Pelvic organ prolapse (POP) is a common condition affecting million women around the world (1), and its pathophysiology is still largely unknown. Genitourinary prolapse occurs when the pelvic muscles and tissue become weak and cannot hold pelvic organs - uterus, vagina, bladder, and rectum - in place. Approximately 11% of women experience some form of pelvic prolapse and some require surgical treatment (2, 3).

It has been recognized that the process of pregnancy, labor, and delivery is associated with the development of prolapse (3, 4). During pregnancy, the resting diameter of the vaginal wall is increased and may distend up to three times in diameter compared with vaginal tissues from non-pregnant animals. These changes, however, come at the expense of vaginal wall strength; the pregnant vagina endured less maximal stress (5).

The mechanisms by which pregnancy, parturition, and aging lead to failure of pelvic organ support, however, are unknown as well as the mechanisms that mediate the delayed manifestations of childbirth-associated injuries.

Neuromuscular damage that occurs to the pelvic floor during childbirth is often associated with prolapse, although this damage is not detected in all POP cases (1, 6, 7).

Prolonged stretch, mechanical stress, and hypoxia within the vaginal wall may produce secondary effects and contribute to progressive deterioration of pelvic organ support, although they are not always related to its primary pathogenesis (8). Support of the pelvic viscera is maintained by fibromuscular connective tissues of the female pelvic floor and a group of skeletal muscles known as the levator ani. However, defects in the levator ani do not correlate with POP in many women (9-12). The pelvic floor is a structure designed to keep pelvic organs within the body still allowing passage through the urethra, vagina and the anal canal. It consists of striated muscles and fibrous connective tissue distributed within the muscles and surrounding the organs forming fascias and ligaments. The pelvic floor connective tissue has been named the endopelvic fascia and one of its roles is to maintain the positions of organs adjacent to the vagina. Therefore, POP can be considered a hernia in the endopelvic fascia (13, 14).

A potential role of fibromuscular connective tissue in the pathophysiology of POP has been proposed by Boreham et al. (15, 16) and other authors (17). Identification of positive family history provides evidence that a genetic component exists (18, 19). Observations of lower rates of prolapse in African-American women have led to hypothesise the existence of a genetic susceptibility/protection toward the disease (20). However, only one genetic variant that confers risk for POP has been reported (18). Anyway, females with little or no risk factors develop prolapse, while other females with multiple risk factors do not. Therefore, some women may have a predisposition for prolapse in the setting of equivalent risk factors. Genetic analysis of the inheritance pattern...
within these families demonstrated that POP segregated as a dominant character with incomplete penetrance in these families. Both maternal and paternal transmissions were observed (18, 19). It was hypothesized that abnormalities in extracellular matrix metabolism and repair could be a possible genetic explanation for POP development. Female pelvic tissues are rich in elastic fibers that turn over slowly in most adult tissues but undergo massive remodelling in the reproductive organs through pregnancy and birth (21, 22). Current research suggests that the vagina and its supportive tissues actively remodel themselves in response to different environmental stimuli. The literature has many shortcomings due to restricted access to tissue, absence of longitudinal data, and limited animal models. Nevertheless, recent studies indicate that within prolapsed tissue both collagen and elastin metabolism is altered. Thus, not only the synthesis of structural proteins but also the balance between the activity of the major proteolytic enzymes and the inhibitors of proteolysis are important components to consider when studying the pathogenesis of POP (23). Connective tissue, mainly consisting of collagen and structural glycoproteins, is an important part of the supportive structures of the genitourinary region. Relatively few data have been published with respect to the role of elastin and glycoproteins in POP. An altered turnover of connective tissue in the uterosacral ligaments might be responsible for the presence of pelvic floor relaxation in postmenopausal women. There is a complex architecture of the extracellular matrix in the uterosacral ligaments, with marked differences in tenasin and elastin expression between postmenopausal women with or without POP (24). To date, basic science research into the pathogenesis of prolapse has been limited. Several immunohistochemical studies investigated the expression of matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-2 (MMP-2) in the uterosacral ligaments biopsies of women with and without genital prolapse. These data show a significant increase in MMP-1 expression in uterosacral ligament tissue from women with POP, but not in MMP-2 expression. These results suggest that MMP-1 may be a potential marker of collagen degradation. Increased MMP-1 expression in uterosacral ligaments is associated with urogenital prolapse (25). The uterosacral ligaments are an important part of the pelvic support system and connective tissue alterations are thought to contribute to the development of POP. No difference in MMP-1 expression between women with POP and those without was reported by Gabriel et al., that on the contrary, hypothesize a close relation between MMP-2 expression and the presence of POP. These findings strongly support the hypothesis that POP development is due to an increased MMP-2 expression in uterosacral ligaments (26). Moreover, abnormal expression of various structural proteins is thought to be one of the possible cause that could develop POP. Recent data identified genes predisposing women to this anatomic alteration, but no specific mutations have been discovered in cohort of families with familial stress incontinence (19, 27).

Our study is the first to investigate by PCR array the expression profile in the tissue of endopelvic fascia.

### Materials and Methods

#### Patients and tissue collection

Twenty patients with anterior vaginal prolapse halfway past hymen with cystocele (grade ≥ 3 of Baden–Walker half way system classification (28) and twenty women without anterior vaginal prolapse, were enrolled at the Department of Clinical Sciences of Polytechnic University of Marche, Ancona, Italy. Two groups are matched for age, menopausal status, parity and body mass index. Exclusion criteria were prior pelvic reconstruction surgery, chronic debilitating disease, autoimmune and connective tissue disorders or cancer. Cases were frequency matched to controls based on menopausal status to minimize the confounding differences of age and menopausal physiology between the POP and the control groups. The procedure for this research project conforms to the previsions of the declaration of Helsinki and the enrolled women gave their written informed consent with guarantees of confidentiality. Punch biopsies, 6 mm in diameter and 10 mm in depth, were collected from pubo-cervical fascia during the procedure for cystocele repair, which consist of plication of the pubo-cervical fascia in the midline, therefore reducing the cystocele. The tissue of control group was obtained during hysterectomy surgery. Studies conducted with vaginal tissues from women with or without POP reveal information only about the tissue differences at the time of surgery but little regarding the pathogenesis of prolapse. Prolonged stretch, mechanical stress, and hypoxia within the vaginal wall may produce secondary effects that may contribute to progressive deterioration of pelvic organ support, but may be unrelated to its primary pathogenesis. Therefore, great attention was focused on tissue sample collection and biopsies that interested all thickness of vaginal wall were not taken into consideration. The tissues were taken and immediately snap frozen in liquid nitrogen and stored at -80°C until use.

#### RNA extraction

An aliquot of the frozen tissue (5-10mg) was homogenized in a lysis buffer, and the total RNA was then extracted using a RNeasy Micro Kit (Qiagen, Hiden, Germany). RNA samples were tested by ultraviolet absorption at 260nm in order to determine the RNA concentration. The quality and concentration of the RNA samples were further confirmed by electrophoresis on denatured 1% agarose gel.

#### Synthesis of cDNA for PCR array

Total RNA was used as a template for cDNA synthesis using the RT² First Strand kit (SuperArray Bioscience Corporation, Frederick, MD). A 1.5μg of total RNA was pre-warmed with Genomic DNA Elimination Mixture in a final volume of 10μl in a thermal cycler at 42°C for 5min. It was then chilled on ice immediately for at least one minute and added to RT cocktail in a final volume of 20μl. The RT cocktail contains specific RT buffer, primer mix, and the reverse transcriptase enzyme. The reaction was incubated at 42°C for 15min, heated at 95°C for 5min, and then added to 91μl RNase-free H₂O (Diluted First Strand cDNA Synthesis Reaction). It was kept on ice until the following PCR assay.
step.

PCR array analysis

For each array, 102 µl of the Diluted First Strand cDNA Synthesis Reaction were added to 1280 µl of the 2X SABiosciences RT2 qPCR Master Mix. Twenty-five microliters of the experimental cocktail were added to each well of the PCR Array. For our study we used RT2 Profiler PCR Array System-Human Extracellular Matrix and Adhesion Molecules PCR arrays (PAHS-013A), obtained from SuperArray Bioscience Corporation (Frederick, MD). For the PCR assay, the thermal cycler was programmed as follows: 95°C, 10 min; 40 cycles of (95°C, 15 sec; 60°C, 1 min). A melting curve program ran immediately after the above cycling program. Procedure for melting curve analysis was followed as recommended by the manufacturer. We chose β-actin as housekeeping gene for data normalization. Relative mRNA expression was calculated by

\[ 2^{-\Delta\Delta CT} \]

where \( \Delta CT = CT (\text{Gene of Interest}) - CT (\text{β-actin}) \) and \( \Delta \Delta CT = \text{mean} - \Delta CT (\text{Pathological samples}) - \text{mean} - \Delta CT (\text{Normal samples}) \). The parameter CT (threshold cycle) was defined as the cycle number at which the first detectable increase above the threshold in fluorescence was observed. Genes with a ratio of 0.5 or less and 2 or greater were defined as down-regulated and up-regulated, respectively.

Using the RT2 Profiler PCR Array data analysis tool supplied by SuperArray Bioscience Corporation, we examined in Real-Time PCR assay only those genes whose difference was statically significant (\( p \leq 0.05 \)) between pathological and control samples.

Synthesis of cDNA for Real-Time PCR

Total RNA was isolated from the tissue samples as described above. Two micrograms of RNA were reverse transcribed in a total volume of 25 µl for 60 min at 37°C with M-MLV Reverse Transcriptase (Promega, Madison, WI) using random nonamers in order to obtain cDNA.

Real-Time quantitative PCR

Genes listed below (Table 1) have been selected for further evaluation. cDNA was used for Real-Time quantitative PCR and generated as described previously. To avoid false-positive results attributable to the amplification of contaminating genomic DNA in the cDNA preparation, the primers were selected to flank an intron, and PCR efficiencies were tested and found to be close to 1. Primer sequences used for expression analysis of selected genes are reported below (Table 1). The genes were run in duplicate using SYBR Green chemistry. All samples were tested in triplicate using β-actin as the reference gene (Table 1) for data normalization to correct for variations in RNA quality and quantity. Direct detection of PCR products was monitored by measuring the fluorescence produced by SYBR Green I dye binding to double-stranded DNA after every cycle. Relative mRNA expression value was calculated as above described.

Western blot

To confirm the above reported results, FBLN5 expression was detected at the protein level by Western blot analysis. Tissue extracts were prepared with lysis buffer (PBS, containing 1% Nonidet P40, 0.1% SDS, 1 mM sodium orthovanadate, 12 mM sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride, 2 µg/ml aprotinin). Samples containing 50 µg proteins were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The blots were incubated with primary antibody against FBLN5.

Proteins were visualized using the enhanced chemiluminescent substrate SuperSignal West Femto Maximum Sensitivity Substrate (Thermoscientific). The intensity of FBLN5 band has been evaluated from images obtained using Quantity One 1-D Analysis Software.

Statistical analysis

All statistical analyses were performed by using the GraphPad Prism software version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). All values were expressed as mean Relative mRNA expression ± Standard Deviation (SD). Differences in gene expression levels between the pathological groups and control group were determined using the Mann-Whitney test. Differences were considered to be significant at \( p < 0.05 \).

Results

PCR array

To explore whether tissue PCR array data can be used to identify new markers for pelvic prolapse, we first analysed the expression of 84 genes involved in human extracellular matrix and adhesion mechanisms in tissue samples taken from pubo-cervical fascia from women with and without POP. A commercially available PCR array designed for exploring changes in the expression of genes important for cell-cell and cell-matrix interactions was used on ten control samples and on ten pathological samples.

PCR array results about the expression level of 84 genes investigated in pathological and control samples are summarized in the Figure 1.

Real-Time PCR

To validate PCR array data and examine gene expression more quantitatively, a Real-Time PCR analysis was performed on twenty control samples and on twenty pathological samples.

Table 1. Sequences of primers used for expression analysis of selected genes.

| Symbol | GeneBank | Description | Sequence (Fw) 5'-3' | Sequence (Rv) 5'-3' |
|--------|----------|-------------|---------------------|---------------------|
| ITGB3  | NM_000212| Integrin beta 3 | GACACGTGAGCTTCAGCAT | AAAAAAGTGACCTTGACGATCA |
| ECM1   | NM_004425| Extracellular matrix protein 1 | GAAAGGAAGAAAGCAGGTC | GGAAGGCAGATTTGGTTCAG |
| FBLN-5 | NM_006329.3 | Fibulin-5 | TGGTGGATGGTGACGGAG | GGAAGGCACTGTTGCTAAGCA |
| ACTB   | NM_001101.3 | Actin, beta | CTCTTCAGCTCCTCCTTCT | AGCAGCTGTGGTGCGTCAG |

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We decided to explore by Real-Time PCR those genes showing between pathological and control samples a statistically significant difference (p<0.05) in expression level and a ratio of 0.5 or less and 2 or greater. Among differentially expressed genes, we selected for Real-Time PCR assay, Expression levels are measured as mean Relative mRNA expression ± Standard Deviation. Mean Relative mRNA expression was calculated as the average of all the Relative mRNA expression values within pathological and control samples, respectively. Mann-Whitney test was used to determine differences between pathological (10 individuals) and control (10 individuals) samples included in the study. Differences were considered statistically significant with a p<0.05. POP, pelvic organ prolapse; CTRL, control.

**Discussion**

POP is common and frequently recurs or continues...
after surgical correction. The aetiology of prolapse is complex and multifactorial, even though several studies in human and experimental animal models suggest that the childbirth-associated trauma plays a significant role. DeLancey et al. have shown that changes in the surrounding pelvic muscle support, i.e. the levator ani, are evident in women with prolapse (29). However, biomechanical properties evaluations in women with prolapse showed different findings. Controversial is the biomechanical role of vaginal tissue in the POP development. Lei et al. reported significant differences in the biomechanical properties between pre- and postmenopausal women with and without prolapse. The authors demonstrated that severe prolapse conferred less elasticity and greater stiffness of the vaginal tissues (30). On the contrary Goh et al. found similar degrees of tissue deformation, on pre- and postmenopausal women with prolapse, even though they did not test tissues from women without prolapse (31). A marked variability in maximal strength and strain with no discernable trends in biomechanical properties of vaginal samples, taken from postmenopausal women with POP, was described (32). The controversial results obtained from these studies could be due to the method of tissue acquisition. When vaginal wall is excised during a prolapse surgery, the vaginal muscularis is often split or separated from the adventitia. The tissue biopsy may be highly variable depending on the ultimate amounts of epithelium, subepithelial connective tissue, and muscularis. In animal models, full thickness vagina can be examined in all cases (5). New methodological approaches, such as array technology, have been suggested to evaluate the variation of biomechanical properties associated with POP. Two studies, performed using RNA isolated from pubococcygeus muscle or vaginal tissue, showed an involvement of actin, myosin, extracellular matrix-related genes as well as chemokines and transcription factors in the pathogenesis of POP (33, 34). Chronic cyclical mechanical stretch applied to cardinal ligament fibroblasts in vitro induced the expression of mechano-responsive genes such as regulators of actin and extracellular matrix remodelling molecules and cell adhesion regulators (35). Recently, Brizzolara et al. investigated gene expression profile in POP cases from samples of fibromuscular connective tissue taken in two regions of uterine attachment in the pelvis (14). Our microarray study analysed the transcription profiles of pubo-coccygeal fascia, identified during a prolapse surgery or during colpohysterectomy. More specifically, we examined the gene expression profile using PCR arrays in ten subjects with POP and ten control subjects. Results obtained in the present study allowed to identify genes differently regulated in POP group compared with control one. A small subset of genes (ECM1, ITGB3 and, FBLN5), were chosen for further evaluation in Real-Time PCR keeping in mind their statistical significance in PCR array data analysis. We set-up the Real-Time PCR experiments to analyse the expression levels of these genes in twenty control samples and twenty samples from patients with POP. Real-Time PCR data confirmed results obtained from PCR array, demonstrating up-regulation for ITGB3 and ECM1 genes, and down-regulation for FBLN5 gene. Our results agree with published data showing reduced expression levels for FBLN5 in POP (8, 36, 37). The FBLN5, a recently discovered integrin ligand, is an essential determinant of elastic fibre organization (38). FBLN5 interacts directly with elastic fibres in vitro, and serves as a ligand for many cell surface integrins through its amino-terminal domain. Thus, FBLN5 may provide anchorage of elastic fibres to cells, thereby acting to stabilize and organize elastic fibres in the skin, lung and vasculature (39). POP in FBLN5−/− mice was remarkably similar to that in primates (8). Recently, it was reported that mice with null mutations in lysyl oxidase-like 1 (LOXL1) develop POP. Elastin is a substrate for lysyl oxidase (LOX) and LOXL1, and the latter interacts with FBLN5 (40). Taken together these results suggest that synthesis and assembly of elastic fibers are crucial for recovery of pelvic organ support after vaginal delivery and that disordered elastic fiber homeostasis is a primary event in the pathogenesis of POP in mice (8). FBLN5 is a calcium-dependent, elastin-binding protein that localizes to the surface of elastic fibres in vivo. This function may be mediated by the tripeptide Arg-Gly-Asp motif in FBLN5, which binds to cell surface integrins, and the Ca2+--binding epidermal growth factor repeat (EGF), repeats, which binds elastin (38). Between the genes, that we found differentially expressed, there is an integrin and, to our knowledge, integrins and growth factor receptors act synergistically to modulate cellular functions. Both alphavbeta3 integrin and the platelet-derived growth factor receptor have been shown to play a positive role in cell migration (41). Cell adhesion is mediated by the specific interactions of cell surface receptors with extracellular glycoproteins. The best characterized cell adhesion receptors are the integrins. Integrin ligands, such as fibronectin, are not passive adhesion molecules but are active participants in the cell adhesive process that leads to signal transduction. The best characterized integrin ligand is fibronectin, a multifunctional glycoprotein comprised of three different types of homologous repeating units. The beta3 integrin intracellular domains are sufficient to initiate signal transduction pathways. Other intracellular responses to cell adhesion include stimulation of migration, the assembly of an F-actin cytoskeleton and specialized structures called focal contacts, changes of cytoplasmic pH and calcium ion concentration, and modulation of proliferation and gene expression (42). Although the transcription factors required for beta3 integrin induction are unknown, the gene promoter has potential binding sites for MZF-1, Sp1, GATA, Myb, Ets, and E2F transcription factors (43). Regulation of integrin function is believed to be essential in promoting stable cell adhesion as well as to be required for cell migration. The in vitro study developed by Gaurang et al have, showed a molecular mechanism by which sex steroid signaling leads to regulation of beta3 integrin expression, through homeobox A10 (HOXA10) as an intermediary. They described the characteristics of the regulatory element by which HOXA10 regulates beta3 integrin (44). In addition, elastin degradation produces peptides displaying a wide range of biological activities. In vitro, these elastin derived peptides (EPs) interact with the elastin receptor complex (ERC), but also bind to alpha(v)beta(3) integrin and galectin-3 (45). So, induction of beta3 integrin expression in women with POP, could lead to the activation of multiple intracellular pathways. Although we are
unable to determine the order of the events that occur in pelvic prolapse, our data suggest that this specific disease results from fundamental phenotypic changes and gene expression rearrangements. We hypothesize that reduced expression of FBLN5 is the start point. It appears that the pelvic tissue attempts to compensate for a lack of FBLN5 by increasing the expression of both ITGB3 and the ECM1. The up-regulation of ECM1 avoids collagen degradation and extracellular matrix remodelling by inhibiting matrix metalloproteinase-9 (MMP-9) activity (46). Asano et al suggested that the up-regulated expression of αvβ3 contributes to the establishment of autocrine TGF-β loop in scleroderma human fibroblasts identifying integrin as a potent target for the treatment of scleroderma (47). In our case, the ITGB3 protein could induce a significant increase of MMP-2 (48) and transforming growth factor beta 1 (TGF-β1) activity (49) which may stimulate fibroblasts to increase the expression of FBLN5.

PCR array results are in accordance with data present in literature about the effect exercised by ECM1 and ITGB3 proteins on MMP-9 and MMP-2 expression level. The over-expression of ECM1 could be responsible for the decreased expression of MMP-9 and that one of ITGB3 for the increased expression of MMP-2 in pathological samples respect to the control ones (Fig.1). Obviously, more in-depth experiments are needed to confirm these preliminary observations about the role of MMP in pelvic prolapse.

The examination of the fundamental mechanisms important for adhesion of cultured human cells and the resultant signalling processes has the potential of providing an understanding of molecular mechanisms involved in complex pathophysiological processes and serving as the basis for the development of novel therapeutic agents for the treatment of this condition. Definitive evidence that pelvic organ prolapse is the result of a defect in the pelvic floor musculature, connective tissue, or a combination of the two is currently lacking. For this study, patients with anterior prolapse were chosen because the main problem of POP is in cystocele and urinary incontinence. Research on gene expression in the anterior vaginal tissues may indicate new therapeutic strategies beyond current surgical techniques. Further studies are needed to clarify the relationship between different gene expressions and position as well as grade of POP.

Our results, although obtained from analyses performed on a small cohort of patients, seem to be very encouraging and may provide potential targets for therapy in early stages of POP, mitigating the extreme conditions of this clinical phenotype.

Conflict of Interest
The authors declare that they have no conflict of interest.

Ethical approval
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Written consent
Written consent was obtained from all individual participants included in the study.

Author’s contribution
All authors contributed to the intellectual development of this work and approved the final manuscript. FS, AC, and ME were responsible for the experimental design, and coordination of research. MC, AC, and SRG were responsible for the preparation of the manuscript. MC, DS, and VP carried out the experimental work to evaluate the expression profile in pelvic organ prolapse. All authors are responsible and guarantors for this work.

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