele G is the ancestral (guanine nucleotide) mapped to the chromosomal region 21q22.3 [10, 11]. Endostatin is a protein derived from carboxy-terminal proteolytic fragment of collagen XVIII. It is a broad-spectrum angiogenesis and neovascularization inhibitor, and together with collagen XVIII precursor plays a role in the structural organization of basement membranes [12]. Another genomic-linkage study by Allen–Brady et al. identified chromosome 10q24-26 as possibly related to POP in women from Utah with high-risk POP pedigrees [13]. Lysyl oxidase like-4 (*LOXL-4*), an isoenzyme of the LOX family, was one of the candidate genes in this region [9, 13]. The SNP rs2862296 is located in the promoter region of *LOXL-4*, of which wild-type allele is A (adenine nucleotide) [14].

It is believed that common diseases such as POP may be due to genetic variants (SNPs) that frequently occur in multiple genes. Due the importance of the collagen and LOX enzymes in pelvic floor support [8] and findings of previous genetic investigations in Caucasian women [10, 11, 13], we developed this study to test the hypothesis that rs2236479 (*COL18A1*) and rs2862296 (*LOXL-4*) polymorphisms may be associated with POP in the Brazilian population, which is influenced by various genetic backgrounds.

Methods

The local Research and Ethics Board of the Federal University of São Paulo approved this case–control study (CEP: 47208/12).

Study population and assessment

Women who attended the General Gynecology Clinic and the Urogynecologic Clinic at Federal University of São Paulo, Brazil, were consecutively assessed for eligibility between July 2012 and July 2016. The patients underwent an interview, clinical examination, gynecological evaluation, and Pelvic Organ Prolapse Quantification (POP-Q) system assessment [1]. Candidate women were invited to contribute to the study, and an informed consent form was subsequently signed by participants. Two groups were formed: the case group consisted of postmenopausal women with stage III or IV POP, while the control group consisted of postmenopausal women with stage 0 or I who had never undergone prior pelvic surgery. Demographic and clinical data were then obtained: age, body mass index (BMI), obstetric history, age at menopause, family history of POP, history of respiratory diseases with chronic cough, and connective tissue or collagen diseases. The Brazilian population is composed of various ethnic backgrounds. Participants were classified as white, black, Asian, or mixed. Mixed includes those with physical features and background that do not fit in any other category, which therefore prevents a precise differentiation among ethnic groups. A substantial part of the Brazilian population resulted from racial miscegenation.

Sampling and genotyping

We collected 10 ml of peripheral blood from each participant for genetic evaluation, which was then sent to the laboratory for analysis. Genomic DNA was extracted from peripheral leukocytes and purified using a commercially available kit (IllustraTM blood genomic Prep Mini Spin Kit, GE Healthcare, Buckinghamshire, UK). Identification of rs2236479 (*COL18A1*) and rs2862296 (*LOXL-4*) polymorphisms was done using real-time reverse transcriptase polymerase chain reaction (RT-PCR) (Step One Plus Real Time PCR System, Applied Biosystems, Foster City, CA, USA) using 400 ng DNA for each reaction. For amplification, Taqman® Universal PCR Master Mix II (Applied Biosystems) and commercially obtained test solution containing the polymorphism were used. Fifty cycles of fluorescent amplification were performed in the reactions. At the conclusion of the RT-PCR reaction, genotyping results were plotted in a graph of the Step One Plus Real Time PCR System software and exported to an Excel file.

Statistical analysis

Sample and effect sizes were not calculated due to common issues on studies of genetic population: allele frequencies are largely unknown, as were SNPs in our study; common diseases such as POP may be due to genetic variants (SNPs) that frequently occur in multiple genetic factors, each contributing only small or moderate effects to disease manifestation. Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 24.0. Categorical demographic variables were described in proportions and compared using the χ^2 test. Variables with normal distribution were described in mean and standard deviation (SD), and the variables with nonnormal distribution were described in median and 25th and 75th percentiles (P25 and P75). Comparisons between groups were made using Student's t test for two independent samples and the Mann–Whitney test, respectively. To verify that no evolutive factor affected the study population and that allele frequencies did not alter along the generations, Hardy–Weinberg equilibrium was calculated using the χ^2 test, with a significance level of 5%. Association between polymorphism genotype and POP was estimated as odds ratio (OR) and respective 95% confidence intervals (CI 95%) using binary

logistic regression models. The nonadjusted model was without adjustment for possible confounding variables; the adjusted model was adjusted for age, parity, number of normal births, and weight of the largest newborn. Women with undetermined genotype were excluded from the analysis. Since this proportion was relatively low (< 3%), it is expected that these exclusions would not have a relevant impact on results. To verify whether variables that were different between groups at baseline could be considered independent risk factors for the development of the disease, OR and respective 95% CI were calculated using binary logistic regression models with adjustment for variables of interest. The level of significance was 5% ($p \leq 0.05$).

Results

A total of 532 women were assessed: 285 (53.6%) cases and 247 (46.4%) controls. Eight patients (2 cases and 6 controls) refused to participate. The clinical and demographic characteristics are described in Table 1. Univariate analyses have shown that mean age, parity, number of vaginal deliveries, weight of the largest newborn, prevalence of varicose veins and UI, and family history of POP were higher in the POP group. There were no statistical differences between groups with regard to ethnicity, age at menopause, BMI, alterations related to collagen (such as hernias and collagenosis), and chronic cough.

According to logistic regression analysis that included all variables not homogeneous between groups at baseline, age, parity, number of vaginal deliveries, and family history of POP were positively and significantly associated with POP in the unadjusted model. After adjustment for all variables, significant associations were only found for age, number of vaginal births, and family history of POP. The highest association was related to POP family history [OR = 2.55; CI 95% (1.43–4.55)], followed by vaginal delivery [OR = 1.66; CI 95% (1.36–2.03)], and age [OR = 1.10; CI 95% (1.07–1.14)] (Table 2).

The study population was stable (not in evolution) according to allelic frequencies. Hardy–Weinberg equilibrium was reached by calculating the χ^2, with p value referring to 1 degree of freedom. The frequency of homozygous (wild-type or polymorphic variant) genotypes was similar in both POP and control groups [17.5% and 15.4% for (AA) *COL18A1*, and 18.9% and 20.6% for (GG) *LOXL-4*, respectively]. Heterozygous (GA) *COL18A1* and (AG) *LOXL-4* genotypes were predominant (Table 1).

Results of the multivariate logistic regression analysis concerning the association between *COL18A1* and *LOXL-4* genotypes and POP are described in Table 3. Two regression models were calculated: the first with no adjustment for possible confounding factors; the second with adjustment for age, parity, number of normal deliveries, and weight of the largest newborn. In both analyses, there were no significant associations between possible genotypes and POP phenotype. The adjusted model showed OR = 0.76 (95% CI 0.43–1.35) in women presenting homozygous polymorphic variant for *LOXL-4* (GG) and OR = 0.69 (95% CI 0.41–1.17) in women presenting homozygous polymorphic variant *COL18A1* (AA). Those results suggest lack of association between rs2236479 (*COL18A1*) and rs2862296 (*LOXL-4*) polymorphisms and POP stages III or IV.

Discussion

POP constitutes a "hidden epidemic," with profound functional consequences for affected women. It is believed that POP development is a result of multiple risk factors acting on the pelvic floor [15]. In our study, we identified aging as an independent risk factor for POP: a 1-year increase in age corresponded to a 10% increase in the risk of POP development. Corroborating our finding, an Italian study found OR for POP of 1.3 (95% CI 1.1–1.5) and 1.7 (95% CI 1.5–2.0), respectively, for women aged 52–55 and ≥56 years compared with women aged ≤51 years [16].

Vaginal delivery is associated with POP and may confer a 4- to 11-fold increase in POP risk [4, 17]. In our study, we verified that the increase of one vaginal delivery can lead to a 66% increase in the risk of POP development. A systematic review showed that women with POP are substantially more likely to have family members with the same condition compared with women without POP, demonstrating that a positive family history is an important risk factor [18]. Our study showed that women with a positive family history had a 2.55-fold higher risk of developing the dysfunction compared with patients without a family history.

Predisposition to POP may potentially occur at the genetic level as a result of the millions of alleles that provide each person with their phenotypic individuality [5]. In recent years, different groups have searched for genetic markers of POP using GWAS, linkage, and SNP studies [6, 10, 11, 13]. Potential candidates are genes related to ECM metabolism, which have previously been identified in pelvic floor tissues of women with POP [6]. Both endostatin and collagen XVIII appear to interfere with growth factors, participate in structural organization of basement membranes, and act in collagen remodeling during a wound-healing process [12]. In the pelvic floor, endostatin and collagen XVIII may play a role in tissue organization and response to both minor and major insults, such as vaginal delivery. We chose to study the rs2236479 polymorphism of the *COL18A1* gene based on findings from the GWAS by Allen–Brady et al. [9] that investigated African American and Hispanic women from the Women's Health Initiative Hormone Therapy Study, which described positive association between SNP and POP in a Caucasian population of women with a high risk for POP. However, Giri et al. [19] could not reproduce these findings, and found OR close to the null for both groups of women with

Table 1 Clinical and demographic characteristics of the study groups

	POP *n* = 285 (53.6%)	Controls *n* = 247 (46.4%)	*P* value
Age (years)	67.4 (9.3)	60.3 (6.5)	<0.001*
Ethnicity			
White	153 (54.8)	131 (53.7)	0.828**
Black	20 (7.2)	23 (9.4)	
Mixed	101 (36.2)	86 (35.2)	
Asiatic	5 (1.8)	4 (1.6)	
Unknown	6	3	
Age at menopause (years)	48.5 (5.3)	47.7 (6.0)	0.111*
Body mass index (kg/m^2)	28.0 (4.4)	27.8 (4.8)	0.635*
Parity	4.0 (3.0–7.0)	3.0 (2.0–4.0)	<0.001***
Number of normal deliveries[b]	3.0 (2.0–5.0)	1.0 (0.0–2.0)	<0.001***
Weight of largest newborn[b] (g)	3799.7 (620.4)	3493.6 (654.8)	<0.001*
Chronic cough			
No	217 (76.1)	185 (74.9)	0.740**
Yes	68 (23.9)	62 (25.1)	
Conditions related to collagen[a]			
No	259 (90.9)	229 (92.7)	0.443**
Yes	26 (9.1)	18 (7.3)	
Varicose veins			
No	193 (67.7)	192 (77.7)	0.010**
Yes	92 (32.3)	55 (22.3)	
Family history of pelvic organ prolapse			
No	201 (70.5)	198 (80.2)	0.020**
Yes	76 (26.7)	47 (19.0)	
Unknown	8 (2.8)	2 (0.8)	
Urinary incontinence			
No	121 (53.1)	204 (83.6)	<0.001**
Yes	107 (46.9)	40 (16.4)	
Unknown	57	3	
Type of incontinence (*n* = 147)			
Stress urinary incontinence	72 (67.3)	29 (72.5)	0.148**
Mixed urinary incontinence	18 (16.8)	2 (5.0)	
Urgency urinary incontinence	17 (15.9)	9 (22.5)	
LOXL-4 genotype rs2862296			
Wild homozygous (AA)	85 (29.8)	62 (25.1)	0.465**
Heterozygous (AG)	138 (48.4)	130 (52.6)	
Homozygous with variant allele (GG)	54 (18.9)	51 (20.6)	
Undetermined	8 (2.8)	4 (1.6)	
COL18A1 genotype rs2236479			
Wild homozygous (GG)	104 (36.5)	80 (32.4)	0.556**
Heterozygous (GA)	126 (42.0)	124 (50.2)	
Homozygous with variant allele (AA)	50 (17.5)	38 (15.4)	
Undetermined	5 (1.8)	5 (2.0)	

Continuous variables are described by mean and standard deviation and categorical variables by number and proportion

*Student's *t* test, **χ^2 test; ***Mann–Whitney test; $p < 0.005$: statistical significance

[a] Hernias, collagenoses, valvopathies, aneurysms

[b] Results are presented as median (25th–75th percentile)

Table 2 Multivariate logistic regression analysis for association between clinical and demographic characteristics and pelvic organ prolapse (POP)

	Unadjusted model; OR (CI)	Adjusted model; OR (CI)
Age	1.12 (1.08–1.15)*	1.10 (1.07–1.14)*
Parity	1.39 (1.25–1.55)*	0.92 (0.77–1.11)
Number of vaginal deliveries	1.69 (1.48–1.93)*	1.66 (1.36–2.03)*
Weight of the largest newborn	1.00 (1.00–1.00)	1.00 (1.00–1.00)
Family history	2.01 (1.25–3.26)*	2.55 (1.43–4.55)*

Control category used as reference category (absence of POP stages III or IV); model adjusted for all variables

OR odds Ratio, *CI* confidence Interval

*Statistical significance

moderate POP (n = 1274; stage II and III) and controls with no POP (n = 317). Similar to our candidate-gene study, Khadzhieva et al. did not observe significant association between rs2236479 of *COL18A1* and POP development by investigating Russian women with advanced (n = 210) and with no (n = 292) POP [11]. Potential reasons for the different results among those studies are that genetic variants for POP may differ across racial/ethnic populations or power, as the minor allele frequency variants may vary across geographic populations. In addition, the studies are of different sample size, inclusion criteria, and methodologies. Even though we found no association of the *COL18A1* rs2236479 with POP in Brazilian women, attention should be given to the potential protective factor of the polymorphic allele (AA) for POP phenotype, which is a subject for further investigations.

LOX-family genes are plausible functional candidates since they are essential for the mechanical stability and maintenance of the vascular structure without which weakening of the connective tissues occurs [14, 20, 21]. Differently from other members of LOX [20–22], *LOXL-4* has not been well explored in POP investigation. The unique study by Shynlova et al. failed to detect change in *LOXL-4* gene expression in vaginal tissues of postmenopausal patients with severe POP [23]. We investigated *LOXL-4* gene based on the relevant findings of Allen–Brady et al. by studying families of Caucasian women with members affected by POP [13]. Those authors reported that genes related to POP may be on or near chromosome 10q24-26 region, where *LOXL-4* is located. In our study, we found no association between the SNP rs2862296 and POP in Brazilian women. However, our study suggests that the homozygous genotype for the polymorphic allele (GG) may attribute a protective factor for POP occurrence, even though results did not reach statistical significance. This subject requires additional investigations.

Sample size is an important aspect in genetic studies and a large sample is ideal and required [24]. However, most studies included limited participants. By definition, SNPs occur in at least 1% of the population [25], and small samples tend to conclude that values are not significant. We acknowledge that our results may be biased by the small sample size and that our findings should be interpreted with caution.

The Brazilian population is influenced by a great ethnic miscegenation, which may have impacted on our results. As genetic changes are population-dependent, it becomes an interesting challenge for future investigations to determine whether ethnic-specific genotype frequencies of SNPs contribute to a higher prevalence of POP. We consider this is an important study, since these polymorphisms of collagen XVIII and *LOXL-4* genes have still been poorly explored in POP. We believe the study can contribute to enhance literature in this field, especially when grouped with other investigations in meta-analyses. However, further studies are needed relating

Table 3 Multivariate logistic regression analysis for association between *LOXL-4* and *COL18A1* genotypes and pelvic organ prolapse (POP)

	Unadjusted model, OR (CI)	Adjusted model, OR (CI)[a]
LOXL-4 genotype rs2862296		
Wild -type homozygous (AA)	1	1
Heterozygous (AG)	0.78 (0.47–1.28)	0.69 (0.34–1.38)
Homozygous with variant allele (GG)	0.78 (0.47–1.28)	0.76 (0.43–1.35)
COL18A1 genotype rs2236479		
Wild-type homozygous (GG)	1	1
Heterozygous (GA)	0.78 (0.53–1.15)	0.66 (0.31–1.38)
Homozygous with variant allele (AA)	1.01 (0.61–1.69)	0.69 (0.41–1.17)

Wild-type homozygous genotype was used as a reference

OR odds ratio, *CI* confidence interval

[a] Model adjusted for age, parity, number of normal births, and weight of the largest newborn

COL18A1 and *LOXL-4* genes to pelvic floor dysfunctions and to address the functionality of those SNPs.

Other SNPs located at 4q21 (rs1455311), 8q24 (rs1036819), 9q22 (rs430794), 15q11 (rs8027714), 20p13 (rs1810636), and 21q22 (rs2236479) have been previously described to be associated with POP in Caucasian high-risk familial cases [10]. Those still require additional replication in Brazilian and other populations. Multicentric collaboration is encouraged to overcome issues of access to a large number of participants and economic burden to encourage genetic screening tests for POP.

Conclusion

Age, number of vaginal deliveries, and family history are associated with POP. Our study suggests a lack of association between polymorphisms rs2236479 of the *COL18A1* and rs2862296 of the *LOXL-4* genes and advanced POP in the Brazilian mixed population.

Author contributions Conception and design: Castro, Bortolini.
Data acquisition: Santos, Pepicelli, Batista, Carvalho.
Data analysis and interpretation: Santos, Pepicelli, Batista, Carvalho, Bortolini.
Manuscript drafting: Santos, Bortolini.
Manuscript revision: Bortolini, Castro.
Supervision: Carvalho, Bortolini, Castro.

Compliance with ethical standards

Conflict of interest None.

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