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Title: The preterm cervix reveals a transcriptomic signature in the presence of premature pre-labor rupture of membranes

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Condensation: The transcriptome of the human uterine cervix reveals a signature in the presence of premature pre-labor rupture of fetal membranes.

Short version of title: The transcriptome of the preterm cervix
Abstract

Background: Premature Pre-labor Rupture of Fetal Membranes (PPROM) accounts for 30% of all premature births and is associated with detrimental long-term infant outcomes. Premature cervical remodeling, facilitated by matrix metalloproteinases (MMPs), may trigger rupture at the zone of the fetal membranes overlying the cervix. The similarities and differences underlying cervical remodeling in PPROM and spontaneous preterm labor with intact membranes (PTL) are unexplored. Objectives: We aimed a) to perform the first transcriptomic assessment of the preterm human cervix to identify differences between PPROM and PTL and b) to compare the enzymatic activities of MMP-2 and 9 between PPROM and PTL. Study Design: Cervical biopsies were collected following PTL (n=6) and PPROM (n=5). Biopsies were also collected from reference groups at term labor (TL; n=12) or term not labor (TNL; n=5). The Illumina HT-12 v4.0 BeadChips microarray was utilized and a novel network graph approach determined the specificity of changes between PPROM and PTL. qRT-PCR and Western blotting confirmed the microarray findings. Immunofluorescence was employed for localization studies and gelatin zymography to assess MMP activity. Results: PRAM1, FGD3 and CEACAM3 were significantly higher whereas NDRG2 lower in the PPROM cervix when compared to the cervix in PTL, TL and TNL. PRAM1 and CEACAM3 were localized to immune cells at the cervical stroma and NDRG2 and FGD3 were localized to cervical myofibroblasts. The activity of MMP-9 was higher (1.22±4.403 fold, p<0.05) in the cervix in PPROM compared to PTL. Conclusions: We identified four novel proteins with a potential role in the regulation of cervical remodeling leading to PPROM. Our findings contribute to the studies dissecting the mechanisms underlying PPROM and inspire further investigations towards the development of PPROM therapeutics.
Keywords: cervix, metalloproteinases, microarray, preterm labor, premature rupture of fetal membranes
Introduction

Preterm birth (PTB), defined as birth before 37 completed weeks of gestation, remains the major cause of neonatal morbidity and mortality affecting approximately 1 million pregnancies each year. PTBs are predominantly spontaneous in nature and only 25% are iatrogenic. Spontaneous PTBs (sPTBs) can be the outcome of spontaneous preterm labor with intact membranes (PTL; 45% of all sPTBs) or preterm pre-labor rupture of membranes (PPROM; 30% of all sPTBs). Although PTL is likely to follow PPROM, PTL and PPROM can present as separate entities due to differences in their initiating triggers and the underlying pathways leading to premature cervical remodeling.

The pathophysiology of PPROM has been poorly explored. It is believed that the tensile strength of the fetal membranes can be reduced by premature cervical dilation, which can expose the weakest zone of the fetal membranes to vaginal microorganisms and reduce the underlying tissue support. Indeed, microbial invasion of the amniotic cavity (MIAC) is present in approximately 30–40% of patients with PPROM. It is noteworthy that premature cervical remodeling in the absence of infection can also result in unscheduled rupture of fetal membranes. What triggers these cervical changes in the absence of infection and how these fine-tune the timing of rupture is currently unknown. Genetic factors have been proposed to predispose women to PPROM and a recent systematic review reported that specific polymorphisms were associated with PPROM in blood, amnion and buccal swabs. From these a main regulation axis for PPROM was proposed consisting of pathways regulating hematologic/coagulation function disorder, local inflammation, collagen metabolism and matrix degradation. Notably, pregnant women with Ehlers-Danlos syndrome, an inherited connective tissue disorder resulting from mutations in genes responsible for collagen structure and/or synthesis, have increased risk for PPROM. A proteomic study...
of the human placenta additionally demonstrated an association of PPROM with alterations in structural/cytoskeletal components of cells and impaired regulation of energy metabolism and oxidative stress. 

In light of the detrimental impact of PPROM on long-term infant outcomes, the early and accurate prediction of the condition could allow for timely intervention in order to improve perinatal outcomes and reduce obstetric complications, such as chorioamnionitis, neonatal sepsis or cord prolapse. Assessment of the cervical length and detection of biomarkers in biological fluids of symptomatic women serves to confirm suspected cases of PTL and MIAC-associated PPROM but a test which predicts PPROM before it occurs is yet to be developed.

Understanding the differences and similarities in the underlying pathologies associated with PPROM and PTL will allow new avenues for research and treatment. Herein we hypothesized that different cervical remodeling events facilitate PPROM and PTL. We set out to explore whether these different events would manifest as a PPROM-specific gene signature. To our knowledge this is the first genome-wide approach study utilizing human cervical biopsies to study PPROM and PTL as individual groups.

**Materials and Methods**

**Human cervical biopsies**

Cervical biopsies were collected at the Karolinska Hospital during 2006-2008 following the informed consent and approval of the local Ethics Committee. Biopsies were taken directly (within 30 minutes) after vaginal delivery or caesarean section (CS) transvaginally (at 12
o’clock position) from anterior cervical lip with scissors and tweezers. A total of 28 women
were recruited: 6 undergoing spontaneous preterm labor (PTL), 5 with preterm premature
rupture of membranes (PPROM) followed by labor, 12 undergoing normal term labor (TL)
and 5 who delivered at term prior to the onset of labor (TNL). Preterm delivery was defined
as delivery before the 37th week of gestation. Women in the PTL, PPROM and TL groups
were in active labor and demonstrated a ripe cervix, with dilatation of more than 4 cm. All
except two of these subjects delivered vaginally. One woman in the PTL group delivered by
emergency CS due to breech presentation and one in the TL group due to protracted labor.
PPROM was defined as a rupture of membranes at least one hour before onset of contractions
2. TNL samples were obtained from women undergoing planned CS with unripe cervix. None
of the subjects had clinical signs of infection or chorioamnionitis nor suffered from pre-
eclampsia, diabetes or other systemic disease. There were no significant differences between
the groups of pregnant women with respect to maternal age, parity or previous preterm births.
For clinical data of the recruited subjects consult Table 1 Supplemental.

Sample processing

The samples were processed for RNA and protein extraction or fixed as detailed in
Supplemental Material and Methods 1.

Illumina HT-12 v4.0 BeadChip expression microarray

A total of 23 samples were QC analyzed using the arrayQualityMetrics package in
Bioconductor 21 and no outliers were identified. The samples were split randomly over the
Illumina HT-12 v4.0 BeadChips to minimize any effect of inter-chip variability. The chips
were imaged using a BeadArray Reader and raw data were obtained with Illumina
BeadStudio software. Raw and processed data are available at www.ebi.ac.uk/arrayexpress/ under accession number E-MTAB-5354.

**Microarray analysis**

Fios Genomics Ltd (Bioquarter, Edinburgh, UK) performed the statistical analysis of the array as described in Supplemental Material and Methods 2.

**Network graph analysis**

Normalized expression data generated by microarray analysis were further filtered to include only the genes up- or down-regulated genes (p<0.05, fold-change = any) in at least at 1 out of 6 comparisons in order to eliminate the noise created by genes with conserved expression. That final dataset was used as an input for Biolayout Express3D (BLE) analysis software to create sample-sample and a gene-gene network graphs as previously described and further detailed in Supplemental Material and Methods 3.

**QRT-PCR**

Quantitative RT-PCR (singleplex) was performed to validate the differences identified in the microarray and BLE analysis. The original samples used in the microarray were used for the validation, in addition to 5 new TL samples. Details about the assay are available in Supplemental Material and Methods 4.

**Western blotting and Immunofluorescence**

Western blotting and immunofluorescence were used to quantify and localize PRAM1, FGD3, CEACAM3 and NDRG2 proteins in the cervix as described in Supplemental Material and Methods 5.
**Gelatin Zymography**

A total of 20 µg protein was loaded onto precast 10% Novex® gelatin-containing gels (Thermo Scientific, Wilmington, DE, USA) and separated by electrophoresis. Subsequently, the gels were incubated with Novex® renaturing and Novex® developing buffer according to manufacturers’ protocol (Thermo Scientific, Wilmington, DE, USA). Staining was then performed using the Novex® SimplyBlue SafeStain solution until the sites of membrane degradation by MMP-2 or MMP-9 manifested as bands on the zymographs. Zymography bands were quantified using Adobe Photoshop’s CS6 histogram function.

**Statistics**

Graphpad Prism 6 (La Jolla, CA 92037 USA) was used for the statistical analysis of the qRT-PCR, Western blotting and Zymography data. For qRT-PCR, the thresholds for the gene of interest (GOI) and actin-β (ACTB) were set in the linear phase of the exponential region of the amplification curves. The cycle number at which the PCR signal crossed a set threshold was used to determine relative gene expression. The average comparative cycle threshold (Ct) values for the GOI and ACTB were used to calculate ΔCt and the number was normalized (ΔΔCt) to the PPROM group. ΔΔCt values were used for statistical analysis and data were plotted as fold change (2^(-ΔΔCt)). For Western blotting, the intensity of band fluorescence was analyzed and the readout value for statistical analysis was the raw ratio of fluorescence intensity value of protein of interest (POI) and α-Tubulin (POI: α-Tubulin). For zymography, the readout for statistical analysis was the raw pixel number for each band. All data were initially analyzed for normal distribution using the Kolmogorov-Smirnov test. Western blotting (raw fluorescence ratio) and qRT-PCR (ΔΔCt) data were analyzed with one-way ANOVA Dunnett’s test to compare each group to PPROM. Zymography data (raw pixel
number) were analyzed with one-way ANOVA Tukey’s test. Significance was set at p<0.05. Error bars denote standard error of the mean (SEM).

Results

Microarray identified gene expression differences between PPROM and PTL.

A sample-sample network graph followed by Markov Cluster Algorithm (MCLi=19.3) analysis was generated from normalized microarray data (Figure 1A, B, C) to understand the relationship between samples at a finer level. The proximity of samples implied similarity in genetic signature (Figure 1A) and MCL analysis of the samples identified four clusters (Figure B). When nodes were coloured according to their group status (Figure 1C) it became evident that all 5 TNL samples belonged to MCL cluster i, where they shared cluster membership with 2 PTL samples. Additionally, MCL cluster ii contained 5 out of 7 TL samples, which shared cluster membership with 4 PTL samples. 3 out of 5 PPROM samples formed their own cluster (MCL cluster iii) and 1 PPROM sample clustered with 2 TL samples to form MCL cluster iv. One PPROM sample did not cluster with others, suggesting it did not genetically identify with other samples. Importantly, PPROM and PTL samples did not share cluster membership and 60% of PPROM samples clustered together suggesting a distinct genetic signature specific to the PPROM pathology. Indeed, a strict cut-off revealed that 44 genes were differentially expressed between the PPROM and PTL groups (Figure 1D) out of which 32 were significantly up-regulated and 12 down-regulated (Figure 2A). A list of these genes is shown in Table 1. A heatmap analysis (Figure 2B) allowed for visual identification of the genes with a conserved PPROM-specific high or low expression across all PPROM samples when compared to all other samples (i.e. FGD3, LILRA5, NDRG2, PRAM1, CD300LF, CEACAM3, PPDPF, RNA28S). Significantly changed genes in the
PPROM-PTL comparison were analyzed for enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway membership (Table 2) and Gene Ontology (GO) terms (Table 3). ‘Osteoclast differentiation’ was the only overexpressed KEGG pathway in the PPROM group, when compared to PTL, with 5 significant genes up-regulated and 19 GO terms associated with immunity were enriched.

Pathological gene signature associated with PPROM.

The normalized microarray data for the 30 up- and 9 down-regulated genes in the PPROM-PTL comparison were used as input to generate two gene-gene network graphs, where each node represented a gene. MCL analysis (MCLi = 1.3) was performed to give an unbiased assessment of how the up-regulated (Figure 3A) and down-regulated genes (Figure 3B) clustered. We identified 6 MCL clusters for the up-regulated and 3 for the down-regulated genes (Figure 3C) and the average (mean) gene expression profile for each cluster was examined to detect a PPROM-specific signature (Figure 3D-L). As with the heatmap, we identified the clusters with a high or low averaged expression of genes conserved across all PPROM samples. Analysis of MCL cluster 4 (Figure 3G) and 5 (Figure 3H) revealed that the averaged expression of genes in MCL cluster 4 (STK4, CEACAM3, FGD3) and MCL cluster 5 (PRAM1, MYO1F) was higher in the PPROM samples when compared with PTL, TL and TNL samples. MCL cluster interpretation relied on visual observation and no statistics were applied at that stage. From the down-regulated MCL clusters, MCL 8 showed a low averaged expression for NDRG2 and ACOT13 in the PPROM samples (Figure 3K). None of the other clusters suggested trends worthy of further investigation. From the pool of 7 genes identified (STK4, CEACAM3, FGD3, PRAM1, MYO1F, NDRG2 and ACOT13), statistical significance between PPROM compared to PTL, TL and TNL was reached for CEACAM3 (Figure 4A), PRAM1 (Figure 4D), FGD3 (Figure 4G), and NDRG2 (Figure 4J) as reported by traditional
microarray analysis performed by Fios Genomics, which was further validated with qRT-PCR and Western blotting. Specifically, the mRNA concentration of CEACAM3 (Figure 4B) was 2.17±0.17 fold lower in the PTL group, 1.79±0.12 fold lower in the TL group and 3.97±0.03 fold lower in the TNL group when compared to PPROM. These values for PRAM1 (Figure 4E) were 2.55±0.17 fold for PTL, 1.85±0.35 fold for TL and 4.8±0.1 fold for TNL. The concentration of FGD3 mRNA (Figure 4H) was also 3.34±0.11 fold lower in PTL, 3.29±0.08 fold lower in TL and 2.7±0.18 fold lower in TNL when compared to PPROM. In contrast, the mRNA of NDRG2 was in lower concentration in the PPROM cervix when compared to PTL (-4.16±0.57), TL (-3.62±0.63) and TNL (-4.0±0.42) groups (Figure 4K). These changes were confirmed in the protein level. CEACAM3 (Figure 4C) and FGD3 (Figure 4I) were significantly higher in the PPROM group when compared to the other groups. CEACAM3 was 2.57±0.06 fold lower in the PTL cervix, 2.65±0.07 fold lower in the TL cervix and 2.77±0.07 fold lower in the TNL cervix. These values for FGD3 were 1.88±0.09 for PTL, 2.02±0.18 for TL and 2.58±0.24 for TNL. PRAM1 (Figure 4F) was significantly higher in PPROM compared to PTL (2.97±0.15) and TL (3.5±0.08) but not TNL. NDRG2 (Figure 4L) protein was significantly lower in the PPROM group when compared to PTL (-6.78±0.5) and TL (7.0±0.54) but not TNL group.

PPROM-specific markers were localized to immune cells and vascular myofibroblasts. We explored the localization of PRAM1, CEACAM3, FGD3 and NDRG2 within the cervical tissue. Although the literature suggests that PRAM1 is predominantly expressed in granulocytes it did not co-localize with the established granulocyte membrane marker CEACAM3 (Figure 5D). Instead, PRAM1 was localized to the cytoplasm of a subset of immune CD45 positive cells (Figure 5H) resident in the cervical stroma (Figure 5C, F, I, M). Notably, all PRAM1 positive cells stained for CD45, suggesting that these are immune
cells. We confirmed that PRAM1 positive cells were neither macrophages (Figure 5K) nor neutrophils (Figure 5O). Positive, albeit marginal, NDRG2 staining was evident in the nuclei of the endocervical epithelial cells (Figure 6C), which were positive for pan-cytokeratin (Figure 6B). Strong NDRG2 staining (Figure 6G) was detected in the cytoplasm of endocervical glands (Figure 6F) and myofibroblasts surrounding blood vessels in the cervical stroma (Figure 6D). A double staining with Von Willebrand factor (vWF), a marker expressed in the endothelial cells of the vasculature, confirmed the blood vessel status (Figure 6J). FDG3 was also expressed in the cytoplasm of myofibroblasts (Figure 6P) surrounding vWF positive blood vessels (Figure 6N). We found that NDRG2 and FGD3 shared the same localization within myofibroblasts (Figure 6T).

GO terms for PRAM1, CEACAM3, FGD3 and NDRG2.

All GO enriched terms for the PPROM-specific markers can be found in Table 4.

The activity of Matrix Metalloproteinase 9 (MMP-9) was higher in the PPROM cervix.

Gelatin zymography revealed that the activity of MMP-9 (Figure 7A), but not MMP-2 (Figure 7B), was significantly higher in the PPROM cervix. Specifically, the activity of MMP-9 was higher 1.22±4.403 fold in PPROM when compared to PTL (p<0.05), 1.25±4.328 fold compared to TL (p<0.05) and 1.57±6.600 fold compared to TNL (p<0.001) (Figure 7A).

Comment

This is the first transcriptomic study of the preterm human cervix, which examined PTL and PPROM as two separate pathologies and compared gene expression in the two groups. According to a recent systematic review, only 4% of all transcriptomic studies in term and
preterm human pregnancies have utilized cervical tissue and, strikingly, none of these has examined PPROM individually \(^{24}\). Several genetic polymorphisms associated with PPROM have been identified in the placenta, membranes and maternal/fetal blood [reviewed in \(^{6}\)] and smaller-scale studies also demonstrated the presence of PPROM-associated inflammatory markers in the amniotic fluid \(^{25,26}\), fetal membranes \(^{27-29}\) and maternal serum \(^{30}\). All these studies combined with recent proteomic \(^{16}\) and epigenetic \(^{31}\) reports of a PPROM signature in the placenta and maternal blood have established the hypothesis that PPROM and PTL may have distinct underlying pathologies. It remained to be deduced whether a PPROM signature would be detected in the cervix. We hypothesized that the cervix might initiate rupture of the fetal membranes at their contact site through PPROM-specific cervical remodeling events. Our findings support this hypothesis and demonstrate that PPROM is associated with expression of key proteins, which may facilitate the organization of the cervical extracellular matrix (ECM) and indirectly accelerate membranes rupture.

The GO terms for the overexpressed genes in PPROM, when compared to PTL (Table 3), were predominantly related to immunity, for example ‘immune system processes’, ‘immunity mediated by myeloid leukocytes’ and ‘immunity mediated by neutrophils’. This is perhaps not surprising because physiological cervical remodeling is accompanied by infiltration of leukocyte subpopulations and neutrophils, which work to achieve the rigidity of the cervix \(^{32,33}\). In line with our findings, a study in the mouse cervix proved that the overarching mechanism underlying cervical remodeling-associated immune cell influx is similar in term and preterm parturition and only marginal differences occur whereby the mediators and effector cells involved may differ \(^{34}\). Our findings provide the first evidence to suggest that the immunity modulators employed to mediate cervical remodeling may be additionally different between the preterm subgroups PPROM and PTL. Immune modulators stimulate
immune and other cells in the cervical stroma to produce cytokines and MMPs to degrade the ECM as part of the remodeling process. MMP-2 and MMP-9 are gelatinases both capable of degrading collagens type I and III, the main constituents of the cervical ECM. MMP-2 and MMP-9 concentration is reportedly elevated in the amniotic fluid of PPROM pregnancies. Both MMP-2 and MMP-9 are produced by human cervical fibroblasts and MMP-9 by vascular fibroblasts and neutrophil granulocytes. To contribute to the notion that the facilitators of ECM degradation may differ between PPROM and PTL or TL in the cervix, we performed an assay to assess MMP-2 and MMP-9 activity. Indeed, the activity of MMP-9 was increased solely in PPROM.

Out of the 44 differentially expressed genes between the PPROM and PTL groups identified with traditional array analysis, our network graph analysis followed by validation, brought forward 4 key proteins that were over- or under-expressed only in the PPROM cervix. Although these proteins are novel to the parturition field, there is some evidence to support that they might be involved in the activation of a pathological cascade, which delivers a “rupture” signal to the weakest zone of fetal membranes overlying the cervix. Specifically, NDRG2 may be switched off in cervical myofibroblasts to promote the production of MMP-9 and accelerate a PPROM-specific remodeling process. Down-regulation of NDRG2 has been previously associated with an increase in the gelatinolytic activities of MMP-2 and MMP-9. In adenocarcinomic human alveolar basal epithelial cell line and more reports have shown direct inhibition of MMP-9 activity by NDRG2. In support of this hypothesis, cathepsin D (CTSD), which is also down-regulated in PPROM compared to PTL (Table 1) and shares GO terms with NDRG2 (Table 4), is additionally a negative regulator of MMP-2 and MMP-9 in endometriotic lesions. CEACAM3, a membrane granulocyte protein involved in neutrophil activation, and FGD3 may also work together towards enhancement of MMP-
9 activity in PPROM. It is not unlikely that aberrant infiltrating neutrophil-granulocytes
overexpress CEACAM3 to promote their activation and stimulate MMP-9 secretion. In
support of this notion, genes that share GO terms with CEACAM3 (Table 4) have also been
associated with MMP actions. For example, the osteoclast-associated markers OSCAR and
SIRPA and TREM-1 have all been implicated in MMP-9-mediated responses 49-52.
CEACAM3 shares cluster membership with FGD3 (Figure 3C), suggesting similar regulation
in gene expression, which itself may imply similar functions. FGD3 may control MMP-9
activity in the PPROM cervix by promoting filopodia formation on the plasma membranes of
myofibroblasts 53. It is well established that proteins of the same family with FGD3 organize
such formations on plasma membranes to release MMPs and in turn induce degradation of
the surrounding stroma 54,55. Remarkably, blockade of filopodia formation by flavoinoids has
been shown to decrease the release of MMP-2 in cancer 56. Electron microscopy studies could
help investigate filopodial formations on cells in PPROM. PRAM1, which shared GO terms
with FGD3 (Table 4), is thought to be predominantly expressed in granulocyte-neutrophils
where it acts as an adaptor protein critical for select integrin functions 57. Integrins are
transmembrane receptors that bridge cell-ECM interactions and activate MMPs 58. A
proteolytic role for integrins has been described in the initiation of labor, whereby they
regulate release of MMP-9 in human fetal membranes 59. Although we did not detect PRAM1
in elastase positive neutrophils or in CEACAM3 positive granulocytes (Figure 5), the
likelihood of PRAM1 regulating integrin functions in the cytoplasm of an alternative immune
cell population in the cervix deserves addressing.

Employing a genome-wide approach has identified key genes associated with PPROM, and
provided an insight into a potential mechanism regulating physiological cervical remodeling.
Analysis of the two top clusters of the up-regulated genes in PPROM (Figure 3D, E)
demonstrated that the genes within these clusters were overexpressed both in PPROM and, surprisingly, in TL. The first overexpressed cluster contained various genes involved in bone marrow-derived cell migration (ARHGAP9, FGR, NFE2) and SLC43A2, the gene coding an essential transporter of Branched Chain Amino Acids (BCAAs). We propose a new mechanism to contribute to cervical remodeling in TL and PPROM, whereby the increase of BCAAs in the cervix triggers the recruitment of bone marrow-derived cells in order to stimulate MMP-induced degradation. Consistent with our hypothesis, MMP-2 and MMP-9 increase in response to exogenous BCAAs in the hippocampus of rats and bone marrow-derived cells have been also shown to secrete MMPs. A similar mechanism for cervical remodeling in TL and PPROM involving bone marrow recruited cells can be further evidenced by KEGG analysis, where ‘Osteoclast differentiation’ pathway is enriched not only in PPROM-PTL comparison (Table 2) but also in TL-PTL (Table 3 Supplemental). Osteoclasts are bone marrow-derived cells traditionally involved in the degradation of bone matrix and have been described to secrete MMP-2 and MMP-9. Further work is required to prove whether bone marrow-derived osteoclasts or osteoclast-like cells mediate MMPs-induced degradation of ECM as part of physiological cervical remodeling cascade. It is noteworthy that only 16 genes were differentially expressed between PPROM and TL, in contrast to 1285 genes in the TNL-TL comparison. The notion that PPROM and TL might share some similar pathways for cervical remodeling was additionally supported by the sample-sample network graph (Figure 1C). In that graph PPROM and TL samples belonged to the same ‘loose’ local structure whereas the TNL samples belong to a separate ‘tight’ structure.

Our study could benefit from a larger sample size but human cervical biopsies are extremely hard to obtain especially in relation to preterm delivery, which explains why so few studies
are conducted on the human preterm cervix. Moreover, the biopsies were collected postpartum and thus postpartum repair mechanism might be reflected in our results. However, it is not practically and ethically possible to obtain cervical biopsies during vaginal delivery and the material used in our study was collected within 30 minutes after delivery. Animal research, for example CRISPR experiments could be useful in future studies, to identify the phenotype associated with knock out or knock in of the genes we suggest are important.

In summary, we have, for the first time identified a gene expression signature involved with PPROM. It is tempting to hypothesize that the PPROM-specific proteins identified herein act as contributors in a pathway whereby MMP-9 facilitates ECM degradation in the cervix to signal a ‘rupture’ message to the overlying membranes. Our work supports the growing body of evidence suggesting that premature labor is a multifactorial disorder with different pathways involved for PPROM and PTL.
Acknowledgments

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factors associated with spontaneous preterm birth and pre-labor preterm rupture of

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Tables

Table 1: List of up and down-regulated genes

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Footnote Table 1: Adj.P.Val: at the adjusted p-value < 0.01, FC: fold change >= 2

Table 2: KEGG pathway enrichment analysis of the up and down-regulated genes that mapped to significant features at adjusted p<0.05.

<table>
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<tr>
<th>Name of KEGG pathway</th>
<th>Pvalue</th>
<th>Genes</th>
<th>No. Sig. Genes % Sig. Genes</th>
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<td>LILRA2, LILRA5, LILRA6, OSCAR, SIRPA</td>
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Table 3: GO term enrichment analysis of the up and down-regulated genes that mapped to significant features at adjusted p<0.001

<table>
<thead>
<tr>
<th>Rank</th>
<th>GO Term</th>
<th>p-value</th>
<th>Gene 1</th>
<th>Log2 Fold Change</th>
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<tbody>
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<td>Retinol metabolism</td>
<td>2.03E-02</td>
<td>PKM</td>
<td>1.8</td>
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<td>Type II diabetes mellitus</td>
<td>2.08E-02</td>
<td>PKM</td>
<td>1.6</td>
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<tr>
<td>4</td>
<td>Glycolysis / Gluconeogenesis</td>
<td>3.20E-02</td>
<td>PKM</td>
<td>1.2</td>
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<tr>
<td>5</td>
<td>Central carbon metabolism in cancer</td>
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<td>6</td>
<td>Glucagon signaling pathway</td>
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</table>

Table 4: Report of the GO terms containing the features PRAM1, FGD3, CEACAM3 and NDRG2 amongst other genes that mapped to significant features at adjusted p<0.01.
Footnote Table 4: BP: Biological Process, MF: Molecular Function, CC: Cellular Component
Figure legends

Figure 1: Sample-sample network graph of all samples used for the microarray and the comparisons performed between the groups. A 2D representation of sample clustering in a 3D graph. Each node represents a different sample and edges are coloured to reflect the Pearson correlation that they represent. Red and blue edges denote high correlation and low correlation respectively. The same graph is coloured by A. no cluster (r=0.91), B. unbiased MCL cluster number (MCL 19.3) C. group status. D: Table shows all the comparisons performed between groups and the number of significant array features at adjusted p-value < 0.01 and fold change >= 2. TL = Term Labor (n=7), TNL = Term Non-Labor (n=5), PTL=Preterm Labor (n=6), PPROM=Preterm Premature Rupture of Membranes (n=5).

Figure 2: PPROM vs PTL comparison. A. Volcano plot and B. heatmap showing the 30 features significant up-regulated (red dots) and 9 down-regulated (blue dots) at adjusted p-value < 0.01 and fold change >= 2 in the PPROM group compared to PTL group. A heatmap shows how genes and samples cluster based on similar expression levels. The bars at the top indicate the sample group (dark green = TNL, dark blue = PTL, light green = TL, light blue = PPROM). Normalized expression values are indicated on a color scale with red denoting high expression and blue low expression.

Figure 3: Probe-probe network cluster analysis. Probe-probe network graph of the up-regulated (A) and down-regulated (B) genes in the PPROM-PTL comparison. Each node represents a gene and nodes are coloured according to membership of different MCL (MCLi = 1.3) clusters. C: The genes belonging to each cluster are shown in the MCL gene clusters table. The Pareto scaled graphs show the mean expression profiles of MCL clusters 1 (D), 2
across all samples (n=23), including the samples in the TL and TNL groups. Samples are plotted on the x-axes. Genes with similar expression pattern across all samples are members of the same cluster. Each bar represents the average expression of all genes that cluster together in that sample. The error bar for each sample denotes the SD extrapolated from the expression of all cluster genes in that sample.

PPROM n=5, PTL n=6, TL n=7, TNL n=5.

Figure 4: Validation of microarray analysis. A, D, G, J: Tables show the fold-changes (FC) and adjusted p values (Adj.P.Val) across all comparisons for the 4 selected genes CEACAM3, PRAM1, FGD3 and NDRG2 as reported by FIOS genomics statistical analysis. qRT-PCR validated that CEACAM3 (B), PRAM1 (E) and FGD3 (H) were up-regulated, and NDRG2 (K) down-regulated in the PPROM group when compared to all other groups. Data analyzed using one-way ANOVA Dunnett’s test. qRT-PCR samples: PPROM n=5, PTL n=6, TL n=12, TNL n=5. Western blotting analysis confirmed that CEACAM3 (C) and FGD3 (I) were in higher concentration in the PPROM cervix compared to all other groups. PRAM1 (F) and NDRG2 (L) changes were also significant between PPROM and PTL/TL but not in TNL. Data analyzed using one-way ANOVA Dunnett’s test. Western blotting samples: PPROM n=4, PTL n=4, TL n=4, TNL n=4. Error bars denote ±SEM. *p<0.05, **p <0.01, ***p<0.001.

Figure 5: Localization of PRAM1 and CEACAM3 in the PPROM human cervix. PRAM1 and CEACAM3 positive cells were identified at the cervical stroma. PRAM1 was localized to the cytoplasm and CEACAM3 to the membrane of cells. CEACAM3 (B) and PRAM1 (C) did not co-localize (D). Double staining for PRAM1 (F) and CD45 (G) identified double positive population (H). PRAM1 cells (I), did not co-localize (K) with the
macrophage marker CD68 (J). PRAM1 cells (M) did not co-localize (O) with neutrophil

Elastase (N). All scale bars 50 µm. Images representative of n=4.

Figure 6: Localization of FGD3 and NDRG2 in the human cervix. Marginal NDRG2

staining (C) was detected to the nuclei of endocervical epithelial cells stained positive for

AE1/AE3 (D). D: NDRG2 staining was evidently stronger in cells surrounding blood vessels

(indicated with asterisks). A co-staining for vWF (J; an endothelial cell marker) and NDRG2

(K) confirmed that NDRG2 is localized to the cytoplasm of myofibroblasts surrounding

blood vessels (L). NDRG2 was also localized to the cytoplasm of endocervical glandular
cells (G) as was evident by co-localization (H) with AE1/AE3 (F). FGD3 was expressed

solely in the cytoplasm of myofibroblasts (O) and co-localized with NDRG2 (T). Scale bars

50 µm/ 100 µm as shown in each picture. Images representative of n=4. A-L: PTL cervix, M-
P: PPROM cervix, Q-T: TL cervix. vWF: Von Willebrand factor, AE1/AE3: Pan

Cytokeratin.

Figure 7: MMP-2 and MMP-9 activity in the human cervix. Gelatin zymography was

performed on protein extracted from the cervix of women with PPROM (n=4), PTL (n=4),

TL (n=4) and TNL (n=4). A: The activity of MMP-9 (82 kDa) was significantly higher in the

PPROM cervix when compared to the other groups (*p=0.05, ***p=0.001 comparison). B:

The activity of MMP-2 was similar in PPROM, PTL and TL but significantly lower in TNL

when compared to the other groups (****p<0.0001). Data analyzed using one-way ANOVA

Tukey’s test.
A

B

C

D

<table>
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<tr>
<th>Comparison</th>
<th>Significant genes</th>
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