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

Sofia Makieva, PhD, Aurelija Dubicke, PhD, MD, Sara F. Rinaldi, PhD, Emma Fransson, PhD, Gunvor Ekman-Ordeberg, PhD, MD, Jane E. Norman, MD

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

3

4 Sofia Makieva, PhD ^{1*}, Aurelija Dubicke, PhD, MD ^{2*}, Sara F Rinaldi, PhD ¹, Emma
5 Fransson, PhD ², Gunvor Ekman-Ordeberg, PhD, MD ², Jane E Norman, MD ¹
6 Edinburgh, UK

7 Stockholm, Sweden

8

9 ¹Tommy's Centre for Maternal and Fetal Health, MRC Centre for Reproductive Health,
10 EH16 4TJ Edinburgh UK

11 ²Department of Women's and Children's Health, Karolinska Institute, 17176 Stockholm,
12 Sweden

13 * Authors contributed equally

14

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19 **Corresponding author's details**

20 Name: Sofia Makieva

21 Address: 47 Little France Crescent, QMRI, EH16 4TJ, Edinburgh, UK

22 Telephone: +44 0131 226613 Email: makievasofia@gmail.com

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28 presence of premature pre-labor rupture of fetal membranes.

29 **Short version of title:** The transcriptome of the preterm cervix

30

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31 **Abstract**

32 **Background:** Premature Pre-labor Rupture of Fetal Membranes (PPROM) accounts for 30%
33 of all premature births and is associated with detrimental long-term infant outcomes.
34 Premature cervical remodeling, facilitated by matrix metalloproteinases (MMPs), may trigger
35 rupture at the zone of the fetal membranes overlying the cervix. The similarities and
36 differences underlying cervical remodeling in PPRM and spontaneous preterm labor with
37 intact membranes (PTL) are unexplored. **Objectives:** We aimed a) to perform the first
38 transcriptomic assessment of the preterm human cervix to identify differences between
39 PPRM and PTL and b) to compare the enzymatic activities of MMP-2 and 9 between
40 PPRM and PTL. **Study Design:** Cervical biopsies were collected following PTL (n=6) and
41 PPRM (n=5). Biopsies were also collected from reference groups at term labor (TL; n=12)
42 or term not labor (TNL; n=5). The Illumina HT-12 v4.0 BeadChips microarray was utilized
43 and a novel network graph approach determined the specificity of changes between PPRM
44 and PTL. qRT-PCR and Western blotting confirmed the microarray findings.
45 Immunofluorescence was employed for localization studies and gelatin zymography to assess
46 MMP activity. **Results:** PRAM1, FGD3 and CEACAM3 were significantly higher whereas
47 NDRG2 lower in the PPRM cervix when compared to the cervix in PTL, TL and TNL.
48 PRAM1 and CEACAM3 were localized to immune cells at the cervical stroma and NDRG2
49 and FGD3 were localized to cervical myofibroblasts. The activity of MMP-9 was higher
50 (1.22±4.403 fold, p<0.05) in the cervix in PPRM compared to PTL. **Conclusions:** We
51 identified four novel proteins with a potential role in the regulation of cervical remodeling
52 leading to PPRM. Our findings contribute to the studies dissecting the mechanisms
53 underlying PPRM and inspire further investigations towards the development of PPRM
54 therapeutics.

55 **Keywords:** cervix, metalloproteinases, microarray, preterm labor, premature rupture of fetal

56 membranes

57

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58 Introduction

59

60 Preterm birth (PTB), defined as birth before 37 completed weeks of gestation, remains the
61 major cause of neonatal morbidity and mortality affecting approximately 1 million
62 pregnancies each year ¹. PTBs are predominantly spontaneous in nature and only 25% are
63 iatrogenic ². Spontaneous PTBs (sPTBs) can be the outcome of spontaneous preterm labor
64 with intact membranes (PTL; 45% of all sPTBs) or preterm pre-labor rupture of membranes
65 (PPROM; 30% of all sPTBs) ². Although PTL is likely to follow PPRM, PTL and PPRM
66 can present as separate entities due to differences in their initiating triggers and the
67 underlying pathways leading to premature cervical remodeling ³.

68

69 The pathophysiology of PPRM has been poorly explored. It is believed that the tensile
70 strength of the fetal membranes can be reduced by premature cervical dilation, which can
71 expose the weakest zone of the fetal membranes to vaginal microorganisms and reduce the
72 underlying tissue support ⁴. Indeed, microbial invasion of the amniotic cavity (MIAC) is
73 present in approximately 30–40% of patients with PPRM ⁵. It is noteworthy that premature
74 cervical remodeling in the absence of infection can also result in unscheduled rupture of fetal
75 membranes. What triggers these cervical changes in the absence of infection and how these
76 fine-tune the timing of rupture is currently unknown. Genetic factors have been proposed to
77 predispose women to PPRM and a recent systematic review ⁶ reported that specific
78 polymorphisms were associated with PPRM in blood ⁷⁻⁹, amnion ^{10, 11} and buccal swabs ^{12,}
79 ¹³. From these a main regulation axis for PPRM was proposed consisting of pathways
80 regulating hematologic/coagulation function disorder, local inflammation, collagen
81 metabolism and matrix degradation. Notably, pregnant women with Ehlers-Danlos syndrome,
82 an inherited connective tissue disorder resulting from mutations in genes responsible for
83 collagen structure and/or synthesis, have increased risk for PPRM ^{14, 15}. A proteomic study

84 of the human placenta additionally demonstrated an association of PPRM with alterations
85 in structural/cytoskeletal components of cells and impaired regulation of energy metabolism
86 and oxidative stress¹⁶.

87

88 In light of the detrimental impact of PPRM on long-term infant outcomes¹⁷, the early and
89 accurate prediction of the condition could allow for timely intervention in order to improve
90 perinatal outcomes and reduce obstetric complications, such as chorioamnionitis, neonatal
91 sepsis or cord prolapse. Assessment of the cervical length and detection of biomarkers in
92 biological fluids of symptomatic women serves to confirm suspected cases of PTL and
93 MIAC-associated PPRM^{18,19} but a test which predicts PPRM before it occurs is yet to be
94 developed.

95

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

102

103 **Materials and Methods**

104

105 ***Human cervical biopsies***

106 Cervical biopsies were collected at the Karolinska Hospital during 2006-2008 following the
107 informed consent and approval of the local Ethics Committee. Biopsies were taken directly
108 (within 30 minutes) after vaginal delivery or caesarean section (CS) transvaginally (at 12

109 o'clock position) from anterior cervical lip with scissors and tweezers. A total of 28 women
110 were recruited: 6 undergoing spontaneous preterm labor (PTL), 5 with preterm premature
111 rupture of membranes (PPROM) followed by labor, 12 undergoing normal term labor (TL)
112 and 5 who delivered at term prior to the onset of labor (TNL). Preterm delivery was defined
113 as delivery before the 37th week of gestation. Women in the PTL, PPPROM and TL groups
114 were in active labor and demonstrated a ripe cervix, with dilatation of more than 4 cm. All
115 except two of these subjects delivered vaginally. One woman in the PTL group delivered by
116 emergency CS due to breech presentation and one in the TL group due to protracted labor.
117 PPRM was defined as a rupture of membranes at least one hour before onset of contractions
118 ². TNL samples were obtained from women undergoing planned CS with unripe cervix. None
119 of the subjects had clinical signs of infection or chorioamnionitis nor suffered from pre-
120 eclampsia, diabetes or other systemic disease. There were no significant differences between
121 the groups of pregnant women with respect to maternal age, parity or previous preterm births.
122 For clinical data of the recruited subjects consult Table 1 Supplemental.

123

124 *Sample processing*

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

127

128 *Illumina HT-12 v4.0 BeadChip expression microarray*

129 A total of 23 samples were QC analyzed using the arrayQualityMetrics package in
130 Bioconductor ²¹ and no outliers were identified. The samples were split randomly over the
131 Illumina HT-12 v4.0 BeadChips to minimize any effect of inter-chip variability. The chips
132 were imaged using a BeadArray Reader and raw data were obtained with Illumina

133 BeadStudio software. Raw and processed data are available at www.ebi.ac.uk/arrayexpress/
134 under accession number E-MTAB-5354.

135 *Microarray analysis*

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

138

139 *Network graph analysis*

140 Normalized expression data generated by microarray analysis were further filtered to include
141 only the genes up- or down-regulated genes ($p < 0.05$, fold-change = any) in at least at 1 out of
142 6 comparisons in order to eliminate the noise created by genes with conserved expression.
143 That final dataset was used as an input for Biolayout Express3D (BLE) analysis software to
144 create sample-sample and a gene-gene network graphs as previously described^{22, 23} and
145 further detailed in Supplemental Material and Methods 3.

146

147 *QRT-PCR*

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

152

153 *Western blotting and Immunofluorescence*

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

157

158 Gelatin Zymography

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

166

167 Statistics

168 Graphpad Prism 6 (La Jolla, CA 92037 USA) was used for the statistical analysis of the qRT-
169 PCR, Western blotting and Zymography data. For qRT-PCR, the thresholds for the gene of
170 interest (GOI) and actin-β (ACTB) were set in the linear phase of the exponential region of
171 the amplification curves. The cycle number at which the PCR signal crossed a set threshold
172 was used to determine relative gene expression. The average comparative cycle threshold
173 (Ct) values for the GOI and ACTB were used to calculate ΔCt and the number was
174 normalized ($\Delta\Delta\text{Ct}$) to the PPROM group. $\Delta\Delta\text{Ct}$ values were used for statistical analysis and
175 data were plotted as fold change ($2^{(-\Delta\Delta\text{Ct})}$). For Western blotting, the intensity of band
176 fluorescence was analyzed and the readout value for statistical analysis was the raw ratio of
177 fluorescence intensity value of protein of interest (POI) and α -Tubulin (POI: α -Tubulin). For
178 zymography, the readout for statistical analysis was the raw pixel number for each band. All
179 data were initially analyzed for normal distribution using the Kolmogorov-Smirnov test.
180 Western blotting (raw fluorescence ratio) and qRT-PCR ($\Delta\Delta\text{Ct}$) data were analyzed with one-
181 way ANOVA Dunnett's test to compare each group to PPROM. Zymography data (raw pixel

182 number) were analyzed with one-way ANOVA Tukey's test. Significance was set at $p < 0.05$.
183 Error bars denote standard error of the mean (SEM).

184

185 **Results**

186

187 **Microarray identified gene expression differences between PPRM and PTL.**

188 A sample-sample network graph followed by Markov Cluster Algorithm (MCL τ =19.3)
189 analysis was generated from normalized microarray data (**Figure 1A, B, C**) to understand the
190 relationship between samples at a finer level. The proximity of samples implied similarity in
191 genetic signature (**Figure 1A**) and MCL analysis of the samples identified four clusters
192 (**Figure B**). When nodes were coloured according to their group status (**Figure 1C**) it became
193 evident that all 5 TNL samples belonged to MCL cluster i, where they shared cluster
194 membership with 2 PTL samples. Additionally, MCL cluster ii contained 5 out of 7 TL
195 samples, which shared cluster membership with 4 PTL samples. 3 out of 5 PPRM samples
196 formed their own cluster (MCL cluster iii) and 1 PPRM sample clustered with 2 TL
197 samples to form MCL cluster iv. One PPRM sample did not cluster with others, suggesting
198 it did not genetically identify with other samples. Importantly, PPRM and PTL samples did
199 not share cluster membership and 60% of PPRM samples clustered together suggesting a
200 distinct genetic signature specific to the PPRM pathology. Indeed, a strict cut-off revealed
201 that 44 genes were differentially expressed between the PPRM and PTL groups (**Figure**
202 **1D**) out of which 32 were significantly up-regulated and 12 down-regulated (**Figure 2A**). A
203 list of these genes is shown in **Table 1**. A heatmap analysis (**Figure 2B**) allowed for visual
204 identification of the genes with a conserved PPRM-specific high or low expression across
205 all PPRM samples when compared to all other samples (i.e. *FGD3*, *LILRA5*, *NDRG2*,
206 *PRAMI*, *CD300LF*, *CEACAM3*, *PPDPF*, *RNA28S*). Significantly changed genes in the

207 PPRM-PTL comparison were analyzed for enrichment of Kyoto Encyclopedia of Genes
208 and Genomes (KEGG) pathway membership (**Table 2**) and Gene Ontology (GO) terms
209 (**Table 3**). ‘Osteoclast differentiation’ was the only overexpressed KEGG pathway in the
210 PPRM group, when compared to PTL, with 5 significant genes up-regulated and 19 GO
211 terms associated with immunity were enriched.

212

213 **Pathological gene signature associated with PPRM.**

214 The normalized microarray data for the 30 up- and 9 down- regulated genes in the PPRM-
215 PTL comparison were used as input to generate two gene-gene network graphs, where each
216 node represented a gene. MCL analysis (MCL_i =1.3) was performed to give an unbiased
217 assessment of how the up- regulated (**Figure 3A**) and down-regulated genes (**Figure 3B**)
218 clustered. We identified 6 MCL clusters for the up- and 3 for the down-regulated genes
219 (**Figure 3C**) and the average (mean) gene expression profile for each cluster was examined to
220 detect a PPRM-specific signature (**Figure 3D-L**). As with the heatmap, we identified the
221 clusters with a high or low averaged expression of genes conserved across all PPRM
222 samples. Analysis of MCL cluster 4 (**Figure 3G**) and 5 (**Figure 3H**) revealed that the
223 averaged expression of genes in MCL cluster 4 (*STK4*, *CEACAM3*, *FGD3*) and MCL cluster
224 5 (*PRAMI*, *MYO1F*) was higher in the PPRM samples when compared with PTL, TL and
225 TNL samples. MCL cluster interpretation relied on visual observation and no statistics were
226 applied at that stage. From the down-regulated MCL clusters, MCL 8 showed a low averaged
227 expression for *NDRG2* and *ACOT13* in the PPRM samples (**Figure 3K**). None of the other
228 clusters suggested trends worthy of further investigation. From the pool of 7 genes identified
229 (*STK4*, *CEACAM3*, *FGD3*, *PRAMI*, *MYO1F*, *NDRG2* and *ACOT13*), statistical significance
230 between PPRM compared to PTL, TL and TNL was reached for *CEACAM3* (**Figure 4A**),
231 *PRAMI* (**Figure 4D**), *FGD3* (**Figure 4G**), and *NDRG2* (**Figure 4J**) as reported by traditional

232 microarray analysis performed by Fios Genomics, which was further validated with qRT-
233 PCR and Western blotting. Specifically, the mRNA concentration of CEACAM3 (**Figure**
234 **4B**) was 2.17 ± 0.17 fold lower in the PTL group, 1.79 ± 0.12 fold lower in the TL group and
235 3.97 ± 0.03 fold lower in the TNL group when compared to PPRM. These values for
236 PRAM1 (**Figure 4E**) were 2.55 ± 0.17 fold for PTL, 1.85 ± 0.35 fold for TL and 4.8 ± 0.1 fold
237 for TNL. The concentration of FGD3 mRNA (**Figure 4H**) was also 3.34 ± 0.11 fold lower in
238 PTL, 3.29 ± 0.08 fold lower in TL and 2.7 ± 0.18 fold lower in TNL when compared to
239 PPRM. In contrast, the mRNA of NDRG2 was in lower concentration in the PPRM
240 cervix when compared to PTL (-4.16 ± 0.57), TL (-3.62 ± 0.63) and TNL (-4.0 ± 0.42) groups
241 (**Figure 4K**). These changes were confirmed in the protein level. CEACAM3 (**Figure 4C**)
242 and FGD3 (**Figure 4I**) were significantly higher in the PPRM group when compared to the
243 other groups. CEACAM3 was 2.57 ± 0.06 fold lower in the PTL cervix, 2.65 ± 0.07 fold lower
244 in the TL cervix and 2.77 ± 0.07 fold lower in the TNL cervix. These values for FGD3 were
245 1.88 ± 0.09 for PTL, 2.02 ± 0.18 for TL and 2.58 ± 0.24 for TNL. PRAM1 (**Figure 4F**) was
246 significantly higher in PPRM compared to PTL (2.97 ± 0.15) and TL (3.5 ± 0.08) but not
247 TNL. NDRG2 (**Figure 4L**) protein was significantly lower in the PPRM group when
248 compared to PTL (-6.78 ± 0.5) and TL (7.0 ± 0.54) but not TNL group.

249

250 **PPROM-specific markers were localized to immune cells and vascular myofibroblasts.**

251 We explored the localization of PRAM1, CEACAM3, FGD3 and NDRG2 within the cervical
252 tissue. Although the literature suggests that PRAM1 is predominantly expressed in
253 granulocytes it did not co-localize with the established granulocyte membrane marker
254 CEACAM3 (**Figure 5D**). Instead, PRAM1 was localized to the cytoplasm of a subset of
255 immune CD45 positive cells (**Figure 5H**) resident in the cervical stroma (**Figure 5C, F, I,**
256 **M**). Notably, all PRAM1 positive cells stained for CD45, suggesting that these are immune

257 cells. We confirmed that PRAM1 positive cells were neither macrophages (**Figure 5K**) nor
258 neutrophils (**Figure 5O**). Positive, albeit marginal, NDRG2 staining was evident in the nuclei
259 of the endocervical epithelial cells (**Figure 6C**), which were positive for pan-cytokeratin
260 (**Figure 6B**). Strong NDRG2 staining (**Figure 6G**) was detected in the cytoplasm of
261 endocervical glands (**Figure 6F**) and myofibroblasts surrounding blood vessels in the
262 cervical stroma (**Figure 6D**). A double staining with Von Willebrand factor (vWF), a marker
263 expressed in the endothelial cells of the vasculature, confirmed the blood vessel status
264 (**Figure 6J**). FDG3 was also expressed in the cytoplasm of myofibroblasts (**Figure 6P**)
265 surrounding vWF positive blood vessels (**Figure 6N**). We found that NDRG2 and FGD3
266 shared the same localization within myofibroblasts (**Figure 6T**).

267

268 **GO terms for PRAM1, CEACAM3, FGD3 and NDRG2.**

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

270

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

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

276

277 **Comment**

278

279 This is the first transcriptomic study of the preterm human cervix, which examined PTL and
280 PPROM as two separate pathologies and compared gene expression in the two groups.
281 According to a recent systematic review, only 4% of all transcriptomic studies in term and

282 preterm human pregnancies have utilized cervical tissue and, strikingly, none of these has
283 examined PPROM individually²⁴. Several genetic polymorphisms associated with PPROM
284 have been identified in the placenta, membranes and maternal/fetal blood [reviewed in⁶] and
285 smaller-scale studies also demonstrated the presence of PPROM-associated inflammatory
286 markers in the amniotic fluid^{25, 26}, fetal membranes²⁷⁻²⁹ and maternal serum³⁰. All these
287 studies combined with recent proteomic¹⁶ and epigenetic³¹ reports of a PPROM signature in
288 the placenta and maternal blood have established the hypothesis that PPROM and PTL may
289 have distinct underlying pathologies. It remained to be deduced whether a PPROM signature
290 would be detected in the cervix. We hypothesized that the cervix might initiate rupture of the
291 fetal membranes at their contact site through PPROM-specific cervical remodeling events.
292 Our findings support this hypothesis and demonstrate that PPROM is associated with
293 expression of key proteins, which may facilitate the organization of the cervical extracellular
294 matrix (ECM) and indirectly accelerate membranes rupture.

295

296 The GO terms for the overexpressed genes in PPROM, when compared to PTL (Table 3),
297 were predominantly related to immunity, for example ‘immune system processes’, ‘immunity
298 mediated by myeloid leukocytes’ and ‘immunity mediated by neutrophils’. This is perhaps
299 not surprising because physiological cervical remodeling is accompanied by infiltration of
300 leukocyte subpopulations and neutrophils, which work to achieve the rigidity of the cervix³².
301³³. In line with our findings, a study in the mouse cervix proved that the overarching
302 mechanism underlying cervical remodeling-associated immune cell influx is similar in term
303 and preterm parturition and only marginal differences occur whereby the mediators and
304 effector cells involved may differ³⁴. Our findings provide the first evidence to suggest that
305 the immunity modulators employed to mediate cervical remodeling may be additionally
306 different between the preterm subgroups PPROM and PTL. Immune modulators stimulate

307 immune and other cells in the cervical stroma to produce cytokines and MMPs to degrade the
308 ECM as part of the remodeling process^{35,4}. MMP-2 and MMP-9 are gelatinases both capable
309 of degrading collagens type I and III, the main constituents of the cervical ECM³⁶. MMP-2
310 and MMP-9 concentration is reportedly elevated in the amniotic fluid of PPRM pregnancies
311²⁵. Both MMP-2 and MMP-9 are produced by human cervical fibroblasts²⁰ and MMP-9 by
312 vascular fibroblasts^{37,38} and neutrophil granulocytes³⁹⁻⁴¹. To contribute to the notion that the
313 facilitators of ECM degradation may differ between PPRM and PTL or TL in the cervix,
314 we performed an assay to assess MMP-2 and MMP-9 activity. Indeed, the activity of MMP-9
315 was increased solely in PPRM.

316

317 Out of the 44 differentially expressed genes between the PPRM and PTL groups identified
318 with traditional array analysis, our network graph analysis followed by validation, brought
319 forward 4 key proteins that were over- or under- expressed only in the PPRM cervix.
320 Although these proteins are novel to the parturition field, there is some evidence to support
321 that they might be involved in the activation of a pathological cascade, which delivers a
322 “rupture” signal to the weakest zone of fetal membranes overlying the cervix. Specifically,
323 NDRG2 may be switched off in cervical myofibroblasts to promote the production of MMP-9
324 and accelerate a PPRM-specific remodeling process. Down-regulation of NDRG2 has been
325 previously associated with an increase in the gelatinolytic activities of MMP-2 and MMP-9⁴²
326 in adenocarcinomic human alveolar basal epithelial cell line and more reports have shown
327 direct inhibition of MMP-9 activity by NDRG2⁴³⁻⁴⁵. In support of this hypothesis, cathepsin
328 D (CTSD), which is also down-regulated in PPRM compared to PTL (Table 1) and shares
329 GO terms with NDRG2 (Table 4), is additionally a negative regulator of MMP-2 and MMP-9
330 in endometriotic lesions⁴⁶. CEACAM3, a membrane granulocyte protein involved in
331 neutrophil activation^{47,48}, and FGD3 may also work together towards enhancement of MMP-

332 9 activity in PPROM. It is not unlikely that aberrant infiltrating neutrophil-granulocytes
333 overexpress CEACAM3 to promote their activation and stimulate MMP-9 secretion. In
334 support of this notion, genes that share GO terms with *CEACAM3* (Table 4) have also been
335 associated with MMP actions. For example, the osteoclast-associated markers *OSCAR* and
336 *SIRPA* and *TREM-1* have all been implicated in MMP-9-mediated responses ⁴⁹⁻⁵².
337 CEACAM3 shares cluster membership with FGD3 (Figure 3C), suggesting similar regulation
338 in gene expression, which itself may imply similar functions. FGD3 may control MMP-9
339 activity in the PPROM cervix by promoting filopodia formation on the plasma membranes of
340 myofibroblasts ⁵³. It is well established that proteins of the same family with FGD3 organize
341 such formations on plasma membranes to release MMPs and in turn induce degradation of
342 the surrounding stroma ^{54,55}. Remarkably, blockade of filopodia formation by flavonoids has
343 been shown to decrease the release of MMP-2 in cancer ⁵⁶. Electron microscopy studies could
344 help investigate filopodial formations on cells in PPROM. PRAM1, which shared GO terms
345 with FGD3 (Table 4), is thought to be predominantly expressed in granulocyte-neutrophils
346 where it acts as an adaptor protein critical for select integrin functions ⁵⁷. Integrins are
347 transmembrane receptors that bridge cell-ECM interactions and activate MMPs ⁵⁸. A
348 proteolytic role for integrins has been described in the initiation of labor, whereby they
349 regulate release of MMP-9 in human fetal membranes ⁵⁹. Although we did not detect PRAM1
350 in elastase positive neutrophils or in CEACAM3 positive granulocytes (Figure 5), the
351 likelihood of PRAM1 regulating integrin functions in the cytoplasm of an alternative immune
352 cell population in the cervix deserves addressing.

353

354 Employing a genome-wide approach has identified key genes associated with PPROM, and
355 provided an insight into a potential mechanism regulating physiological cervical remodeling.
356 Analysis of the two top clusters of the up-regulated genes in PPROM (Figure 3D, E)

357 demonstrated that the genes within these clusters were overexpressed both in PPRM and,
358 surprisingly, in TL. The first overexpressed cluster contained various genes involved in bone
359 marrow-derived cell migration (*ARHGAP9*, *FGR*, *NFE2*) and *SLC43A2*, the gene coding an
360 essential transporter of Branched Chain Amino Acids (BCAAs). We propose a new
361 mechanism to contribute to cervical remodeling in TL and PPRM, whereby the increase of
362 BCAAs in the cervix triggers the recruitment of bone marrow-derived cells in order to
363 stimulate MMP-induced degradation. Consistent with our hypothesis, MMP-2 and MMP-9
364 increase in response to exogenous BCAAs in the hippocampus of rats⁶⁰ and bone marrow-
365 derived cells have been also shown to secrete MMPs⁶¹⁻⁶³. A similar mechanism for cervical
366 remodeling in TL and PPRM involving bone marrow recruited cells can be further
367 evidenced by KEGG analysis, where ‘Osteoclast differentiation’ pathway is enriched not
368 only in PPRM-PTL comparison (Table 2) but also in TL-PTL (Table 3 Supplemental).
369 Osteoclasts are bone marrow-derived cells traditionally involved in the degradation of bone
370 matrix⁶⁴ and have been described to secrete MMP-2 and MMP-9^{62, 63}. Further work is
371 required to prove whether bone marrow-derived osteoclasts or osteoclast-like cells mediate
372 MMPs-induced degradation of ECM as part of physiological cervical remodeling cascade. It
373 is noteworthy that only 16 genes were differentially expressed between PPRM and TL, in
374 contrast to 1285 genes in the TNL-TL comparison. The notion that PPRM and TL might
375 share some similar pathways for cervical remodeling was additionally supported by the
376 sample-sample network graph (Figure 1C). In that graph PPRM and TL samples belonged
377 to the same ‘loose’ local structure whereas the TNL samples belong to a separate ‘tight’
378 structure.

379

380 Our study could benefit from a larger sample size but human cervical biopsies are extremely
381 hard to obtain especially in relation to preterm delivery, which explains why so few studies

382 are conducted on the human preterm cervix. Moreover, the biopsies were collected
383 postpartum and thus postpartum repair mechanism might be reflected in our results.
384 However, it is not practically and ethically possible to obtain cervical biopsies during vaginal
385 delivery and the material used in our study was collected within 30 minutes after delivery.
386 Animal research, for example CRISPR experiments could be useful in future studies, to
387 identify the phenotype associated with knock out or knock in of the genes we suggest are
388 important.

389
390 In summary, we have, for the first time identified a gene expression signature involved with
391 PPROM. It is tempting to hypothesize that the PPROM-specific proteins identified herein act
392 as contributors in a pathway whereby MMP-9 facilitates ECM degradation in the cervix to
393 signal a 'rupture' message to the overlying membranes. Our work supports the growing body
394 of evidence suggesting that premature labor is a multifactorial disorder with different
395 pathways involved for PPROM and PTL.

396

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399

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613 **Tables**614 **Table 1:** List of up and down-regulated genes

	Symbol	FC	Adj.P.Val
Up	PRAM1	2.094	1.36E-04
	SIRPA	2.101	1.36E-04
	CEACAM3	2.412	2.92E-04
	CD300LF	2.232	1.82E-03
	LILRA2	2.598	2.42E-03
	FGD3	2.735	2.42E-03
	OSCAR	2.168	2.65E-03
	TREM1	3.826	2.65E-03
	OSCAR	2.351	2.65E-03
	STK4	2.023	2.65E-03
	NUDT11	2.233	3.09E-03
	LILRA6	2.522	3.09E-03
	MAMLD1	2.821	3.23E-03
	ASGR1	2.063	3.42E-03
	MYO1F	2.004	3.77E-03
	MMP25	3.117	3.77E-03
	TMEM71	2.269	4.96E-03
	CSF3R	4.164	4.96E-03
	FGR	3.036	6.00E-03
	PRDM8	2.577	6.00E-03
	NLRP12	2.211	6.00E-03
	FGR	2.668	6.00E-03
	NFE2	4.23	6.29E-03
	FKBP1A	2.25	6.38E-03
	SLC43A2	2.067	7.72E-03
	CLEC5A	2.374	7.87E-03
	LILRA5	2.781	7.87E-03
ARHGAP9	2.107	8.72E-03	
GK	2.837	9.61E-03	
CYTH4	2.437	9.66E-03	
Down	NDRG2	-3.551	7.52E-04
	PPDPF	-5.093	3.09E-03
	RNU4ATAC	-3.67	3.23E-03
	PKM	-3.278	6.00E-03
	ACOT13	-2.171	6.00E-03
	CTSD	-2.031	6.38E-03
	RETSAT	-2.565	7.00E-03
	RNA28S5	-11.005	7.87E-03
	RNA28S5	-6.788	8.72E-03

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

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618 **Table 2:** KEGG pathway enrichment analysis of the up and down-regulated genes that

619 mapped to significant features at adjusted p<0.05.

620

	Name of KEGG pathway	Pvalue	Genes	No. Sig. Genes	% Sig. Genes
Up	Osteoclast differentiation	4.34E-06	LILRA2, LILRA5, LILRA6, OSCAR, SIRPA	5	4.1

Down	Pyruvate metabolism	2.03E-02	PKM	1	2.8
	Retinol metabolism	2.03E-02	RETSAT	1	2.8
	Type II diabetes mellitus	2.08E-02	PKM	1	2.7
	Glycolysis / Gluconeogenesis	3.20E-02	PKM	1	1.8
	Central carbon metabolism in cancer	3.42E-02	PKM	1	1.6
	Glucagon signaling pathway	4.74E-02	PKM	1	1.2

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624 **Table 3:** GO term enrichment analysis of the up and down-regulated genes that mapped to625 significant features at adjusted $p < 0.001$

	Ontology	Name	Pvalue	Genes	No. Sig. Genes	% Sig. Genes
Up	BP	immune system process	3.44E-06	CD300LF, CLECSA, CSF3R, FGR, FKBP1A, LILRA2, LILRA5, LILRA6, MYO1F, NLRP12, PRAM1, SIRPA, STK4, TREM1	14	0.7
	BP	defense response	3.38E-04	CLECSA, CSF3R, FGR, LILRA2, LILRA5, MMP25, MYO1F, NLRP12, TREM1	9	0.8
	BP	myeloid leukocyte mediated immunity	3.73E-06	FGR, MYO1F, PRAM1, TREM1	4	7.4
	BP	cytokine secretion	6.77E-05	CLECSA, FGR, NLRP12, TREM1	4	3.6
	BP	protein secretion	4.86E-04	CLECSA, FGR, NLRP12, TREM1	4	2.1
	BP	leukocyte mediated immunity	6.62E-04	FGR, MYO1F, PRAM1, TREM1	4	2
	BP	neutrophil mediated immunity	1.07E-05	MYO1F, PRAM1, TREM1	3	13.6
	BP	leukocyte degranulation	8.96E-05	FGR, MYO1F, PRAM1	3	6.8
	BP	regulated secretory pathway	1.75E-04	FGR, MYO1F, PRAM1	3	5.5
	BP	myeloid cell activation involved in immune response	1.75E-04	FGR, MYO1F, PRAM1	3	5.5
	BP	positive regulation of cytokine secretion	2.50E-04	CLECSA, FGR, NLRP12	3	4.8
	BP	positive regulation of protein secretion	7.97E-04	CLECSA, FGR, NLRP12	3	3.3
	BP	regulation of cytokine secretion	8.75E-04	CLECSA, FGR, NLRP12	3	3.2
	BP	neutrophil degranulation	1.72E-04	MYO1F, PRAM1	2	20
	BP	neutrophil activation involved in immune response	2.52E-04	MYO1F, PRAM1	2	16.7
	BP	neutrophil activation	8.72E-04	MYO1F, PRAM1	2	9.1
	BP	regulation of myeloid leukocyte mediated immunity	9.54E-04	FGR, PRAM1	2	8.7
	BP	regulation of leukocyte degranulation	9.54E-04	FGR, PRAM1	2	8.7
	BP	regulation of regulated secretory pathway	9.54E-04	FGR, PRAM1	2	8.7
	Down	MF	pyruvate kinase activity	8.24E-04	PKM	1
MF		all-trans-retinol 13,14-reductase activity	4.12E-04	RETSAT	1	100

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628 Footnote Table 3: BP: Biological Process, MF: Molecular Function

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631 **Table 4:** Report of the GO terms containing the features PRAM1, FGD3, CEACAM3 and632 NDRG2 amongst other genes that mapped to significant features at adjusted $p < 0.01$.

633

	Gene	Ontology	Name	Pvalue	Genes	No. Sig. Genes	% Sig. Genes		
Up	PRAM1	BP	response to stimulus	1.12E-02	ARHGAP9, ASGR1, CLEC5A, CSF3R, CYTH4, FGD3, FGR, FKBP1A, LILRA2, LILRA5, MMP25, MYO1F, NFE2, NLRP12, PRAM1, SIRPA, STK4, TREM1	18	0.3		
		BP	immune system process	3.44E-06	CD300LF, CLEC5A, CSF3R, FGR, FKBP1A, LILRA2, LILRA5, LILRA6, MYO1F, NLRP12, PRAM1, SIRPA, STK4, TREM1	14	0.7		
		BP	cell communication	2.44E-02	ARHGAP9, ASGR1, CLEC5A, CSF3R, CYTH4, FGD3, FGR, FKBP1A, LILRA2, NFE2, NLRP12, PRAM1, STK4, TREM1	14	0.3		
		BP	immune response	6.07E-03	CLEC5A, FGR, FKBP1A, LILRA5, MYO1F, PRAM1, TREM1	7	0.6		
		BP	secretion by cell	1.64E-03	CLEC5A, FGR, MYO1F, NLRP12, PRAM1, TREM1	6	0.9		
		BP	secretion	3.04E-03	CLEC5A, FGR, MYO1F, NLRP12, PRAM1, TREM1	6	0.8		
		BP	myeloid leukocyte mediated immunity	3.73E-06	FGR, MYO1F, PRAM1, TREM1	4	7.4		
		BP	leukocyte mediated immunity	6.62E-04	FGR, MYO1F, PRAM1, TREM1	4	2		
		BP	regulation of secretion	1.01E-02	CLEC5A, FGR, NLRP12, PRAM1	4	0.9		
		BP	immune effector process	1.74E-02	FGR, MYO1F, PRAM1, TREM1	4	0.8		
		BP	leukocyte activation	2.33E-02	FGR, FKBP1A, MYO1F, PRAM1	4	0.7		
		BP	regulation of immune response	4.03E-02	FGR, FKBP1A, MYO1F, PRAM1	4	0.6		
		BP	neutrophil mediated immunity	1.07E-05	MYO1F, PRAM1, TREM1	3	13.6		
		BP	leukocyte degranulation	8.96E-05	FGR, MYO1F, PRAM1	3	6.8		
		BP	regulated secretory pathway	1.75E-04	FGR, MYO1F, PRAM1	3	5.5		
		BP	myeloid cell activation involved in immune response	1.75E-04	FGR, MYO1F, PRAM1	3	5.5		
		BP	myeloid leukocyte activation	2.02E-03	FGR, MYO1F, PRAM1	3	2.4		
		BP	leukocyte activation involved in immune response	2.95E-03	FGR, MYO1F, PRAM1	3	2.1		
		BP	cell activation involved in immune response	2.95E-03	FGR, MYO1F, PRAM1	3	2.1		
		BP	exocytosis	1.66E-02	FGR, MYO1F, PRAM1	3	1.1		
		MF	lipid binding	4.65E-02	ARHGAP9, CYTH4, PRAM1	3	0.7		
		BP	neutrophil degranulation	1.72E-04	MYO1F, PRAM1	2	20		
		BP	neutrophil activation involved in immune response	2.52E-04	MYO1F, PRAM1	2	16.7		
		BP	neutrophil activation	8.72E-04	MYO1F, PRAM1	2	9.1		
		BP	regulation of myeloid leukocyte mediated immunity	9.54E-04	FGR, PRAM1	2	8.7		
		BP	regulation of leukocyte degranulation	9.54E-04	FGR, PRAM1	2	8.7		
		BP	regulation of regulated secretory pathway	9.54E-04	FGR, PRAM1	2	8.7		
		BP	granulocyte activation	1.04E-03	MYO1F, PRAM1	2	8.3		
		BP	integrin-mediated signaling pathway	1.16E-02	FGR, PRAM1	2	2.4		
		BP	regulation of exocytosis	1.30E-02	FGR, PRAM1	2	2.3		
		BP	regulation of leukocyte mediated immunity	1.80E-02	FGR, PRAM1	2	1.9		
		BP	regulation of neutrophil degranulation	8.01E-03	PRAM1	1	25		
		BP	regulation of neutrophil activation	1.00E-02	PRAM1	1	20		
		FGD3		BP	response to stimulus	1.12E-02	ARHGAP9, ASGR1, CLEC5A, CSF3R, CYTH4, FGD3, FGR, FKBP1A, LILRA2, LILRA5, MMP25, MYO1F, NFE2, NLRP12, PRAM1, SIRPA, STK4, TREM1	18	0.3
				BP	cell communication	2.44E-02	ARHGAP9, ASGR1, CLEC5A, CSF3R, CYTH4, FGD3, FGR, FKBP1A, LILRA2, NFE2, NLRP12, PRAM1, STK4, TREM1	14	0.3
				BP	intracellular signal transduction	2.54E-02	ARHGAP9, CYTH4, FGD3, FGR, FKBP1A, NLRP12, STK4, TREM1	8	0.4
				BP	positive regulation of molecular function	1.45E-02	ARHGAP9, CYTH4, FGD3, FGR, FKBP1A, NLRP12, STK4	7	0.5
				BP	regulation of phosphate metabolic process	2.58E-02	ARHGAP9, CYTH4, FGD3, FGR, FKBP1A, NLRP12, STK4	7	0.5
				BP	regulation of phosphorus metabolic process	2.69E-02	ARHGAP9, CYTH4, FGD3, FGR, FKBP1A, NLRP12, STK4	7	0.5
				BP	positive regulation of catalytic activity	2.40E-02	ARHGAP9, CYTH4, FGD3, FGR, NLRP12, STK4	6	0.5
				BP	regulation of intracellular signal transduction	3.39E-02	ARHGAP9, CYTH4, FGD3, FGR, FKBP1A, NLRP12	6	0.5
				BP	regulation of hydrolase activity	4.74E-02	ARHGAP9, CYTH4, FGD3, FKBP1A, NLRP12	5	0.5
				BP	regulation of small GTPase mediated signal transduction	4.97E-02	ARHGAP9, CYTH4, FGD3	3	0.7
				BP	positive regulation of GTPase activity	4.71E-02	ARHGAP9, CYTH4, FGD3	3	0.7
				BP	regulation of cell shape	1.73E-02	FGD3, FGR	2	2
				CC	ruffle	3.15E-02	FGD3, FGR	2	1.5
				MF	guanyl-nucleotide exchange factor activity	3.71E-02	CYTH4, FGD3	2	1.2
BP	regulation of Cdc42 GTPase activity			3.94E-02	FGD3	1	5		
BP	regulation of Cdc42 protein signal transduction			4.52E-02	FGD3	1	4.3		
CEACAM3				CC	membrane part	6.82E-03	ASGR1, CD300LF, CEACAM3, CLEC5A, CSF3R, FGR, FKBP1A, LILRA2, LILRA5, LILRA6, MMP25, OSCAR, SIRPA, SLC43A2, TMEM71, TREM1	16	0.4
				CC	integral component of membrane	6.91E-03	ASGR1, CD300LF, CEACAM3, CLEC5A, CSF3R, LILRA2, LILRA5, LILRA6, MMP25, OSCAR, SIRPA, SLC43A2, TMEM71, TREM1	14	0.4
				CC	intrinsic component of membrane	8.61E-03	ASGR1, CD300LF, CEACAM3, CLEC5A, CSF3R, LILRA2, LILRA5, LILRA6, MMP25, OSCAR, SIRPA, SLC43A2, TMEM71, TREM1	14	0.4
				CC	extracellular vesicular exosome	4.17E-03	ACOT13, CTSD, NDRG2, PKM	4	0.2
Down	NDRG2			CC	extracellular membrane-bounded organelle	4.17E-03	ACOT13, CTSD, NDRG2, PKM	4	0.2
				CC	extracellular organelle	4.17E-03	ACOT13, CTSD, NDRG2, PKM	4	0.2
				CC	membrane-bounded vesicle	9.41E-03	ACOT13, CTSD, NDRG2, PKM	4	0.1
		BP	regulation of platelet-derived growth factor production	1.45E-03	NDRG2	1	33.3		
		BP	platelet-derived growth factor production	1.45E-03	NDRG2	1	33.3		

634

635

636 Footnote Table 4: BP: Biological Process, MF: Molecular Function, CC: Cellular Component

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638

639 **Figure legends**

640

641 **Figure 1: Sample-sample network graph of all samples used for the microarray and the**
642 **comparisons performed between the groups.** A 2D representation of sample clustering in a
643 3D graph. Each node represents a different sample and edges are coloured to reflect the
644 Pearson correlation that they represent. Red and blue edges denote high correlation and low
645 correlation respectively. The same graph is coloured by **A.** no cluster ($r=0.91$), **B.** unbiased
646 MCL cluster number (MCL 19.3) **C.** group status. **D:** Table shows all the comparisons
647 performed between groups and the number of significant array features at adjusted p-value <
648 0.01 and fold change ≥ 2 . TL = Term Labor (n=7), TNL = Term Non-Labor (n=5),
649 PTL=Preterm Labor (n=6), PPRM=Preterm Premature Rupture of Membranes (n=5).

650

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

658

659 **Figure 3: Probe-probe network cluster analysis.** Probe-probe network graph of the up-
660 regulated (**A**) and down-regulated (**B**) genes in the PPRM-PTL comparison. Each node
661 represents a gene and nodes are coloured according to membership of different MCL (MCL_i
662 = 1.3) clusters. **C:** The genes belonging to each cluster are shown in the MCL gene clusters
663 table. The Pareto scaled graphs show the mean expression profiles of MCL clusters 1 (**D**), 2

664 (E), 3 (F), 4 (G), 5 (H), 6 (I), 7 (J), 8 (K), 9 (L) across all samples (n=23), including the
665 samples in the TL and TNL groups. Samples are plotted on the x-axes. Genes with similar
666 expression pattern across all samples are members of the same cluster. Each bar represents
667 the average expression of all genes that cluster together in that sample. The error bar for each
668 sample denotes the SD extrapolated from the expression of all cluster genes in that sample.
669 PPR0M n=5, PTL n=6, TL n=7, TNL n=5.

670

671 **Figure 4: Validation of microarray analysis. A, D, G, J:** Tables show the fold-changes
672 (FC) and adjusted p values (Adj.P.Val) across all comparisons for the 4 selected genes
673 CEACAM3, PRAM1, FGD3 and NDRG2 as reported by FIOS genomics statistical analysis.
674 qRT-PCR validated that *CEACAM3* (B), *PRAM1* (E) and *FGD3* (H) were up-regulated, and
675 *NDRG2* (K) down-regulated in the PPR0M group when compared to all other groups. Data
676 analyzed using one-way ANOVA Dunnett's test. qRT-PCR samples: PPR0M n=5, PTL n=6,
677 TL n=12, TNL n=5. Western blotting analysis confirmed that CEACAM3 (C) and FGD3 (I)
678 were in higher concentration in the PPR0M cervix compared to all other groups. PRAM1 (F)
679 and NDRG2 (L) changes were also significant between PPR0M and PTL/TL but not in TNL.
680 Data analyzed using one-way ANOVA Dunnett's test. Western blotting samples: PPR0M
681 n=4, PTL n=4, TL n=4, TNL n=4. Error bars denote \pm SEM. *p<0.05, **p <0.01,
682 ***p<0.001.

683

684 **Figure 5: Localization of PRAM1 and CEACAM3 in the PPR0M human cervix.**
685 PRAM1 and CEACAM3 positive cells were identified at the cervical stroma. PRAM1 was
686 localized to the cytoplasm and CEACAM3 to the membrane of cells. CEACAM3 (B) and
687 PRAM1 (C) did not co-localize (D). Double staining for PRAM1 (F) and CD45 (G)
688 identified double positive population (H). PRAM1 cells (I), did not co-localize (K) with the

689 macrophage marker CD68 (**J**). PRAM1 cells (**M**) did not co-localize (**O**) with neutrophil
690 Elastase (**N**). All scale bars 50 μ m. Images representative of n=4.

691

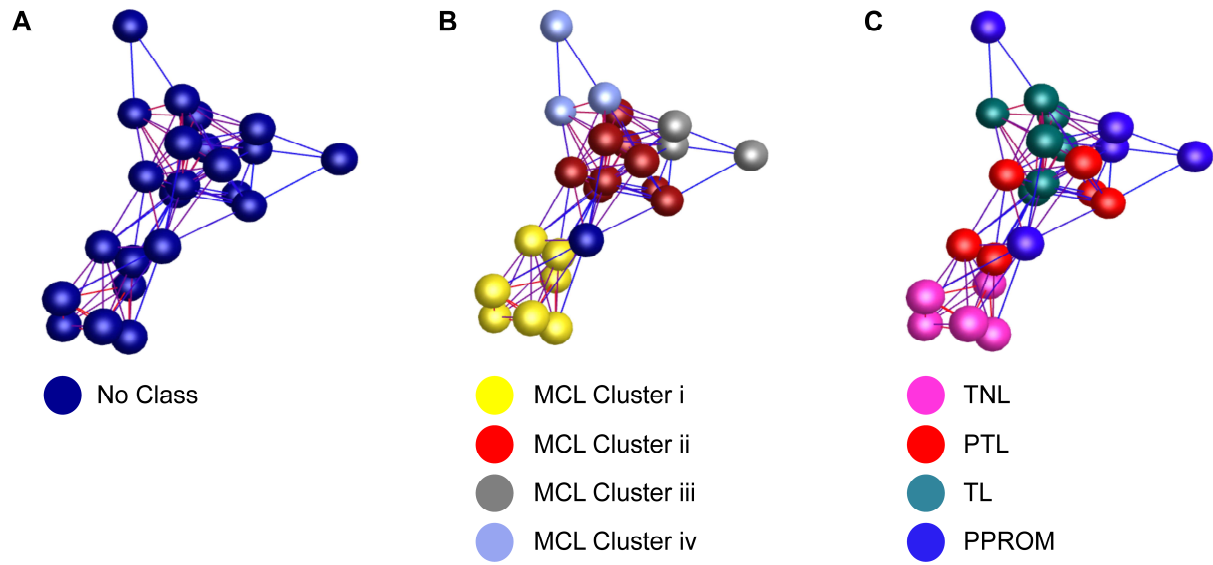
692 **Figure 6: Localization of FGD3 and NDRG2 in the human cervix.** Marginal NDRG2
693 staining (**C**) was detected to the nuclei of endocervical epithelial cells stained positive for
694 AE1/AE3 (**D**). **D**: NDRG2 staining was evidently stronger in cells surrounding blood vessels
695 (indicated with asterisks). A co-staining for vWF (**J**; an endothelial cell marker) and NDRG2
696 (**K**) confirmed that NDRG2 is localized to the cytoplasm of myofibroblasts surrounding
697 blood vessels (**L**). NDRG2 was also localized to the cytoplasm of endocervical glandular
698 cells (**G**) as was evident by co-localization (**H**) with AE1/AE3 (**F**). FGD3 was expressed
699 solely in the cytoplasm of myofibroblasts (**O**) and co-localized with NDRG2 (**T**). Scale bars
700 50 μ m/ 100 μ m as shown in each picture. Images representative of n=4. **A-L**: PTL cervix, **M-**
701 **P**: PPROM cervix, **Q-T**: TL cervix. vWF: Von Willebrand factor, AE1/AE3: Pan
702 Cytokeratin.

703

704 **Figure 7: MMP-2 and MMP-9 activity in the human cervix.** Gelatin zymography was
705 performed on protein extracted from the cervix of women with PPROM (n=4), PTL (n=4),
706 TL (n=4) and TNL (n=4). **A**: The activity of MMP-9 (82 kDa) was significantly higher in the
707 PPROM cervix when compared to the other groups (*p=0.05, ***p=0.001 comparison). **B**:
708 The activity of MMP-2 was similar in PPROM, PTL and TL but significantly lower in TNL
709 when compared to the other groups (****p<0.0001). Data analyzed using one-way ANOVA
710 Tukey's test.

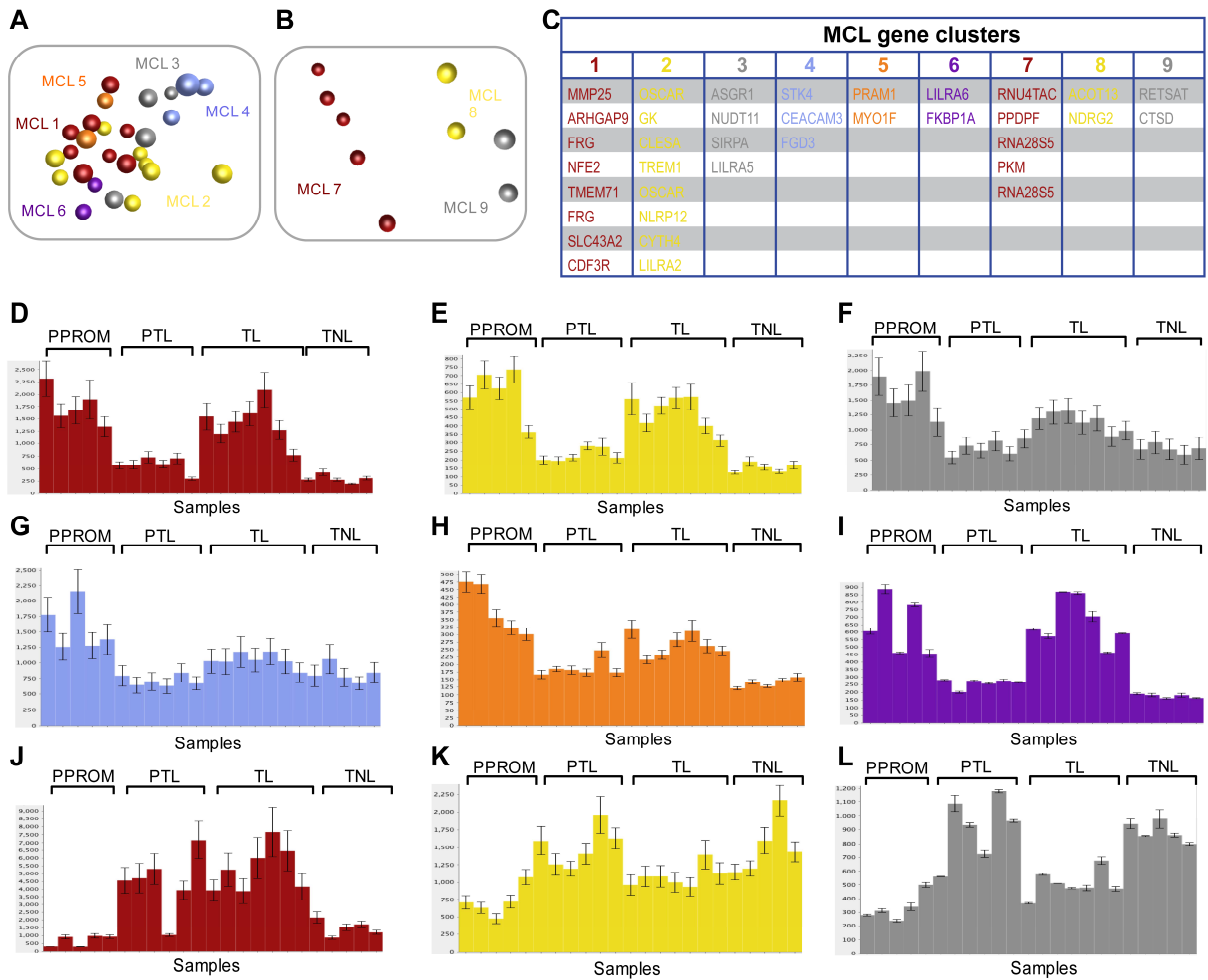
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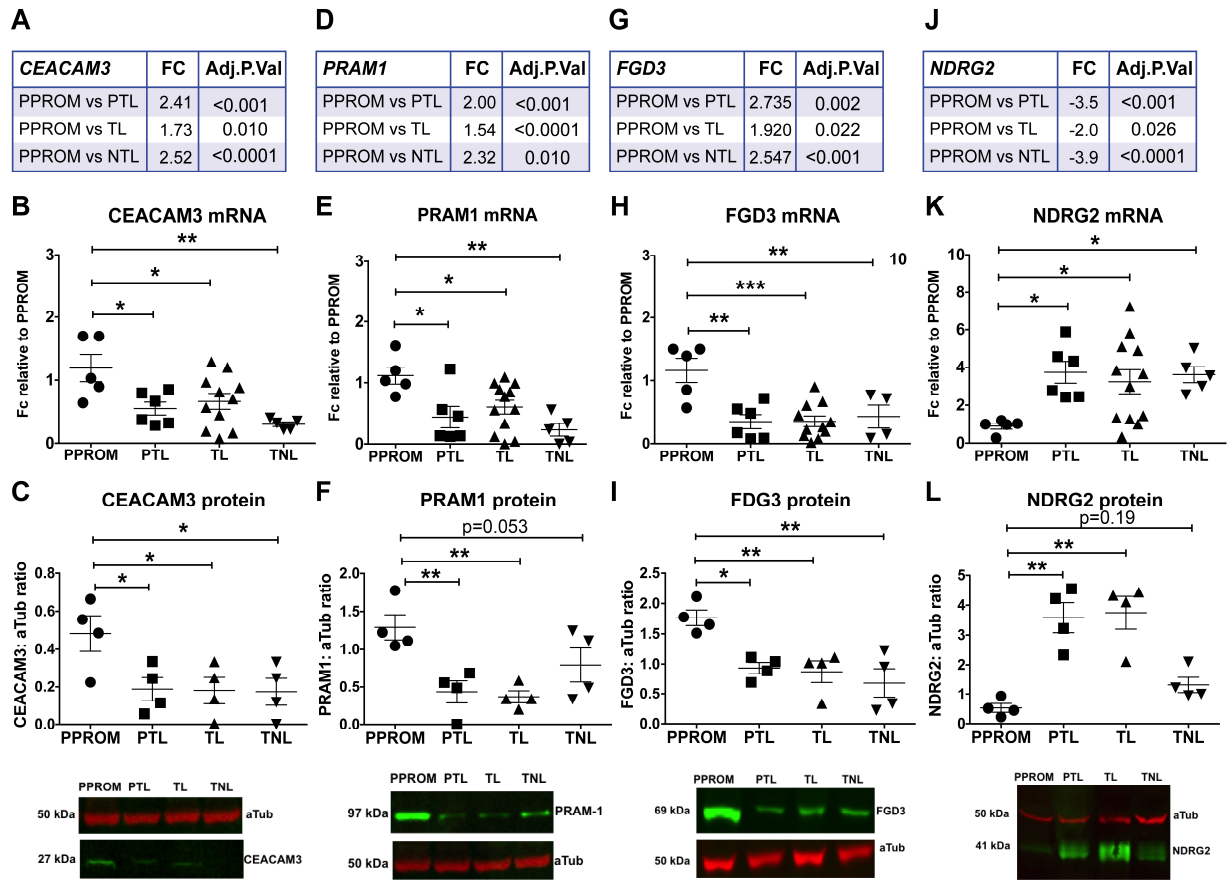


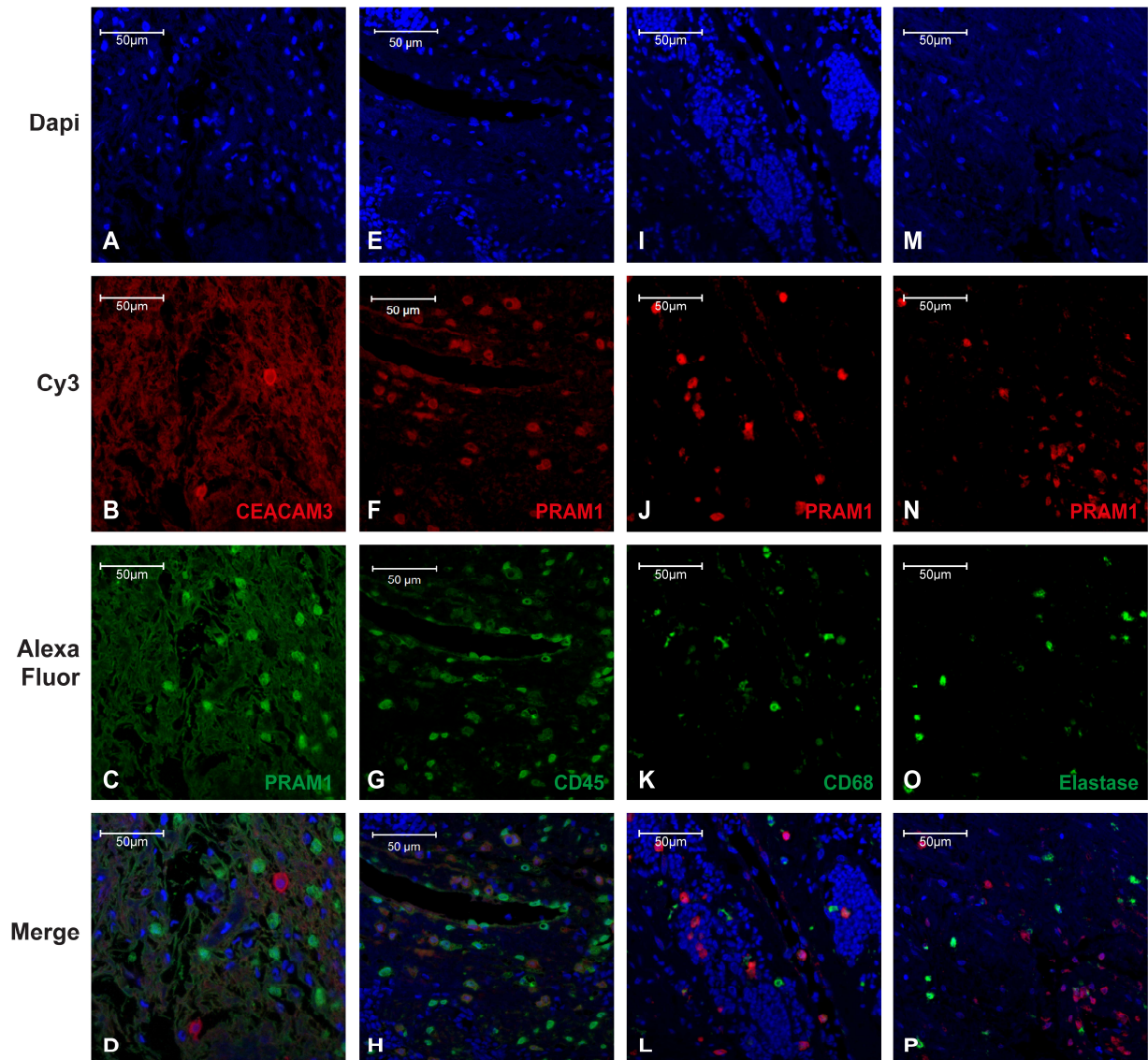
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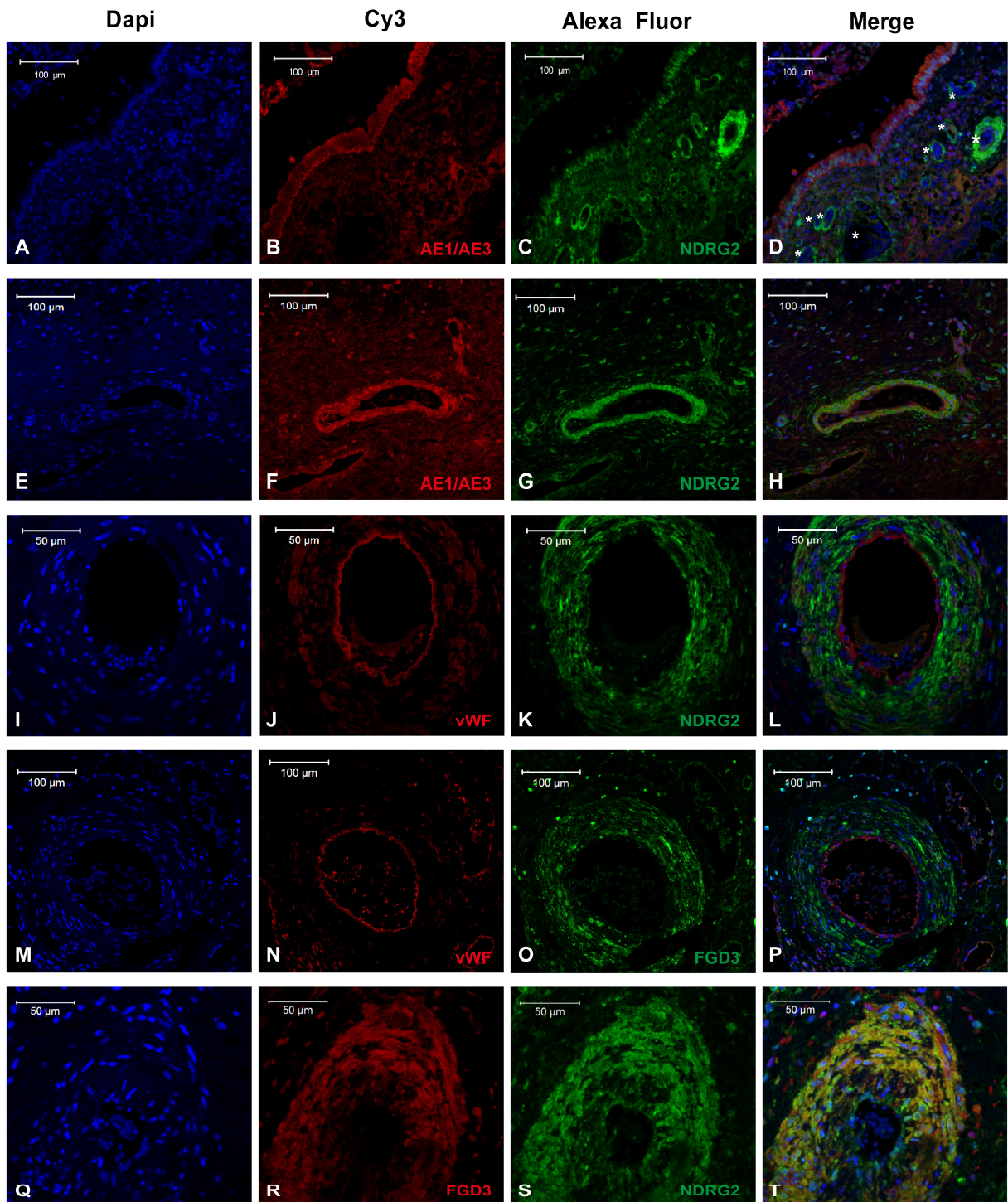
Comparison	Significant genes
TL-TNL	1285
TL-PTL	19
TL-PPROM	16
TNL-PTL	93
TNL-PPROM	886
PPROM-PTL	44

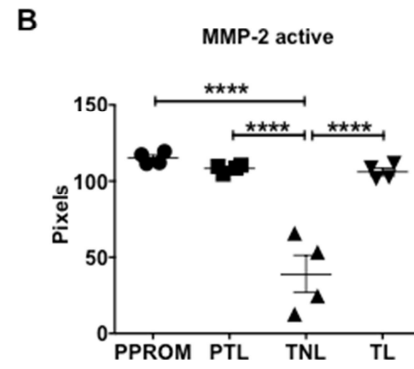
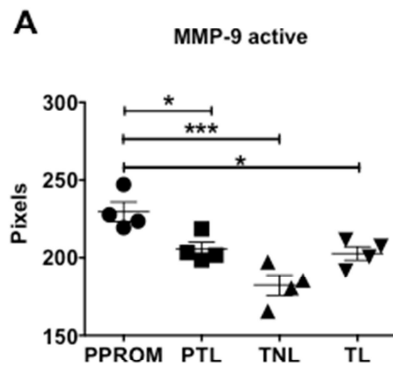
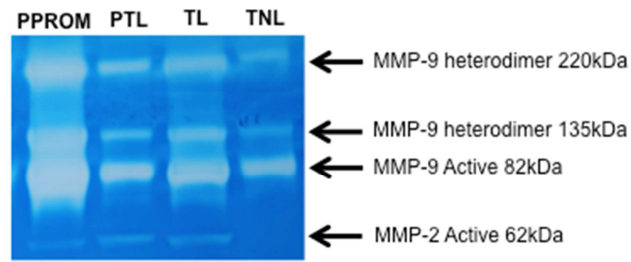


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