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CHAPTER 5

Microarray analysis of the anterior vaginal wall from premenopausal patients with pelvic organ prolapse

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ABSTRACT

Objective: Pelvic organ prolapse (POP) is a common multifactorial disease in a heterogeneous population of women. Due to this heterogeneity, the underlying molecular mechanisms contributing to the pathogenesis of POP are still unclear. We sought to identify dysregulations in POP related pathways by comparing gene expression profiles of prolapsed and non-prolapsed anterior vaginal wall tissues within the same patient.

Study design: Biopsies were collected from 12 premenopausal women undergoing prolapse surgery (cystocele POP-Q stage ≥ 2). A full thickness anterior vaginal wall sample was taken from the POP site during anterior colporrhaphy. An additional sample was taken from the non-prolapsed apex of the anterior vaginal cuff. Microarray analysis was performed using whole genome GE 4x44K microarrays. Beside a paired significance analysis of microarray (SAM), also cluster analysis of differentially expressed genes were performed.

Results: In both the SAM and the supervised cluster analysis, a set of genes could be identified as being specific for the 'diseased' prolapsed anterior vaginal wall. Ontology analysis revealed that these genes were involved in signal transduction and transcriptional regulation, mainly AP-1 and FRA-related pathways. Cluster analysis showed that patients could be divided in an 'ECM/ integrin' pathway subgroup and a 'muscle cell/contraction' pathway subgroup. This division was already present in the non-prolapsed tissue. Quantitative PCR confirmed results.

Conclusion: Prolapsed anterior vaginal wall tissue shows dysregulation of generic pathways related to signal transduction and transcription. We provided evidence for inter-individual differences in both non-prolapsed and prolapsed tissues reflecting either ECM/integrin or muscle cell/contraction dysfunctioning, implying different failure mechanisms leading to POP.

INTRODUCTION

With the ageing population in the Western world, pelvic organ prolapse (POP) is a serious public health problem, affecting the quality of life of millions of women. Women with POP suffer from chronic pelvic pain and pressure, urinary and faecal incontinence, sexual dysfunction and social isolation[1,2]. Although pregnancy and vaginal childbirth are considered the major causes for the development of POP, these risk factors do not fully explain the origin and progression of all cases of POP. In fact POP has been observed in nulliparous women, on the other hand, many multiparous women do not develop any pelvic floor dysfunction[3,4]. Other risk factors that are thought to be responsible for the development of POP include physiological ageing, menopause, factors associated with elevated intra-abdominal pressure (*e.g.* obesity, chronic constipation and/or coughing), connective tissue disorders, ethnic background, family history and genetic predisposition[5,6]. The extensive list of risk factors illustrates that POP should be considered as a multifactorial disease. Inherent to any multifactorial conditions in humans, the bias is introduced by individual genetic variation, life style differences, and the influence of external environmental factors. Due to this heterogeneity, the underlying affected biological processes contributing to the pathogenesis of POP are still unclear.

Over the past decades, the molecular and biochemical characteristics of POP have been extensively studied. The main observation is a weakening of the connective tissue at the pelvic support level; the ligaments, endopelvic fascia and the levator muscle[7]. One hypothesis is that a parturition-related injury that causes denervation of the pelvic floor, consequently weakens the levator ani muscles, resulting in overloads on the uterosacral, cardinal ligaments and endopelvic fascia. This might lead to secondary failure of the fascia and the development of POP[8-10]. Other studies suggest that a primary defect in the connective tissues with tissue failure of the fascia may play a role. Conclusive evidence that POP is the result of a defect in the pelvic floor musculature, connective tissue, or a combination of the two is currently lacking. Moreover, there is an ongoing discussion about whether the changes in the different components of the connective tissues in POP are a cause or are only an effect of POP[11].

A better understanding of the pathophysiology of POP is clinically relevant for identifying women at risk, as well as for the development of interventional therapies. The advances of microarray technology offer new opportunities to study molecular mechanisms involved in different physiological and pathophysiological conditions. The number of microarray studies described in literature with respect to POP is low. These studies have addressed gene expression changes related to POP, focusing on the uterosacral or round ligaments in uterine prolapse in pre- and post-menopausal women[12-15]. However, due to a lack of standardization of the sample-sites and collection techniques, as well as the mixed demographic characteristics of the study population regarding menopausal status, age, POP-stage and type, ethnicity and life style, the potential molecular mechanisms behind POP have not been identified yet.

The aim of the current study was to identify POP related dysregulated pathways by comparing gene expression profiles of prolapsed and non-prolapsed anterior vaginal wall tissues from women with cystocele by means of whole-genome microarrays. The study design followed a novel approach where each premenopausal POP patient was her own control to reduce the bias caused by differences between patients.

MATERIALS AND METHODS

Patient selection

This observational study of premenopausal Caucasian women recruited and enrolled patients from the urogynaecology clinic of the Department of Obstetrics and Gynaecology of the Kennemer Gasthuis, Haarlem, The Netherlands between March 2009 until March 2011. We included women undergoing vaginal reconstructive pelvic surgery of the anterior vaginal compartment because of cystocele (POP-Q stage ≤ 2). Exclusion criteria were: previous pelvic surgery, auto-immune and connective tissue diseases, history of endometriosis, history or presence of cancer, adhesions or scarring at the biopsy site, diabetes, chronic inflammatory diseases, chronic infections and steroid use. Signs of POP were recorded during the pelvic examination by the same urogynecologist (MHK), and were described according to the International Continence Society Pelvic Organ Prolapse Quantification (POP-Q)[16]. Standardized demographic and clinical information was recorded and stored in a dedicated database. The study was approved by the medical ethical committee and all patients signed informed consent before participation.

Tissue collection and histology

Tissue collection and histology were performed as previously described[17]. Approximately 1cm^2 of full-thickness anterior vaginal (mid-line) wall biopsy was excised from the vaginal cuff after vaginal hysterectomy (non-POP site). A second full thickness anterior vaginal wall biopsy was taken from the POP site during anterior colporrhaphy (POP-Q, point Ba). All biopsies were large enough to perform histology and immunohistochemistry. Directly after excision, the tissues were divided in two: one portion was immediately frozen in liquid nitrogen and stored at -80˚C, and the other portion was embedded in paraffin for microscopic evaluation. Paraffin-embedded samples were cut into 3µm sections and stained with hematoxylin and eosin. A pathologist, blinded to the source of the samples confirmed that all samples represented full thickness vaginal wall without portions of the cervix or the uterus. The menstrual phase was determined by histological evaluation of the endometrium.

Microarray analysis

RNA extraction

The snap frozen tissues were de-epitheliased and total RNA was extracted with Trizol and according to the manufacturer's protocol (Life Technologies, Paisley, UK). RNA integrity and concentrations were measured on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and Nanodrop spectrophotometer ND-1000 (Fisher Scientific, Waltham, MA, USA). All samples were of good quality with an RNA Integrity Number (RIN-value) between 6.3-8.6.

cRNA synthesis and probe labeling

200 ng total RNA per sample was used as input for amplification and labeled with the Low Input Quick Amp Labeling kit (Agilent Technologies, Palo Alto, CA, USA), including control spikes according to the manufacturer's guidelines. Labeled RNA was purified using the RNeasy Mini Kit (QIAGEN Ltd., Venlo, the Netherlands) yielding 4.7 µg or more of labeled cRNA and specific activities greater than 13.2 pg Cye3 dye/µg cRNA and 12.0 pg Cy5 dye/µg cRNA.

Hybridization and scanning

Labeled cRNA was hybridized onto whole human genome GE 4x44K microarrays according to the manufacturer's protocol (Agilent Technologies). Scanning was performed using a microarray scanner G2505C and data were feature-extracted using Feature Extraction Software (v9.5) according to the manufacturer's protocols (Agilent Technologies). Outlier features on the array were flagged by the software.

Data pre-processing

All analyses of the gene expression microarray data were done within the R statistical software, using the Limma-package. Preprocessing of the gene expression data comprised of RMA background correction, loess within-array normalization and A-quantile between-array normalization[18-20]. Values were log2 transformed. The microarray data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database under accession number GSE53868, http://www.ncbi.nlm.nih.gov/geo/info/linking.html.

Quantitative real time PCR

To confirm the microarray data, seven differentially expressed genes were chosen for quantitative real time polymerase chain reaction (RT-PCR). Total RNA samples used for microarray analysis with a final concentration of 250 ng/ml were reverse-transcribed using SuperScript VILO cDNA synthesis kit (Life technologies). Gene expression of Collagen 1α1(COL1α1), Collagen 3α1(COL 3α1), alpha smooth muscle actin (ACTA2), Desmin (DES), interleukin 6 (IL-6), chemokine receptor type 4 (CXCR4) and tumor necrotic factor

alpha (TNF-α) were normalized to the housekeeping genes: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz), hypoxanthine-guanine phosphoribosyltransferase (HPRT) and human ubiquitin C (hUBC). Genes were evaluated using the primers listed in table 1 (Life technologies), with the SYBR Green Reaction Kit and measured by RT-PCR in a Light Cycler 480 device following suppliers' specifications (Roche, Mannheim, Germany). Gene expression levels were normalized using a factor derived from the equation (*Ywhaz x HPRT x hUBC*)^(1/3). Crossing points were assessed using the Light Cycler software (version 4; Roche) and plotted versus serial dilutions of cDNA derived from a human universal reference total RNA (Clontech Laboratories, Palo Alto, CA, USA).

Table 1. Primer sequences used for RT-PCR

Target gene		Oligonucleotide sequence	Annealing temperature (°C)	Product Size (bp)
Col 1α1	Forward	5' TCCAACGAGATCGAGATCC 3'	57	191
	Reverse	5' AAGCCGAATTCCTGGTCT 3'		
Col 3α1	Forward	5' GATCCGTTCTCTGCGATGAC 3'	56	279
	Reverse	5' AGTTCTGAGGACCAGTAGGG 3'		
TNF-α	Forward	5' AGAGGGCCTGTACCTCATCT 3'	56	315
	Reverse	5' AGGGCAATGATCCCAAAGTAG 3'		
IL-6	Forward	5' ACAGCCACTCACCTCTTCA 3'	56	207
	Reverse	5' ACCAGGCAAGTCTCCTCAT 3'		
CXCR4	Forward	5' GGCCCTCAAGACCACAGTCA 3'	57	352
	Reverse	5' TTAGCTGGAGTGAAAACTTGAAG 3'		
ACTA2	Forward	5' CCTGACTGAGCGTGGCTATT 3'	56	206
	Reverse	5' GATGAAGGATGGCTGGAACA 3'		
Desmin	Forward	5' TGTGGAGATTGCCACCTAC 3'	57	165
	Reverse	5' CGTGTCTCGATGGTCTTGAT 3'		
Ywhaz	Forward	5' GATGAAGCCATTGCTGAACTTG 3'	56	229
	Reverse	5' CTATTTGTGGGACAGCATGGA 3'		
HPRT	Forward	5' GCTGACCTGCTGGATTACAT 3'	56	260
	Reverse	5' CTTGCGACCTTGACCATCT 3'		
hUBC	Forward	5' GCGGTGAACGCCGATGATTAT 3'	56	202
	Reverse	5' TTTGCCTTGACATTCTCGATGG 3'		

Col1α1, α1(I)procollagen; Col3α1, α1(III)procollagen; TNF-α, tumor necrotic factor-α; IL-6, interleukin 6; CXCR4, chemokine receptor type 4; ACTA2, alpha smooth muscle actin; DES, desmin; Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; HPRT, hypoxanthine-guanine phosphoribosyltransferase; hUBC, human ubiquitin C.

Statistical analysis

Statistical analysis of the microarray data was performed using a Significance Analysis of Microarray (SAM) from Stanford University, CA, version 3.09[21]. Two class paired analysis using SAM at a false discovery rate (FDR) of less than 1% was applied to search for single genes that were significantly differentially expressed between the POP site and the non-POP site. Cluster analysis was used for the categorization of coordinately differentially expressed genes which were selected by filtering for genes with at least two observations with an absolute of more than two and an absolute difference between the maximum and minimum value greater or equal to two. Selected genes were clustered using average linkage clustering[22]. Differentially expressed genes were visualize with Tree View (Eisen Software http://rana.lbl.gov/EisenSoftware.htm). Molecular Signatures Database (MSigDB database v4.0 updated May 31, 2013 GSEA/MSigDB web site v4.01, reference Subramanian, Tamayo, et al. 2005, PNAS 102, 15545-15550) was used to compute the overlap of significantly up- and down-regulated genes, as well as clusters of genes with gene sets encoding canonical pathways, derived from BIOCARTA (www.biocarta.com), KEGG (www.genome.jp/kegg/pathways.html) and REACTOME (www.reactome.org).

Correlation analyses were performed using Prism software version 6.0 (GraphPad Software Inc., San Diego, CA, USA). Data were tested for normal distribution and either a Pearson correlation or a Spearman correlation were performed. Baseline characteristics of POP patients were expressed as mean (SD) or median (interquartile range), where appropriate. P values less than 0.05 were considered significant.

RESULTS

Patient demographics

We recruited 12 premenopausal Caucasian women with pelvic organ prolapse, mean age 42.6 ± 6.1 years, average parity 2.3 ± 0.8 and a mean body mass index (BMI) of 27.2 ± 5.9 kg/m^2. All patients had a cystocele POP-Q stage ≥ 2. Five women had a cystocele stage 2, regarded as a mild POP, seven women suffered from severe cystocele stage > 2 (Table 2). The median stage of prolapse in the apical compartment was 0 (range 0-1) and in the posterior compartment 1 (range 0-3). All women underwent vaginal pelvic reconstructive surgery for the cystocele and tissue from the POP as well as from the non-POP site could be obtained.

Microarray analysis

Differentially regulated processes between POP and non-POP tissues

In order to identify POP-related dysregulated molecular processes, gene expression profiles of the prolapsed anterior vaginal wall and the non-prolapsed precervical anterior vaginal wall were compared using paired *Significant Analysis of Microarrays (SAM).* This analysis revealed 984 significantly different expressed genes with a FDR < 1%; 277 genes were up-

regulated and 707 down-regulated in POP versus non-POP tissue. Ontology analysis of the up-regulated genes at the prolapsed anterior vaginal wall revealed a significant enrichment of more than 20 pathways, among which activating transcription factor 2 (ATF2), transforming growth factor beta (TGF-ß) signaling, FRA and activated protein -1 (AP-1) pathways were the most significant (Table 3). Down-regulated genes were involved in generic transcription pathways. Unpaired *SAM* analysis showed 86 up-regulated and 55 down-regulated genes with a FDR < 1%. These genes were involved in the same pathways than the genes that were identified in the paired analysis.

Table 2. Patient characteristics

Characteristics	**Case (n=12)**
Age, y [a]	42.6 ± 6.1
BMI, kg/m^2 [a]	27.2 ± 5.9
Parity, births[b]	2.2 (1-3)
Vaginal delivery[b]	2 (1-4)
Assisted vaginal delivery[c]	3 (25)
Stress urinary incontinence[c]	8 (67)
POP-Q stage of prolapse[b]	3 (25)
Anterior	2 (2-3)
Apical	0 (0-1)
Posterior	1 (0-3)
Menstrual phase[c]	
Proliferative phase	7 (58)
Secretory phase	5 (42)

BMI, body mass index; POP-Q, POP-Quantification. [a] Data presented as mean ± SD; [b] Data presented as median (interquartile range); [c] Data presented as number of patients (%).

Table 3. Paired statistical analysis of microarray (SAM) POP versus non-POP tissue

Upregulated pathways

Gene Set Name	# Genes in Gene Set (K)	# Genes in Overlap (k)	k/K	P-value	FDR q-value
PID_ATF2_PATHWAY	59	6	0.1017	1.79E-7	1.65E-4
REACTOME_SIGNALING_BY_TGF_BETA_RECEPTOR_COMPLEX	63	6	0.0952	2.66E-7	1.65E-4
PID_FRA_PATHWAY	37	5	0.1351	4.6E-7	1.65E-4
PID_AP1_PATHWAY	70	6	0.0857	5.01E-7	1.65E-4
REACTOME_HEMOSTASIS	466	12	0.0258	6.92E-7	1.83E-4
KEGG_CHRONIC_MYELOID_LEUKEMIA	73	5	0.0685	1.41E-5	3.09E-3
KEGG_MAPK_SIGNALING_PATHWAY	267	8	0.0300	1.77E-5	3.15E-3
REACTOME_TRANSCRIPTIONAL_ACTIVITY_OF_SMAD2_SMAD3_SMAD4_HETEROTRIMER	38	4	0.1053	1.91E-5	3.15E-3
REACTOME_PLATELET_ACTIVATION_SIGNALING_AND_AGGREGATION	208	7	0.0337	2.87E-5	3.43E-3
KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM	85	5	0.0588	2.95E-5	3.43E-3
BIOCARTA_ERYTH_PATHWAY	15	3	0.2000	3.1E-5	3.43E-3
KEGG_TGF_BETA_SIGNALING_PATHWAY	86	5	0.0581	3.12E-5	3.43E-3
BIOCARTA_TGFB_PATHWAY	19	3	0.1579	6.52E-5	6.15E-3
REACTOME_ACTIVATED_AMPK_STIMULATES_FATTY_ACID_OXIDATION_IN_MUSCLE	19	3	0.1579	6.52E-5	6.15E-3
PID_TGFBRPATHWAY	55	4	0.0727	8.35E-5	7.35E-3
BIOCARTA_NFAT_PATHWAY	56	4	0.0714	8.97E-5	7.4E-3
REACTOME_SIGNALING_BY_HIPPO	22	3	0.1364	1.03E-4	7.97E-3
BIOCARTA_CTCF_PATHWAY	23	3	0.1304	1.18E-4	8.18E-3
REACTOME_INTERACTION_BETWEEN_L1_AND_ANKYRINS	23	3	0.1304	1.18E-4	8.18E-3
KEGG_ADIPOCYTOKINE_SIGNALING_PATHWAY	67	4	0.0597	1.81E-4	1.19E-2

Downregulated pathways

Gene Set Name	# Genes in Gene Set (K)	# Genes in Overlap (k)	k/K	P-value	FDR q-value
REACTOME_GENERIC_TRANSCRIPTION_PATHWAY	352	33	0.0938	0.00E+00	0.00E+00
KEGG_LIMONENE_AND_PINENE_DEGRADATION	10	3	0.3000	6.6E-5	4.36E-2

Inter-individual variability at the molecular level in POP women

Although the above identified pathways are relevant for disease, we found no differences in pathways related to collagen, elastin or SMCs between the POP and the non-POP anterior vaginal wall. Since these processes are believed to play a major role in the development of POP we questioned whether this could be a consequence of inter-individual variation of molecular mechanisms involved in POP. Paired analysis might level out such inter-individual differences. To gain more insight in these potential differences, a *hierarchical cluster analysis* was performed. Differentially expressed genes were clustered based on similar expression profiles. A number of 2502 differentially regulated genes were categorized into three clusters representing different biological themes (Fig.1). In line with the SAM analysis, a cluster of 625 differentially expressed genes showed marked variation between POP and non-POP tissue (light blue bar, Fig.1). Pathway analysis of these genes revealed pathways related to cytokine and chemokine receptor pathway, AP-1, FRA and ATF2 pathway and nuclear factor of activated T cells (NFAT) signalling pathway as shown in the SAM analysis. Also peptide ligand binding receptor pathway, class A1 rhodopsinlike receptor pathway, GPRC ligand binding pathway and IL-6 pathway were up-regulated in the prolapsed tissues.

Additionally, two clusters of genes – 1073 genes and 804 genes in the dark blue and yellow marked clusters, respectively (Fig.1) – reflected variance between patients. Pathway analysis of the genes in the dark blue cluster showed that these genes belong to the processes of ECM organization, collagen formation, the integrin 1 pathway, ECM receptor, cytokine-cytokine receptor interaction and cell adhesion pathways. The yellow cluster represents genes involved in smooth and striated muscle contraction, focal adhesion and actin cytoskeletal regulation. This cluster analysis suggests that besides a POP specific gene expression profile, as was also shown in the paired SAM analysis, at least two clusters of genes reflect variance in molecular processes between individuals. A subgroup of patients showed increased expression of genes related to the ECM/integrin pathway cluster whereas another subgroup of patients was characterized by increased expression of genes related to muscle cell contraction. To verify whether this variability in elevated expression for these pathways was already seen in the non-prolapsed precervical anterior vaginal wall tissue, we additionally performed an unsupervised cluster analysis of non-POP expression profiles. This analysis did indeed divide the patient group in two subgroups of patients. One subgroup was characterized by elevated expression of genes reflecting ECM organization, integrin-1 and collagen formation pathways. The other subgroup was characterized by genes such as myosin light chain (MYLK), myosin heavy chain 2 and 3 (MYH2 and 3), and desmin (DES), which are also involved in smooth muscle contraction and actin cytoskeleton pathways (Fig.2A). Analysis of the gene expression of Col1α1 and DES – the representative genes for the ECM cluster and muscle cell cluster – shown in figure 3, illustrates the subdivision into the two clusters on single-gene level. The patients from the muscle cell cluster subgroup were also found to have a high percentage of desmin-positive cells according to data from our previous study on morphometric analysis of the tissue

composition of the vaginal wall tissues of the same study population (Fig.2B).The inter-individual differences were not related to disease severity or menstrual phase (data not shown).

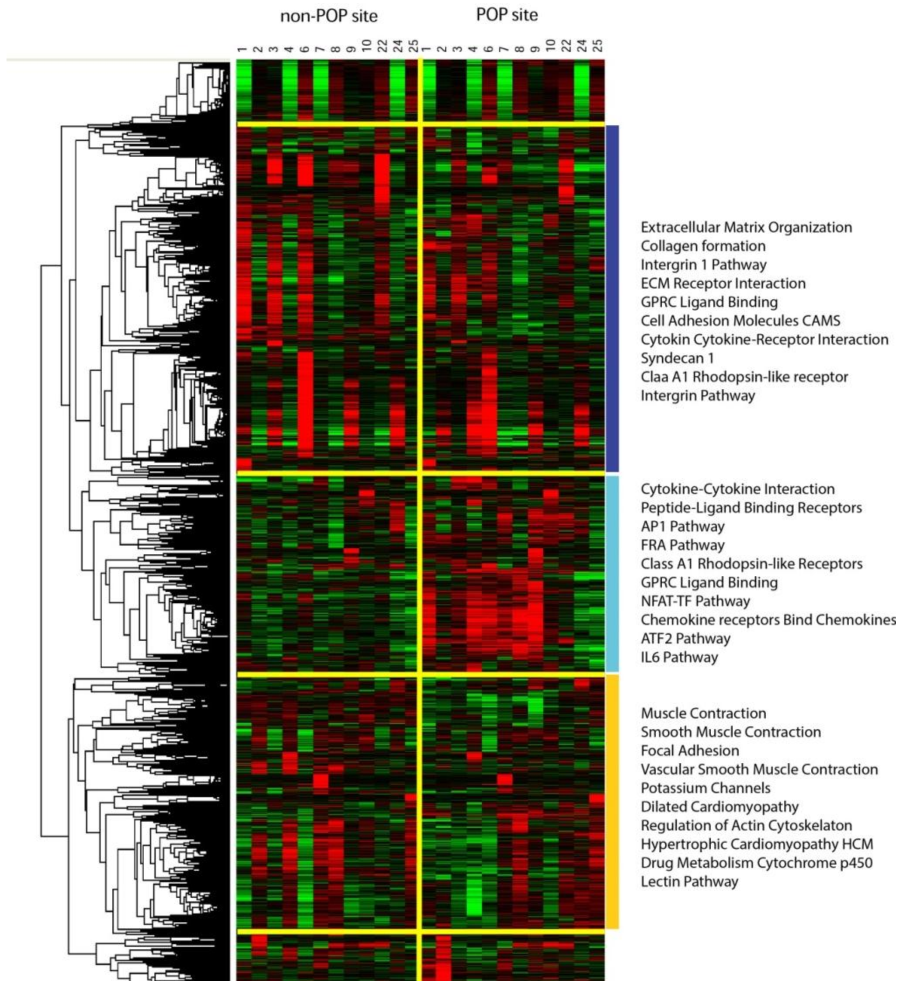
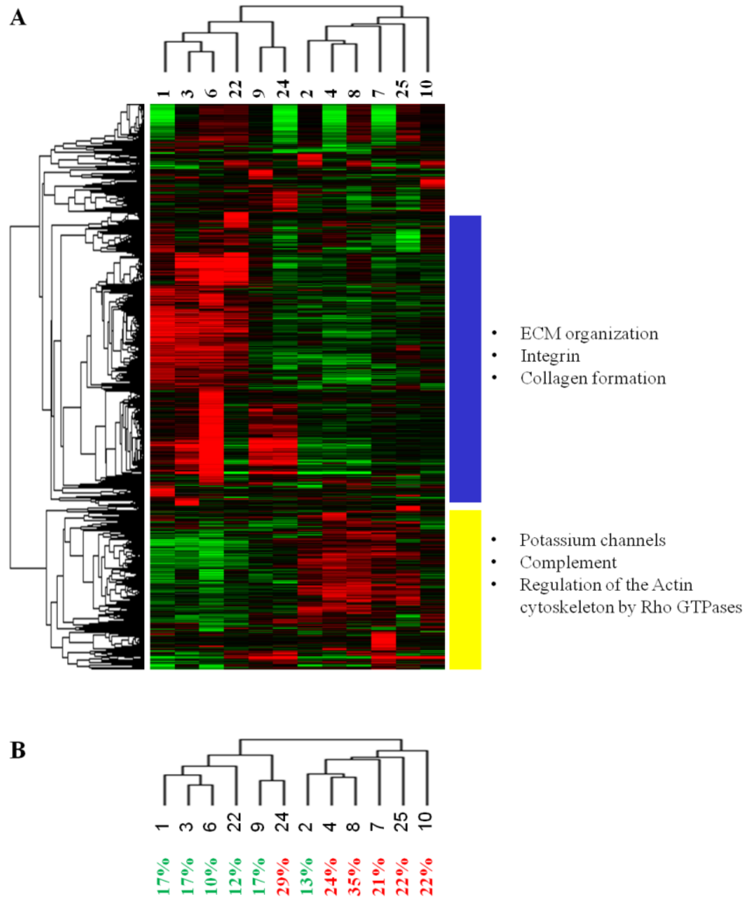


Figure 1. Cluster diagrams of genes that were differentially expressed between precervical non-prolapsed and the prolapsed anterior vaginal wall tissues of 12 premenopausal Caucasian women with POP. Supervised (one-way) hierarchical cluster analysis of gene expression levels was performed. Genes (row), that are increased relatively to the mean are indicated in red, decreased in green and genes that show no difference are black colored. Tissues samples were stratified in precervical non-prolapsed tissue (cervix) and prolapsed anterior vaginal wall tissue. The supervised analysis revealed three clusters of which one cluster was associated with diseased prolapsed tissue (light blue) and two were reflecting inter-individual variance (dark blue and yellow). The 10 most significant pathways are indicated at the right.

Figure 2. Cluster diagrams of genes that were differentially expressed in precervical non-prolapsed tissues between 12 premenopausal Caucasian women. (**A**) The cluster tree dividing the POP patients into two groups is also reflected in the (**B**) percentage of desmin-positive cells found with immunohistochemistry in the non-prolapsed tissues of the same POP patients.



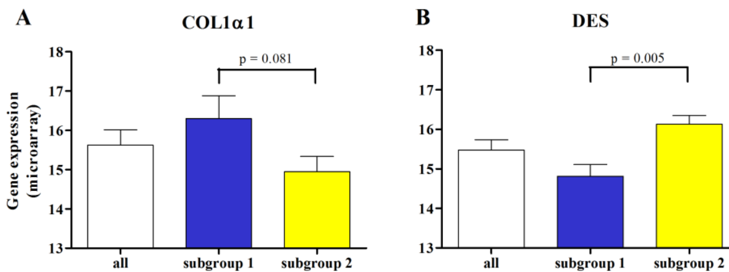


Figure 3. Analysis of the gene expression of collagen I (Col1α1) and desmin (DES) as representative genes for the ECM cluster and muscle cell cluster respectively.

RT PCR

To confirm the microarray results, qPCR based expression analysis was performed for genes representing various processes, *e.g.* IL-6 and CXCR4 for the POP specific cluster; COL1α1, COL3α1 and TNF-α for the ECM subgroup; and ACTA2 and DES for muscle contraction cluster. qPCR confirmed the elevated steady-state mRNA levels for these seven genes as shown in Table 4.

Table 4. Correlation of the microarray results and the quantitative polymerase chain reaction (qPCR). The seven genes represent each cluster shown in Figure 1.

Cluster	Genes	Spearman r	p value
Dark blue			
	COL1α1	0.7687	<0.0001
	COL3α1	0.9139	<0.0001
	TNF-α	0.8113	<0.0001
Light blue			
	IL6	0.913	<0.0001
	CXCR4	0.8009	< 0.0001
Yellow			
	ACTA2	0.7809	<0.0001
	DES	0.9226	<0.0001

Col1α1, α1(I)procollagen; Col3α1, α1(III)procollagen; TNF-α, tumor necrotic factor-α; IL-6, interleukin 6; CXCR4, chemokine receptor type 4; ACTA2, alpha smooth muscle actin; DES, desmin.

DISCUSSION

In this study, disease specific disregulated pathways as well as inter-individual differently regulated pathways were identified. A cluster involved in signal transduction and transcriptional regulation mainly via AP-1 and FRA-related pathways were specifically identified in the prolapsed anterior vaginal wall. Two gene clusters showed variability between patients: an ECM/integrin pathway cluster, and a muscle cell/contraction pathway cluster. The same variability between patients was observed in the precervical non-prolapsed tissues.

With respect to the disease-specific cluster, it appears that the general signal transduction pathways (cytokine-cytokine interaction; peptide ligand binding receptor; class A1rhodopsin like receptor; chemokine receptor; and GPCR ligand binding pathway), together with the transcriptional pathways (AP1; FRA; NFAT-TF; ATF2), are generally involved in inflammatory processes[23-28]. Nevertheless, in our previous histochemical evaluation of the same vaginal wall tissues, we did not find any inflammatory cell infiltration[17]. This phenomenon has been reported before in uterosacral ligaments and round ligaments in women with uterine prolapse[12]. We hypothesize that alternative functions, other than inflammation, exist for these pathways.

The differences in gene expression and pathways between prolapsed and non–prolapsed tissues may reflect an adaptation of the connective tissue to the permanently increased mechanical load on the vaginal wall tissues caused by the prolapse. Interleukin-6 (IL-6, FDR<0.007), for example, is a cytokine secreted by T-cells and macrophages generally to stimulate immune responses. Recent *in vitro* studies have shown that IL-6 is also produced in soft tissues, as excessive mechanical loading on human synovial cells can increase the expression of IL-6[29]. Cytokines and mechanical stretching can also induce smooth muscle de-differentiation and proliferation by the activation of the transcriptional mediators of the AP-1 and FRA pathway. This is initially beneficial to allow tissue adaptation to increasing pressure. Sustained and prolonged exposure to mechanical loading or to stretching, however, can lead to aberrant hyperplasia and hypertrophy and loss of contractility[30,31]. This phenomenon was seen in our previous study, demonstrating a significant increase in the amount of SMCs in the prolapsed site compared to the unaffected site of the same premenopausal patient[17]. Numerous studies investigating mechano-transduction have shown that mechanical loading activates different pathways, affecting the cellular structure and function and causing changes in cell shape, migration, proliferation, contraction and differentiation, in order to adapt to changes in the microenvironment[32-38]. The activated pathways in the prolapsed anterior vaginal wall tissues are all involved in mechano-transduction, indicating that the cells are adapting to the altered micro-environment probably due to the exposure to the overloading that can occur in the prolapsed tissues.

Current literature remains unclear about which components of the connective tissues are affected the most and in which way[39]. The cluster analysis of most dysregulated genes in our study group reveals two clusters of genes reflecting variability between patients: an ECM/integrin pathway cluster (dark blue, Fig.1) and a muscle cell/contraction pathway cluster (yellow, Fig.1). Comparing these two different clusters of genes in the non-prolapsed tissue, they seem to be inversely related to one another, *i.e.* certain patients show up-regulation in the ECM/integrin pathway cluster and down-regulation of SMC pathways, while other patients show the reverse pattern. In women with POP the precervical region which serves as a control region is exposed to only minor changes in mechanical loading and tissue stretching. All tissues need a certain amount of stretching and loading to be able to maintain their structure. We therefore hypothesize that the two different clusters detected may in fact reflect two groups of patients in which one or the other pathway (ECM organization vs. smooth muscle cell contraction) dominates in non-prolapsed tissue in order to adapt to minor changes in the environment. In the ‘ECM group’ the pathways of integrins and cytokines are biomechanically and biochemically activated by strain or load. Integrins mediate the attachment of cells to the ECM and serve as mechanosensors. Such activities can trigger many transcription factors that can activate the gene expression of proteins involved in ECM metabolism – such as collagens, elastin, and metalloproteinases – allowing for rapid and flexible responses to changes in the micro-environment. In the ‘muscle contraction group’ minor changes in the ECM mechanics influence myosin-driven,

actin-mediated contractility of smooth muscle cells, and the modulations of the actin cytoskeleton. The focal adhesion kinase (FAK) pathway is involved in the actin-myosin process and triggers a diverse array of cellular responses by linking to other downstream effectors, including the Rho-family GTPases such as Rho and Rac1and the Ras-MEK-ERK pathway[40,41]. All of these factors contribute to a coordinated cell behaviour in order to maintain tissue homeostasis[42].

We recently reported an increase of desmin-positive cells in prolapsed tissues reflecting the percentage of SMCs in samples from the same study population[17]. Strikingly, when comparing immunohistochemistry data with the current microarray data, we were able to verify that the group of patients that showed an increased percentage of desmin-positive cells were all part of the 'muscle contraction group' identified in this cluster analysis (yellow cluster, Fig.2B).

In the present study, we analyzed 44.000 distinct genes, which is the most widespread microarray analysis possible. We performed an extensive SAM, as well as cluster analysis, increasing the chance to find significant pathways and patterns. We were able to compare these results with our previous study into the morphological features of these tissues. The study set-up with each woman being her own control reduces biases caused by differences between patients. In a previous description of this study group we already showed that the non-prolapsed site of patients did not differ, either histologically or immuno-histochemically from healthy controls[17]. This makes the non-POP site of the patient a true internal control. The study set-up, however, does not allow us to determine whether the differential gene expression was a cause or an effect of prolapse. Actually, only a longitudinal study would be able to answer this question. This is not possible to perform in humans and a good human representative animal model does not yet exist. The apparent small sample size is not an issue in this microarray analysis as the large number of probes (variables), and the relative small number of samples, make the P-value concept and sample size determination, based on classical power considerations, obsolete[12,19,43]. A sample size of 8-15 samples gives near-maximal levels of power and stability[44].

In conclusion, we applied genome-wide gene expression technology to identify molecular mechanisms underlying POP. We found no clear POP related pathway in the prolapsed tissues other than general pathways related to mechanical loading. However, we do provide evidence for inter-individual differences in both control and diseased tissue, reflecting either ECM/integrin or muscle cell/contraction pathways. We therefore hypothesize that women with a predisposition for POP show two different compensatory mechanisms to adapt to physiological changes in mechanical loading. Future research could provide different sets of biomarkers, *i.e.* one related to ECM and another related to cell contraction, differentiation and proliferation. This would help the development of personalized treatments for women with POP.

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