# High-mobility group box protein 1 and its signalling receptors in human preterm and term cervix

Aurelija Dubicke[a*], Peter Andersson[b], Emma Fransson[a], Eva Andersson[a], Angelos Sioutas[a], Anders Malmström[c], Eva Sverremark-Ekström[b] and Gunvor Ekman-Ordeberg[a]

[a]Department of Woman and Child Health, Karolinska Institute, 171 76 Stockholm, Sweden

[b]Department of Immunology, the Wenner-Gren Institute, Stockholm University, 106 91 Stockholm, Sweden

[c]Department of Experimental Medical Science, University of Lund, 221 84 Lund, Sweden

[*]Corresponding author: Tel.: +46851773818; Fax: +468323048; e-mail: Aurelija.Dubicke@ki.se

**Abbreviations**

HMGB1- high mobility group box protein 1, NP-non-pregnant, PPROM- preterm premature rupture of membranes, PTL-preterm labor, RAGE- receptor for advanced glycation end products, TL- term labor, TnotL-term not in labor, TLR- Toll-like receptors

**Abstract**

The objective of this study was to identify the possible changes in mRNA and protein expression of High mobility group box protein 1 (HMGB1) and its suggested receptors - receptor for advanced glycation end-products (RAGE) and Toll-like receptor 2 (TLR2) and TLR4 - in human cervix during pregnancy, term and preterm labor. Cervical biopsies were taken from 58 women: 20 at preterm labor, 24 at term labor, 10 at term not in labor and 4 from non-pregnant women. Real-time RT-PCR was used for mRNA analysis, immunohistochemistry and ELISA for protein analysis. HMGB1, RAGE, TLR2 and TLR4 were localized and had mRNA expression in cervix. There was more extranuclear HMGB1 in the cervical epithelium and stroma in preterm and term labor compared to the term not in labor ($p<0.01$). TLR2 mRNA expression was upregulated 5-fold in term labor and 3-fold in preterm labor compared to term not in labor and non pregnant controls ($p<0.01$). There was a significant lower expression of TLR2 and TLR4 mRNA in preterm labor compared to term ($p<0.05$). Lower mRNA expression of HMGB1 was found in the subgroup with preterm premature rupture of membranes than in the rest of preterm group ($p<0.05$), which was significantly higher than in term labor ($p<0.05$). In conclusion, extranuclear expression of HMGB1 during labor suggests a possible role of HMGB1 during the process of cervical ripening. Changes in mRNA expression of HMGB1, TLR2 and TLR4 in preterm labor, point towards possible differences in the mechanism of cervical ripening at preterm and term.

Keywords: cervical ripening; cervix; HMGB1; preterm birth; receptor for advanced glycation end-products (RAGE); toll-like receptors

## 1. Introduction

The mechanisms underlying the initiation of both preterm and term labor remain largely unknown (Goldenberg et al. 2008). For a successful delivery, uterine contractions must be coordinated with a softening of the cervix. Cervical softening involves intensive remodeling of the extracellular matrix, with extensive changes in concentration and composition of collagens (Uldbjerg et al. 1983) and proteoglycans (Westergren-Thorsson et al. 1998). This process can be also regarded as an inflammatory reaction associated with elevated levels of cytokines at the time of both preterm and term labor (Sennstrom et al. 2000; Tornblom et al. 2005a).

High-mobility group box protein 1 (HMGB1) was discovered as a nuclear protein that migrates quickly during electrophoresis and it was named after this property (Lotze and Tracey 2005). Recently, it was demonstrated that HMGB1 has important extracellular cytokine-like functions and signals tissue injury and initiates inflammation and repair. HMGB1 is nowadays also considered to be an alarmin (Harris and Raucci 2006). It is involved in several diseases, such as lupus erythematosus, arthritis and atherosclerosis (Lotze and Tracey 2005). The receptor for advanced glycation end-products (RAGE) and Toll-like receptor 2 (TLR2) and TLR4 are involved in HMGB1-mediated signaling (Lotze and Tracey 2005). There are three major spliced variants of RAGE: full length, N-terminally truncated, and C-terminally truncated. The C-terminally truncated form of RAGE is secreted from the cell and is named endogenously secreted RAGE (esRAGE). The other forms of soluble RAGE (sRAGE) are cleaved from cell-surface RAGE by matrix metalloproteinases (Koyama et al. 2007).

Little is known regarding HMGB1 involvement in pregnancy and labor, though parturition is an inflammatory process. Human term placenta expresses HMGB1 and RAGE, but labor does not influence their expression (Holmlund et al. 2007). Placental TLR expression has

since been extensively studied in pregnancy and in labor (Patni et al. 2007), but only one study has investigated these receptors in human cervix after vaginal delivery and caesarean section (Hassan et al. 2006). Changes in concentrations of sRAGE and esRAGE in amniotic fluid have been seen in labor and during intra-amniotic infection (Romero et al. 2008). Nevertheless, no studies have been published on HMGB1 and its receptors in human cervix at preterm and term labor.

As labor is associated with a local cervical pro-inflammatory response, we wanted to identify and investigate the possible changes in mRNA and protein expression of HMGB1 and its suggested receptors (RAGE, TLR2 and TLR4) in human cervix during pregnancy, and term and preterm labor.

## 2. Materials and Methods

### 2.1 Patients

Data collection was carried out at Karolinska Hospital, Stockholm, Sweden during 2006-2008. A total of 58 women were included. Women in preterm or term labor with singleton pregnancies were asked to participate in a study on PTB. Women who agreed to undergo cervical biopsy were included: 20 women undergoing preterm labor (PTL), 24 women undergoing normal term labor (TL). As controls, ten women who delivered at term by caesarean section prior to the onset of labor (TnotL) were recruited. Four non-pregnant women (NP) undergoing hysterectomy for benign conditions such as myomas, were used as a reference group, since several new substances were investigated in cervical tissue.

Preterm delivery was defined as delivery before the 37$^{th}$ week of gestation. The labor groups (PTL and TL) were in active labor and demonstrated a ripe cervix, with dilatation more than 4 cm. All except three of these patients were delivered vaginally. One patient in the PTL

group was delivered by emergency caesarean section due to breech presentation and two in the TL group due to protracted labor. Women in the TnotL group had unripe cervices (with a Bishop score of <5 points) and were delivered by caesarean section prior to the onset of labor. The indications were breech presentation, humanitarian, earlier caesarean section or disproportion. None of the women included in the study suffered from pre-eclampsia, diabetes or other systemic disease.

Vaginal and urine cultures were taken from women in the PTL group. Nine of 20 women in this group had negative cultures, six had bacterial growth in at least one of the cultures (three with ureaplasma urealyticum and three with group B streptococcus) and five had candida growth. Ten of the PTL patients had preterm premature rupture of membranes (PPROM) defined as rupture of membranes at least one hour before contractions (Goldenberg et al. 2008). In the PPROM subgroup, four women had negative cultures, four had bacterial growth and two had candida growth in the cultures.

There were no significant differences between the groups of pregnant women with respect to maternal age, parity, previous preterm births or previous caesarean sections. For clinical data see Table 1.

Before the study, the approval of the local Ethics Committee of Karolinska Institute and the informed consent of each subject were obtained.

## 2.2 Sampling procedure

The biopsy was taken as described previously (Dubicke et al. 2008) and divided into three pieces. The samples for mRNA analysis were immediately immersed in RNA*later*® (Ambion Inc, Austin, TX, USA), kept at 4° C for 24 hours and thereafter frozen and stored at -70°. The samples for protein analysis were frozen immediately and stored at –70° C. The biopsies for immunohistochemistry were rinsed in physiological saline solution and fixated in a

4% formaldehyde solution for a maximum of 24 hours, thereafter dehydrated in 70% ethanol solution and embedded in paraffin.

Not all subsequent analyses were performed on all 58 women, due to the limited amount of tissue retrieved from some of them.

**2.3 Tissue homogenization and extraction of RNA**

Tissue was homogenized with a dismembranation apparatus (Retsch KG, Haan, Germany). This was followed by either RNA extraction or protein extraction. Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

**2.4 Treatment with DNAse and reverse transcription (RT)**

The concentration of total RNA obtained was determined employing a NanoDrop$^{TM}$ 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). All samples had an OD260/OD280 ratio higher than 1.8. One μg of total RNA, pre-treated with 1 μl RQ1 RNase-Free DNase (Promega, Madison, WI, USA), was used for RT reaction, which was performed using SuperScript$^{TM}$ RNase H$^{-}$ Reverse Transcriptase (Invitrogen, Carlsbad, California, USA) as described elsewhere (Tornblom et al. 2005a). The cDNA was stored at -70$^{o}$ C prior to use.

**2.5 Real-Time RT-PCR**

The levels of mRNA encoding HMGB1, esRAGE, RAGE, TLR2 and TLR4 were quantified by real-time RT-PCR with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously (Dubicke et al. 2008). Appropriate primers and probes were purchased from commercially available Taqman® gene expression assays (Applied Biosystems). Assay IDs and Reference Sequence database accession numbers are presented in Table 2. For each reaction, 5 μl of diluted cDNA

(corresponding to 10 ng total RNA) was used. 18S, β-actin and cyclophilin A were used as endogenous controls. The geometric mean of these three endogenous controls was used for normalizing the mRNA levels for the gene of interest (Vandesompele et al. 2002). Relative gene expression was calculated using a $\Delta\Delta C_T$ method, where the non-pregnant group was used as a control group. The geometric mean of $C_T$ of endogenous controls was subtracted from the $C_T$ of the respective gene, followed by subtraction of the median $\Delta C_T$ value of the control group, giving the $\Delta\Delta C_T$. The amount of products doubles in each cycle, so the relative gene expression was calculated with the formula $2^{-\Delta\Delta C_T}$, given in the manufacturer's instructions. Serial dilutions of placental cDNA made from purchased total RNA (Ambion, Austin, TX, USA) were used for validation of the experiment.

**2.6 Protein extraction**

For protein extraction, 1 ml of phosphate-buffered saline (PBS), including 0.01% Triton X-100 (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and protease inhibitor cocktail 25 Complete™ (Boehringer Mannheim GmbH, Ingelheim, Germany), was added to tissue homogenates. After centrifugation at 10000g, 4°C for 10 min, the supernatant was retrieved and stored in aliquots at -70 °C until analyzed. Total protein concentration was determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA) according to the manufacturer's instructions.

**2.7 Determination of the protein levels of HMGB1 and sRAGE**

The concentrations of HMGB1 and all soluble forms of RAGE (sRAGE) in the supernatants were determined employing a HMGB1 ELISA kit II (Shino-test Corporation, Kanagawa, Japan) and a Human RAGE ELISA kit (Quantikine®, R&D systems, Minneapolis, MN, USA) respectively according to the manufacturers' instructions. The results were interpolated

168 from the standard reference curve provided with each kit. The sensitivity of the kits was 1
169 ng/ml for HMGB1 and 4 pg/ml for RAGE. The concentrations of HMGB1 and RAGE were
170 normalized against the total protein concentration.
171
172 **2.8 Immunohistochemical staining**
173 For detection of HMGB1, RAGE, TLR2 and TLR4 tissue expression, slides were
174 deparaffinized in Bioclear (Bio Optica, Milan, Italy) and rehydrated with ethanol.
175 Subsequently, antigen was retrieved with microwave irradiation in ethylenediamine
176 tetraacetic acid (EDTA) buffer (pH 9.0) (for HMGB1, RAGE, TLR2) or sodium citrate buffer
177 (pH 6.0) (for TLR4). To block endogenous peroxidase activity, sections were treated with
178 0.3% $H_2O_2$, followed by incubation with 10% normal horse serum or normal goat serum. All
179 antibodies used for staining are presented in Table 2. The primary antibodies were absorbed
180 against 10% human serum for 2 hr before being added to the specimen. Subsequently, the
181 slides were incubated with the respective primary antibody for 45 min at room temperature.
182 Biotin-labeled horse anti-goat or goat-anti rabbit antibodies (Vector Laboratories Inc.,
183 Burlingame, CA, USA) were used for detection. In each assay, controls for specificity of the
184 HMGB1 staining were included, based on parallel stainings with the primary antibodies pre-
185 incubated with blocking peptides specific for each antibody (Santa Cruz Biotechnology Inc.
186 or AbCam). All stainings were developed using an ABC ELITE and a DAB-kit (Vector
187 Laboratories Inc.) according to the instructions of the manufacturer. Sections were
188 counterstained with Mayer's haematoxylin.
189 For all immunohistochemical examinations, the immunoreactivity was checked in the
190 squamous epithelium, the glandular epithelium, the vascular endothelium and five
191 fields in the stroma. A semiquantitative scale from 0 to + + + was used for the evaluating
192 HMGB1 staining and that of the receptors RAGE, TLR2 and TLR4. The evaluation was

performed blindly by two independent investigators (A.D. and E.A.) using conventional light microscopy. The agreement between the investigators was > 90%. The discrepancy was never more than one scale step. The mean was calculated from evaluations of both observers and further used for statistical analysis.

### 2.9 Statistical analysis

Two independent groups were compared using the Mann-Whitney U test. When more than two groups were compared, the Kruskal-Wallis test was applied, followed by multiple comparison with Dunn's correction. Spearman's rho was used for analyzing non-parametric correlations. Fisher's exact test was used to test for non-random associations. In all cases a p-value of $<0.05$ was considered to be statistically significant. All calculations were performed with the STATISTICA 8.0 software (StatSoft Inc, Tulsa, OK, USA) and GraphPad Prism 5.01 (GraphPad Software Inc, CA, USA).

## 3. Results

### 3.1 mRNA expression

The mRNA expression of HMGB1 was 3-fold lower in the laboring groups (PTL and TL) than in the NP group ($p<0.01$) (Figure 1A). The same trend was seen comparing laboring groups to the TnotL group, but not statistically significant. The mRNA expression of RAGE followed a similar pattern, being 3-fold lower in the laboring groups than in the TnotL and the NP groups ($p<0.05$) (Figure 1B). esRAGE mRNA was undetectable in all the samples. In contrast, TLR2 mRNA expression was upregulated 5-fold in TL and 3-fold in PTL compared to TnotL and NP ($p<0.01$) (Figure 1C). There were no significant differences between the laboring groups and the TnotL and NP groups in mRNA expression of TLR4 (Figure 1D).

Comparing PTL and TL groups, no differences were registered in the mRNA expression of HMGB1 or RAGE (Figure 1A-B). However, there was a significant decrease in TLR2 and TLR4 mRNA expression in preterm labor compared to term ($p<0.05$) (Figure 1C-D). The mRNA expression of these two genes showed positive correlation (rho=0.57, $p<0.001$). There was no correlation between expression of HMGB1, RAGE, TLR2, TLR4 and gestational age. Subgroup analysis revealed lower mRNA expression of HMGB1 in the PPROM subgroup than in the rest of the PTL group ($p<0.05$), which was significantly higher than in the TL group ($p<0.05$) (Figure 2A). No statistically significant differences in mRNA expression were detected in the PTL subgroups, with positive vaginal and/or urinary cultures, but we observed a tendency towards lower expression of TLR2 and TLR4 in the group with bacterial growth in the cervical and/or urine cultures (Figure 2B-C).

**3.2 Protein levels of HMGB1 and sRAGE**

As expected, HMGB1 protein concentrations were high in all the samples. The highest HMGB1 concentration was in the NP group ($p<0.05$) (Figure 3A). The protein levels of sRAGE were 3-fold higher in the laboring groups than in the TnotL group ($p<0.05$). In the NP group sRAGE levels were equal to those in the laboring groups and 3-fold higher than in the TnotL group ($p<0.05$) (Figure 3B). No differences were registered between the PTL and the TL group in HMGB1 or sRAGE concentrations. There was no correlation between HMGB1 and sRAGE levels and gestational age. Subgroup analysis revealed no differences associated with positive vaginal or urinary cultures or PPROM.

**3.3 Tissue protein expression**

HMGB1, RAGE, TLR-2 and TLR-4 were all readily identified in the cervical tissue. All these proteins stained positively in squamous epithelium, vascular endothelium, glandular

epithelium and stroma. Overall, HMGB1 was expressed in the nuclei of virtually all cells in the tissue. In the laboring groups, we observed many empty nuclei and HMGB1-positivity close to the nuclear membrane in the squamous epithelium; whereas in the TnotL and the NP groups the nuclear HMGB1 protein expression was more evenly distributed in the entire nucleus (Figure 4A-D). We found empty nuclei in a vast majority of the laboring groups (8 of 9 samples in the PTL group and in 10 of 11 samples in the TL group). This observation was statistically significant compared to the TnotL group, where no empty nuclei were observed in any of the samples ($p<0.01$). More extranuclear staining was also seen in the stroma in the laboring groups (Figure 4F-I). The corresponding negative control sections demonstrated no staining (Figure 4E, J). The staining of HMGB1 was significantly lower in squamous epithelium and stroma in the laboring groups than in the TnotL group ($p<0.05$) (Figure 6A-B). There were large variations in RAGE expression between the patients within the same group and no obvious group-differences were registered (data not shown). There was a tendency towards lower staining of TLR2 in the squamous epithelium in the laboring groups (Figure 5A-D). This difference was statistically significant comparing TL to TnotL and NP ($p<0.05$) (data not shown). The nuclei in the squamous epithelium stained positive for TLR2 in TnotL, whereas in the laboring groups nuclei stained negatively (Figure 5A-D). Also, TLR2 expression in stroma and vessels was significantly lower in the laboring groups than in the TnotL group (Figure 6C, data in stroma). The corresponding negative control section showed no staining (Figure 5E). Further, TLR4 expression in squamous epithelium, stroma and vessels was significantly lower in the laboring groups than in the TnotL and the NP group (Figure 5F-I, Figure 6D data in stroma). The corresponding negative control showed no staining (Figure 5J).

No significant differences were registered in HMGB1, RAGE, TLR2 or TLR4 staining comparing the PTL and TL groups. Subgroup analysis in the PTL group revealed a tendency

to higher TLR4 protein expression in squamous epithelium in the PPROM subgroup than in the rest of the PTL group (p=0.06), and was significantly higher than in the TL group (p=0.04) (Figure 6E). There was a tendency towards higher TLR4 expression in squamous epithelium in the group with bacterial infection than in the non-infected group (p=0.05) (Figure 6F).

## 4. Discussion

We present the novel finding of both nuclear and cytoplasmic localization as well as mRNA expression of HMGB1 in human cervix with distinct changes in the laboring cervix.

HMGB1 has pro-inflammatory properties and its release to the cytoplasm and further to the extracellular milieu is associated with active stages of chronic inflammatory diseases such as rheumatic arthritis, lupus erythematosus and others (Kokkola et al. 2002; Barkauskaite et al. 2007). Cervical ripening and parturition are considered to be inflammatory processes (Sennstrom et al. 2000), but very little is known regarding the role of HMGB1 in the labor process. Holmlund et al. recently demonstrated that placental HMGB1 protein expression did not change during active labor, although there was a tendency towards higher cytoplasmic HMGB1 expression in the decidua of preeclamptic pregnancies (Holmlund et al. 2007). Here, we show release of HMGB1 from the nuclei in the squamous epithelium as well as more cytoplasmic staining in the stroma in the cervix during preterm and term labor. This is in agreement with earlier findings by our group of higher levels of pro-inflammatory cytokines and matrix metalloproteinases (MMPs) in the laboring cervix both at term and at preterm (Tornblom et al. 2005a; Dubicke et al. 2008). The lower HMGB1 protein expression in cervix observed during labor could be explained by a release of HMGB1 from nuclei to the cytoplasm and/or the extracellular space and later to systemic circulation, a phenomenon also seen in sepsis (Sunden-Cullberg et al. 2005). One striking finding here is that in analyzing the

292 subgroups of preterm labor, we found lower mRNA expression of HMGB1 in the PPROM
293 group than in the rest of PTL group, where such expression was higher than in term labor.
294 This suggests that PPROM and PTL could partly involve different mechanisms (Menon et al.
295 2001; Hajek et al. 2008) and that cervical ripening at preterm may differ from that at term.
296 Interestingly, we saw high levels of both mRNA and protein expression of HMGB1 in the
297 non-pregnant cervix. However, we used samples only from four older non-pregnant women,
298 undergoing hysterectomy due to myomas, which could be associated with inflammatory
299 reaction (Miura et al. 2006).
300 We observed the largest changes in HMGB1 immunoreactivity in the cervical squamous
301 epithelium during labor. Earlier studies from our group have identified fetal fibronectine
302 (Sennstrom et al. 1998), interleukin-8 (Sennstrom et al. 2000), MMP-8 (Sennstrom et al.
303 2003), corticotropin-releasing hormone (Klimaviciute et al. 2006) and syndican-1 (Sahlin et
304 al. 2008) in the cervical epithelium. Together, this suggests that cervical epithelium is
305 important in the signaling process during cervical ripening.
306 It has been proposed that HMGB1 signals through receptors including RAGE, TLR2 and
307 TLR4. We detected both mRNA and protein expression of these receptors in human cervix.
308 These findings indicate a functional role of HMGB1 in the cervix. We saw downregulation
309 cell-surface RAGE mRNA during labor, but higher protein levels of sRAGE in laboring
310 cervix compared to elective cesarean section. Soluble RAGE is thought to compete with
311 RAGE and to block inflammatory action of HMGB1 (Geroldi et al. 2006). However, *in vitro*
312 study has shown that sRAGE can also act as a pro-inflammatory and chemotactic molecule
313 (Pullerits et al. 2006). We also found high levels of sRAGE in non-pregnant subjects, higher
314 than in term pregnant women and equal to the levels in the laboring cervix. This finding is in
315 line with findings in the maternal circulation, where higher sRAGE levels were registered in
316 non-pregnant women, lower in pregnancy and again higher in threatening preterm labor

317 (Hajek et al. 2008). Conversely, in amniotic fluid sRAGE levels decrease in spontaneous
318 labor (Romero et al. 2008). This suggests different roles of sRAGE in different parts of the
319 reproductive tract.
320 We found an upregulation of TLR2 mRNA during labor, which is consistent with earlier
321 findings in fetal membranes (Kim et al. 2004). Hassan et al also described a TLR2
322 upregulation in microarray analysis of cervical tissue, but this was not confirmed by real-time
323 RT-PCR (Hassan et al. 2006). Interestingly, we saw lower mRNA expression of TLR2 and
324 TLR4 in the cervix at preterm labor compared to term, which confirms that there could be
325 some differences in the process of cervical ripening at preterm (Tornblom et al. 2005b;
326 Dubicke et al. 2008). Subgroup analysis showed a tendency for further downregulation in the
327 group with bacterial infection. TLR2 and TLR4 could be downregulated in response to
328 stimuli inducing or secreted during preterm pro-inflammatory labor. On the other hand, a
329 lower baseline mRNA expression of TLR2 and TLR4 could render these women more
330 susceptible to preterm labor, as polymorphisms in these genes are associated with impaired
331 receptor function and preterm birth (Lorenz et al. 2002; Krediet et al. 2007). In vitro studies
332 show downregulation of TLR4 in monocytes after stimulation with LPS (Amoudruz et al.
333 2005), which could be a possible mechanism for LPS tolerance. Moreover, lower levels of
334 TLR2 are registered in trophoblasts from placenta with chorioamnionitis (Rindsjo et al.
335 2007). We also saw lower protein expression of TLR2 and TLR4 in the laboring groups.
336 This is in agreement with previous findings of a strong trend towards lower TLR4 expression
337 in placenta during labor (Kim et al. 2005). The lower protein levels of TLR2 and TLR4 could
338 be necessary for successful delivery. However, higher levels of TLR2 and TLR4 are seen in
339 fetal membranes in chorioamnionitis (Kim et al. 2004), suggesting a differential
340 responsiveness to inflammatory and/or infectious stimuli within the placenta.

In the contrast to the reduced TLR4 mRNA expression, we saw a tendency towards higher tissue expression of TLR4 in the squamous epithelium in the group with bacterial infection, which could be due to translocation of TLR4 as a protective mechanism (Adams et al. 2007). In our study, there was a discrepancy between mRNA expression and the protein levels of TLR2 and TLR4. Possible explanations of this could be temporal differences between mRNA synthesis and protein expression, shorter half-life of mRNA than protein and that mRNA was prepared from the whole cervical biopsy and immunoreactivity analyzed separately in epithelium, stroma, vessels and glands.

In conclusion, we have demonstrated that HMGB1 and its receptors RAGE, TLR2 and TLR4 all are expressed and produced in the human cervix. Extranuclear expression of HMGB1 during labor suggests a possible role of HMGB1 during the process of cervical ripening. We have also demonstrated lower mRNA expression of TLR2 and TLR4, but higher expression of HMGB1, in preterm labor compared to term. This suggests possible differences in the mechanism of cervical ripening at preterm and at term.

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471 involution. Biochim Biophys Acta 1406 (2), 203-13.
472
473
474

475

476 **Table 1** Clinical data on women included in the study

| Parameter | Preterm labor (PTL) | Term labor (TL) | Term not in labor (TnotL) | Non-pregnant (NP) |
|---|---|---|---|---|
| N | 20 | 24 | 10 | 4 |
| Age | 31 (24-38) | 31 (23-40) | 33 (26-42) | 46 (37-49) |
| Parity | 0 (0-1) | 0 (0-1) | 0 (0-2) | 0 (0-2) |
| Previous preterm births in the group | 2 | 0 | 0 | 0 |
| Previous caesarian sections in the group | 0 | 0 | 3 | 0 |
| Gestational age in fgw[a] | 34 (25-36) | 40 (38-41) | 39 (37-39) | - |
| Gestational age in days | 239 (175-256) | 282 (266-292) | 272 (264-278) | - |
| Treatment with corticosteroids | 7 | 0 | 0 | - |

477 Note: Data is presented as median (range) if not otherwise stated.
478 [a] full gestational weeks
479

480 **Table 2**. Description of gene expression assays used for Real-time RT-PCR and anti-human
481 antibodies used for immunohistochemical staining.

| | Gene expression assays | | Antibodies | | | |
|---|---|---|---|---|---|---|
| Gene/Protein | Assay ID | RefSeq[a] | Manufacturer | Cat No | Type | conc. μg/ml |
| HMGB1 | Hs01923466_g1 | NM_002128.4 | AbCam (Cambridge, UK) | ab18256 | rabbit | 0.2 |
| RAGE | Hs00153957_m1 | NM_001136.3 | Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA) | sc-8230 | goat | 2 |
| TLR2 | Hs00152932_m1 | NM_003264.3 | Santa Cruz Biotechnology Inc | sc-8689 | goat | 2.7 |
| TLR4 | Hs00152939_m1 | NM_138554.2 | Santa Cruz Biotechnology Inc | sc-8694 | goat | 4 |
| esRAGE | Hs00542590_m1 | NM_172197.1 | | | | |
| 18S[b] | 4319413E | X03205.1 | | | | |
| β-actin[b] | 4352935E | NM_001101.2 | | | | |
| Cyclophilin A[b] | 4326316E | NM_021130.3 | | | | |

482 [a] Reference Sequence database accession numbers
483 [b] 18s, β-actin and cyclophilin A were used as endogenous controls.
484

485
486 **Figure legends**

487 **Figure 1.** mRNA expression of HMGB1 (A), RAGE (B), TLR2 (C) and TLR4 (D) in
488 cervical tissue. Preterm labor (PTL), term labor (TL), term not in labor (TnotL), non-pregnant
489 (NP). The box represents 25-75% of all data with the line through the box representing the
490 median value. The whiskers extend to the range (min/max). *p<0.05, **p<0.01, ***p<0.001

491 **Figure 2.** mRNA expression of HMGB1, TLR2 and TLR4 in the subgroups of preterm labor
492 group. mRNA expression of HMGB1 in preterm premature rupture of membranes (PPROM),
493 the rest of preterm group in labor (PTL) and term in labor group (TL) (A). mRNA expression
494 of TLR2 (B) and TLR4 (C) in the preterm group with bacterial infection (PTL b), with
495 candida infection (PTL c), the group with negative cultures (PTL n) and term in labor group
496 (TL). Every point in the scatter plot represents one sample and the mark is median value.
497 *p<0.05.

498 **Figure 3.** Protein levels of HMGB1 (A) and sRAGE (B) in cervical tissue. Preterm labor
499 (PTL), term labor (TL), term not in labor (TnotL), non-pregnant (NP). The box represents 25-
500 75% of all data with the line through the box representing the median value. The whiskers
501 extend to the range (min/max). *p<0.05, **p<0.01.

502 **Figure 4.** Immunohistochemical staining of HMGB1 in the squamous epithelium (left
503 column) and in the stroma (right column) in cervical tissue at preterm labor (A, F), term labor
504 (B, G), term not in labor (C, H) and non-pregnant state (D, I). Negative controls (E, J).
505 Magnification x400; mark 50 μm (A-E). Magnification x1000; mark 20 μm (F-J).

506 **Figure 5.** Immunohistochemical staining of TLR2 (left column) and TLR4 (right column) in
507 the squamous epithelium in cervical tissue at preterm labor (A, F), term labor (B, G), term not
508 in labor (C, H) and non-pregnant state (D, I). Negative controls (E, J). Magnification x400.
509 Mark 50 μm.

510 **Figure 6.** Quantification of immunohistochemical staining of HMGB1 in the stroma (A) and
511 epithelium (B), TLR2 in the stroma (C) and TLR4 in the stroma (D). Preterm labor (PTL),
512 term labor (TL), term not in labor (TnotL), non-pregnant (NP). The box represents 25-75% of
513 all data with the line through the box representing the median value. The whiskers extend to
514 the range (min/max).
515 TLR4 in the squamous epithelium in preterm premature rupture of membranes (PPROM),
516 the rest of PTL group and TL group (E); in the group with bacterial infection (PTL b), with
517 candida infection (PTL c), the group with negative cultures (PTL n) and TL group (F). Every
518 point in the scatter plot represents one sample and the mark is median value. Semiquantative
519 scale 0 to + + +. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

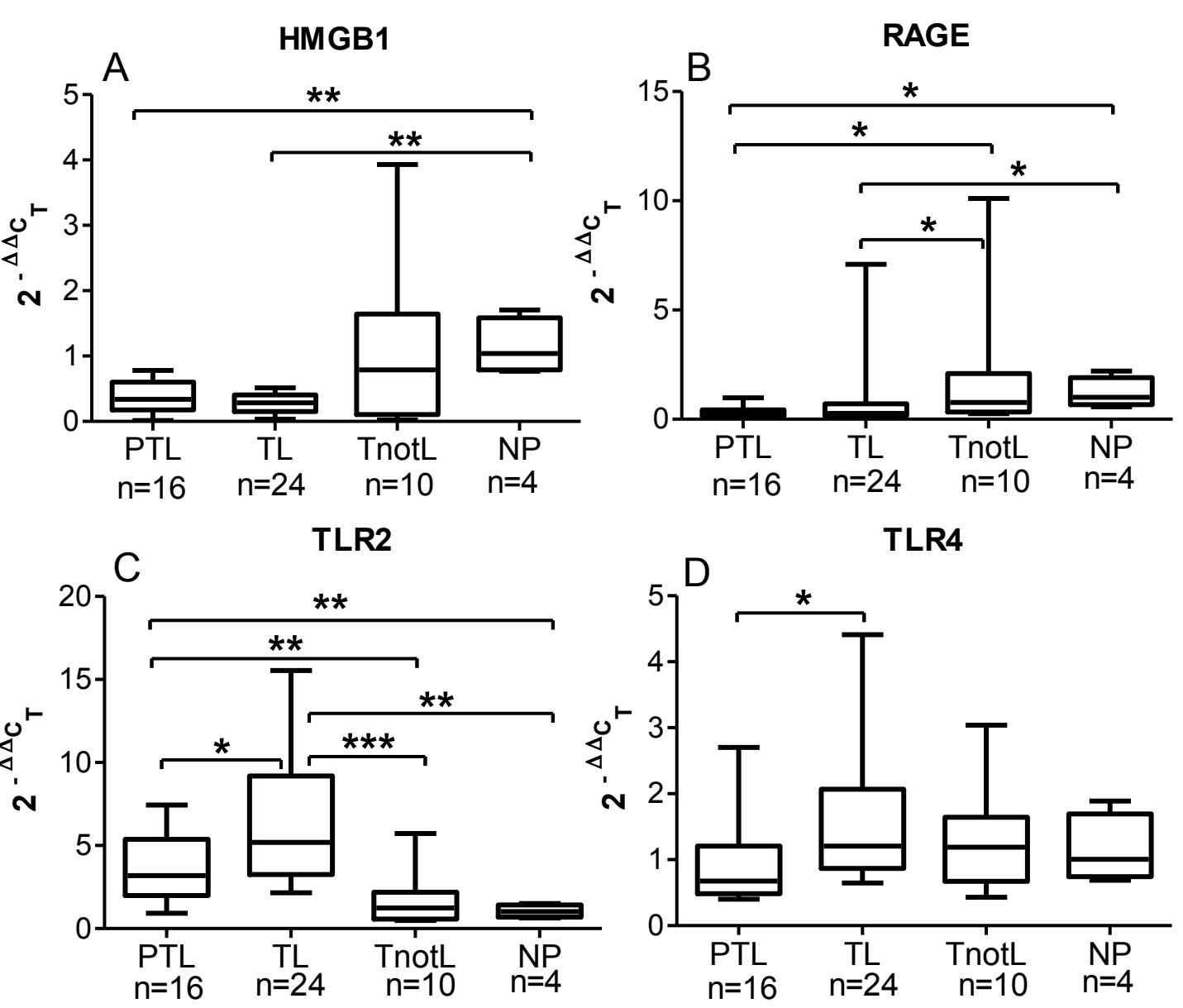
HMGB1
A
**
**
5
4
3
2
1
0
$2^{-\Delta\Delta c_T}$
PTL n=16
TL n=24
TnotL n=10
NP n=4
RAGE
B
*
*
*
*
*
15
10
5
0
$2^{-\Delta\Delta c_T}$
PTL n=16
TL n=24
TnotL n=10
NP n=4
TLR2
C
**
**
*
***
**
20
15
10
5
0
$2^{-\Delta\Delta c_T}$
PTL n=16
TL n=24
TnotL n=10
NP n=4
TLR4
D
*
5
4
3
2
1
0
$2^{-\Delta\Delta c_T}$
PTL n=16
TL n=24
TnotL n=10
NP n=4

Figure 1

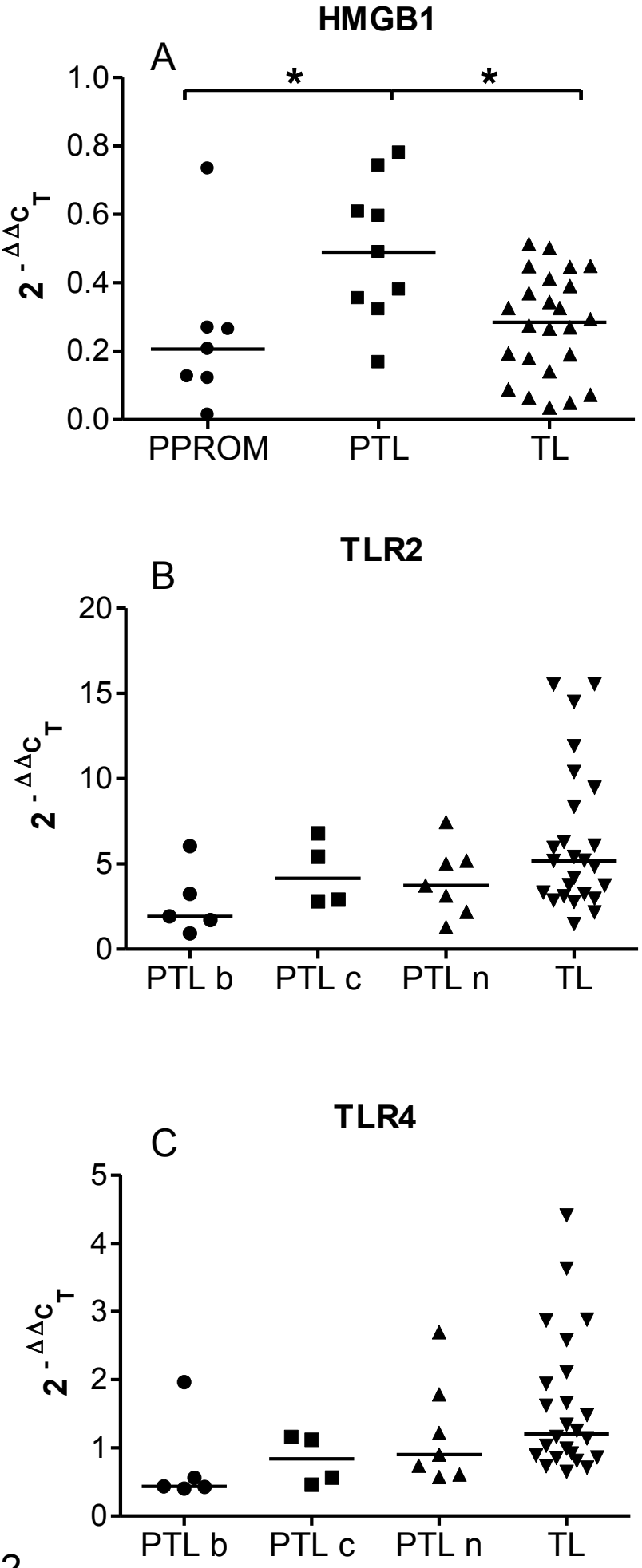
HMGB1
A
*
*
1.0
0.8
0.6
0.4
0.2
0.0
$2^{-\Delta\Delta c_T}$
PPROM
PTL
TL
TLR2
B
20
15
10
5
0
$2^{-\Delta\Delta c_T}$
PTL b
PTL c
PTL n
TL
TLR4
C
5
4
3
2
1
0
$2^{-\Delta\Delta c_T}$
PTL b
PTL c
PTL n
TL

Figure 2

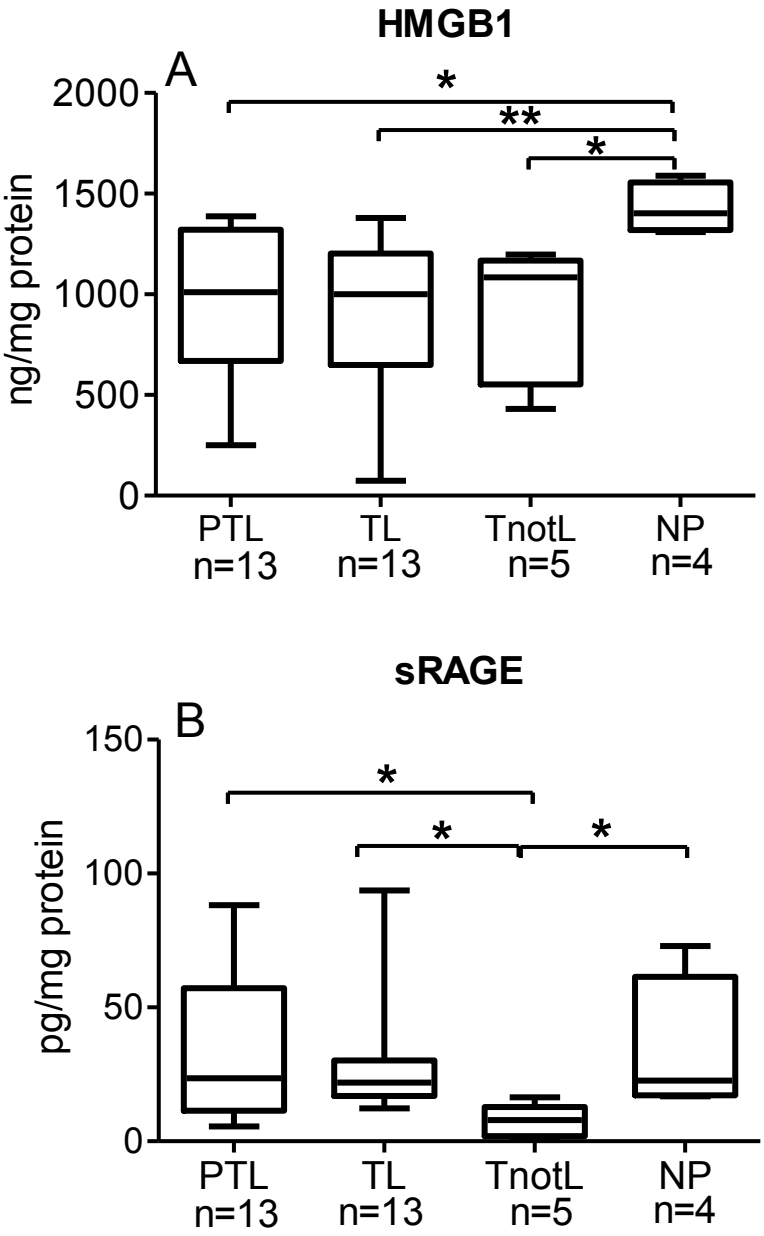
HMGB1
A
2000
1500
1000
500
0
ng/mg protein
*
**
*
PTL
n=13
TL
n=13
TnotL
n=5
NP
n=4
sRAGE
B
150
100
50
0
pg/mg protein
*
*
*
PTL
n=13
TL
n=13
TnotL
n=5
NP
n=4

Figure 3

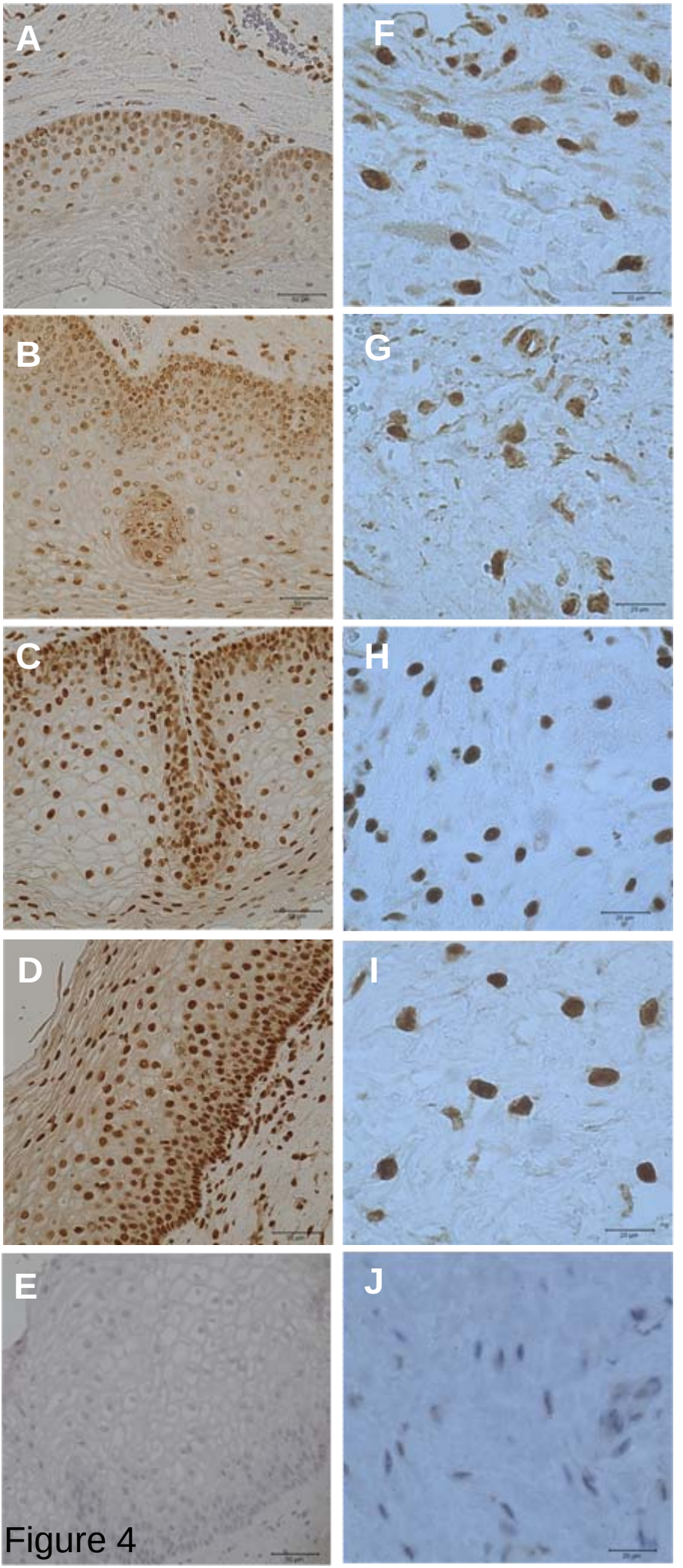


Figure 4

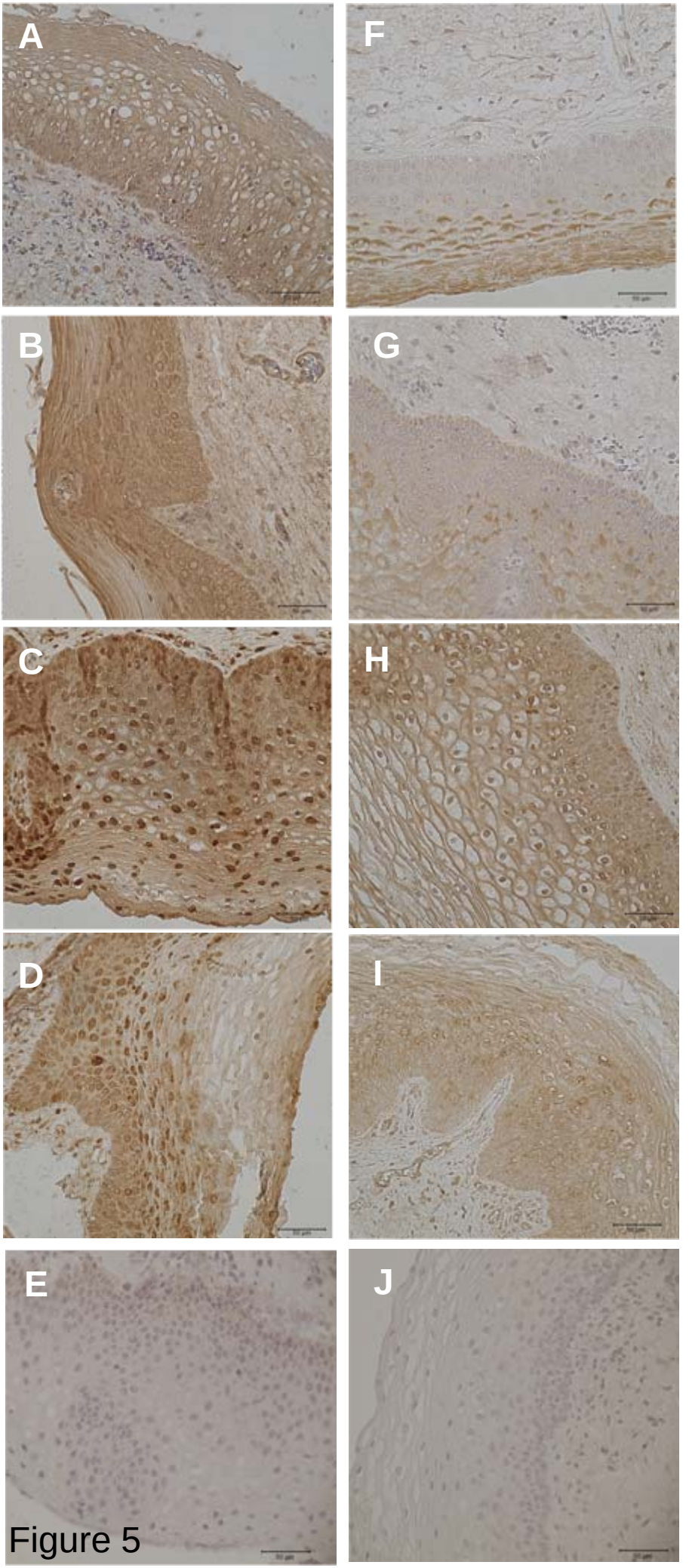


Figure 5

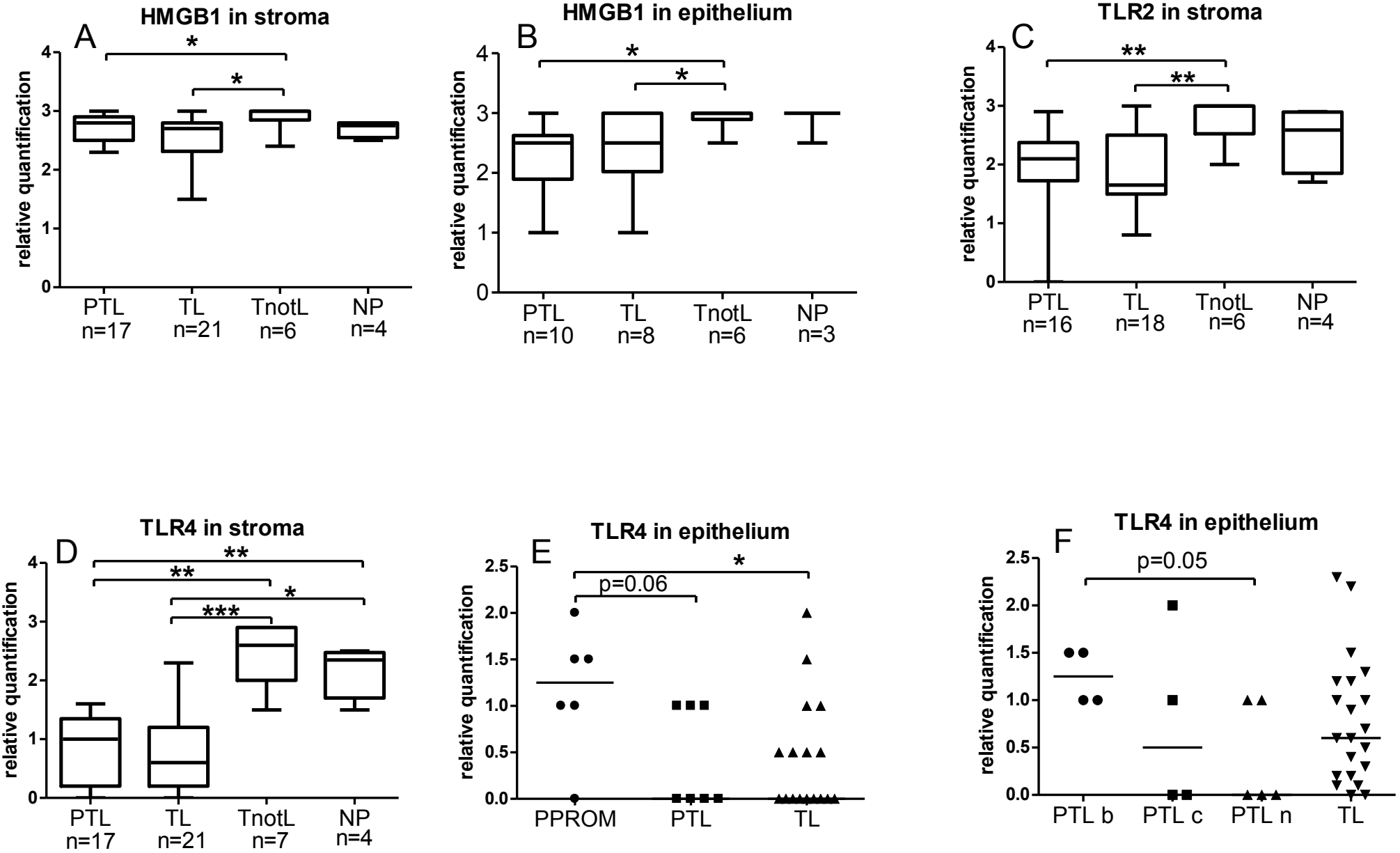
A
HMGB1 in stroma
relative quantification
PTL n=17
TL n=21
TnotL n=6
NP n=4
B
HMGB1 in epithelium
PTL n=10
TL n=8
TnotL n=6
NP n=3
C
TLR2 in stroma
PTL n=16
TL n=18
TnotL n=6
NP n=4
D
TLR4 in stroma
PTL n=17
TL n=21
TnotL n=7
NP n=4
E
TLR4 in epithelium
p=0.06
PPROM
PTL
TL
F
TLR4 in epithelium
p=0.05
PTL b
PTL c
PTL n
TL

Figure 6