RESEARCH ARTICLE

Inflammatory Signalling in Fetal Membranes: Increased Expression Levels of TLR 1 in the Presence of Preterm Histological Chorioamnionitis

Gareth J. Waring1,2, Stephen C. Robson1,2, Judith N. Bulmer2, Alison J. Tyson-Capper2*

1 Directorate of Women’s services, Newcastle Upon Tyne NHS Foundation Trust, Newcastle Upon Tyne, United Kingdom, 2 Institute of Cellular Medicine, Newcastle University, Newcastle Upon Tyne, United Kingdom

* alison.tyson-capper@ncl.ac.uk

Abstract

Histological chorioamnionitis (HCA) is an established marker of ascending infection, a major cause of preterm birth. No studies have characterised the global change in expression of genes involved in the toll-like receptor (TLR) signalling pathways in the presence of HCA in the setting of preterm birth (pHCA). Fetal membranes were collected immediately after delivery and underwent histological staging for inflammation to derive 3 groups; term spontaneous labour without HCA (n = 9), preterm birth <34 weeks gestation without HCA (n = 8) and pHCA <34 weeks (n = 12). Profiling arrays ran in triplicate for each group were used to determine the expression of 84 genes associated with TLR signalling and screen for genes of interest (fold change >2; p<0.1). Expression of identified genes was validated individually for all samples, relative to GAPDH, using RT-PCR. Expression of TLR 1, TLR 2, lymphocyte antigen 96, interleukin 8 and Interleukin-1 receptor-associated kinase-like 2 was increased in pHCA (p<0.05). Degree of expression was positively associated with histological staging of both maternal and fetal inflammation (p<0.05). The inflammatory expression profile at the maternal/fetal interface associated with pHCA, a reflection of ascending infection, is extremely heterogeneous suggesting polymicrobial involvement with activation of a common pathway. Antagonism of TLR 1 and TLR 2 signalling in this setting warrants further assessment.

Introduction

Preterm birth (PTB), birth less than 37 weeks completed gestation, is a major cause of perinatal morbidity and mortality. In the developed world it accounts for 75% of perinatal mortality and >50% long term morbidity [1]. Of the three precursor groups of PTB, spontaneous preterm labour with intact membranes and preterm premature rupture of the membranes (pPROM) are considered spontaneous preterm birth (sPTB). sPTB account for 65–75% of all PTB [1].
Infection is the principle pathologic process with an established causal link to sPTB and a defined molecular pathophysiology. Ascending infection via the vagina and cervix is the most common pathway [2–4]. Histologic chorioamnionitis (HCA) is the most specific and sensitive marker for infection [5–8] and correlates with periventricular leukomalacia (RR 1.9; 95% CI, 1.4–2.5) and cerebral palsy (RR 1.6; 95% CI, 0.9–2.7)[9]. It has been suggested that there is some benefit to HCA in that it is associated with a reduction in the incidence and severity of respiratory distress syndrome, although this is accompanied by an increased risk of bronchopulmonary dysplasia [10].

Chorioamnionitis refers to the presence of acute inflammation in the fetal membranes (chorion and amnion). There is strong relationship between HCA and gestational age at delivery; HCA is identified in 10% of term deliveries, 30% of preterm labour with intact membranes and 50% of pPROM with a highest finding of 80–90% in miscarriages between 20 and 24 weeks [11]. A major drawback in developing new interventions to treat sPTB is our poor understanding of the physiology of human parturition at term. Parturition involves a common pathway which manifests as uterine contractions, cervical ripening and decidual activation, culminating in membrane rupture and birth. This has been observed in both term and preterm birth and whilst the mechanism is not fully understood, the evidence supports the role of inflammatory mediators [12].

The innate immune system is the first line of defence against invading microorganisms at the interface of the maternal and fetal compartments. The system is responsible for establishing and maintaining a suitable microenvironment for pregnancy, recognising ‘infectious non-self’ (microorganisms) and ‘non infectious self’ (mother, placenta, fetus). Microorganisms are identified by pattern recognition receptors such as Toll-like receptors (TLRs) which recognise pathogen associated molecular patterns (PAMPs) unique to the microorganisms. Ligation of TLRs by PAMPs results in an inflammatory response generated against the invading pathogen. Upon ligand recognition TLRs recruit MyD88, an intracellular signalling adaptor protein, leading to a kinase cascade which triggers activation of the nuclear factor-kappa B (NF-κB) signalling pathway. This leads to a rapid change in gene expression producing chemokines, cytokines and antimicrobial peptides [13]. TLR-3 and TLR-4 are also able to signal in a MyD88 independent pathway (TRIF) to trigger an antiviral response [14]. There are 11 known mammalian TLRs (1–11). Expression of TLRs has been investigated in gestational tissue; mRNA expression of TLRs 1–10 and accessory proteins (e.g. CD14) has been described in the placenta [15] and temporal expression changes in TLR-2 and TLR-4 have been reported in term and preterm myometrium [16]. We have shown that TLR-4 and several cofactors of TLR activation (e.g. CD-14, MyD88 and MD-2) are up-regulated in the lower region of the uterus in advancing pregnancy but not the upper region [17]. Less is known about TLR expression and activation in the fetal membranes. Chorioamnionitis at both term and preterm is associated with increased protein expression of TLR-2 and TLR-4. TLR-2 expression has been shown to be restricted to the basal surface of the amniotic epithelial membrane in preterm labour without HCA but diffusely present across the entire epithelial cytoplasm with HCA [18]. TLR-4 has also been shown to translocate from the apical to the basal membrane in the presence of HCA [19]. Studies have traditionally focused on individual inflammatory mediators but with the advent of qPCR array technology it is now possible to examine global changes of many genes simultaneously in a single experiment. This approach has been used to assess the transcriptome of fetal membranes at term [20] and the cytokine expression at term and preterm in fetal membranes has also been reported [21].

In this study we aimed to describe the changes in gene expression in the TLR signalling pathway associated with preterm chorioamnionitis. We used qPCR array technology to screen
84 genes in the signalling pathway and sought to confirm changes in individual genes using RT-PCR.

**Materials and Methods**

**Study design: tissue selection**

A prospective study was designed to examine the differential gene expression in both amnion and chorion with and without preterm HCA. Women admitted in spontaneous labour were recruited and categorised into 3 different groups; term spontaneous labour without chorioamnionitis (TSL−CA), preterm spontaneous labour without chorioamnionitis (PTL−CA), preterm spontaneous labour with chorioamnionitis (PTL+CA). Over a 12 month period, 29 women were consented and recruited into this study. All participants were recruited from the Newcastle Upon Tyne NHS Foundation Trust Hospitals. After histological phenotyping the groups were made up as follows; TSL−CA (n = 9), PTL−CA (n = 8), PTL+CA (n = 12).

Approval for the study was granted by Newcastle and North Tyneside 1 Research Ethics Committee (Ref:10/H0906/71) and Newcastle Upon Tyne NHS Foundation Trust. Written consent was taken from all participants to collect and store the samples and to use medical outcome data from the patient and neonate for the purpose of the study. Labour was defined as the presence of regular spontaneous uterine contractions accompanied by progressive cervical dilation that lead to delivery. Term was defined as a gestational age of ≥37 weeks. Samples were only collected from women delivering <34 weeks’ gestation to capture early preterm birth.

**Tissue preparation**

Chorioamniotic membranes and placenta were collected immediately after delivery from participants. Pieces of chorioamniotic membranes were sterile dissected from the placental edge and placed in sterile ice cold phosphate buffered saline solution (1x PBS). Chorioamniotic membranes were washed thoroughly in 1xPBS to remove blood and debris. 4–5cm pieces of membrane were dissected out, amnion was separated from chorion and decidua parietalis was scraped off the chorion. Amnion and chorion were stored at -70°C. For histological examination of term control placentas, sections of the amniochorioinic membranes, umbilical cord and chorionic plate were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 3μm and stained with haematoxylin and eosin (H&E) for histological assessment. Preterm placentas were sent for routine histopathological assessment. Two membrane rolls (amnion and chorion laeve intact), two cross sections of umbilical cord and two full thickness sections of placental parenchyma including maternal and fetal surfaces were assessed for the presence of maternal and fetal inflammatory responses by a consultant gynaecological/placental histopathologist (JNB) using standard criteria [22]. Maternal inflammatory responses were assessed by the presence of chorionitis and subchorionitis (stage 1 maternal inflammatory response) and chorioamnionitis (stage 2 maternal inflammatory responses). Fetal inflammatory responses were assessed in chorionic arteries and umbilical cord vessels. Samples were designated as showing HCA with a maternal inflammatory response of stage 2 or above.

**RNA isolation and cDNA synthesis**

Total RNA was extracted using QIAzol/TRizol (Qiagen) in accordance with the manufacturer’s protocol. After the ethanol precipitation step RNA was further cleaned using the Qiagen RNeasy mini kit. This included an on-column DNase I treatment step. Integrity, quantity and purity of the RNA was verified by the A260:A230 ratio, A260:A280 ratio and gel
electrophoresis. Single stranded cDNA was synthesised using Qiagen RT² First Strand kit in accordance with the manufacturer’s protocol.

qPCR

PCR array for TLR pathway was performed by using RT²-Profiler PCR array platform (Toll-like Receptor signalling pathway array PAHS-018, SABiosciences, Qiagen). A 96-well plate contains gene-specific primer sets for 84 relevant genes for TLR signalling pathway, 5 housekeeping genes and 2 negative controls. An experimental cocktail of diluted first strand cDNA, nuclease free water and RT² qPCR master mix (Sybr Green) was mixed in accordance with the manufacturer’s protocol. Arrays were performed using ABI StepOnePlus thermocycler. For each of the experimental groups, qPCR arrays were performed in triplicate for both amnion (n = 9) and chorion (n = 9). Genes showing significant change in expression on signalling arrays were validated individually using qPCR. Real-time PCR was performed on cDNA using inventoried TaqMan (Applied Biosystems) and TaqMan Universal Master Mix II (Applied Biosystems). TaqMan GAPDH assay was selected as an endogenous control due to its consistent results as a house keeping gene in the signalling array phase of the study. Each assay was performed in triplicate (3 biological replicates per plate) for both amnion (n = 29) and chorion (n = 29) for each sample.

Statistical analyses

Clinical data was analysed using GraphPad Prism 5 software. Comparison of means was by unpaired t-test with categorical data analysed with Fisher’s exact test. Analysis of both the signalling arrays and the individual gene qPCR was carried out using Sabiosciences PCR array data analysis web portal[23]. To determine genes of interest, volcano plots were used comparing each of the three groups to each other for both amnion and chorion. Students t test was used to test for differential expression when comparing one group to another. A false discovery rate (p) threshold of 0.1 in conjunction with a fold change threshold of 2 to assign gene significance in the array analysis stage[24]. This is to screen the large amount of data generated and hone in on genes of interest. The same test and analysis software was used for the qPCR validation but here we used a threshold of p<0.05 to infer statistical significance. Correlation between gestational age and gene expression was estimated by least squares linear regression modelling using a significance of 0.05. Correlation between staging of inflammation (maternal and fetal) and gene expression was also estimated using least squares linear regression modelling; a p < 0.05 was used to define statistical significance.

Results

Maternal characteristics and fetal outcome data was collected and can be seen in Tables 1 and 2. The characteristics of both preterm birth groups and the term group were very similar and did not differ significantly with the expected exceptions of birth weight, gestational age at delivery and percentage receiving antenatal corticosteroids. Fetal outcomes are displayed in Table 3. A composite measure of immediate problems at birth was similar between both the preterm birth groups. A higher rate of bronchopulmonary dysplasia was found in the PTL⁺CA group however given the numbers this was not statistically significant. There was a single neonatal death, this occurred in the PTL⁺CA group.
TLR profiling array signalling in chorioamnionitis

Fig 1 shows an example of the data generated from the array analysis for amnion from PTL+CA and PTL-CA. In the amnion; comparing PTL+CA and PTL-CA there were 4 genes showing increased expression, while comparing PTL+CA and TSL-CA 7 genes showed a change in expression and comparing PTL+CA and TSL-CA there was 1 gene that changed (fold change +/- 2; \( p < 0.1 \)). In the chorion; comparing PTL+CA and PTL-CA there were 3 genes showing a change in expression, when comparing PTL+CA and TSL-CA 4 genes showed changes and comparing PTL+CA and TSL-CA there were 3 gene showing changes. The full signalling array analysis can be found. (S1, S2 and S3 Tables)

Overall the expression profile, changed for 13 different genes in the presence or absence of HCA. These were designated genes of interest; TLR1, TLR2, TLR4, TLR6, TLR7, IL-8, HMGB1, SIGIRR, MyD88, IRAK2, LY96, SARM1 and TIRAP. To validate the array work expression of each of these individual genes was assessed for all samples in the study. In this validation we did not detect TLR 7 expression and therefore data for TLR 7 is not shown.

Expression profiling in the absence of inflammation

All samples from all three groups in the study were analysed. Firstly we compared PTL-CA and TSL-CA. In the absence of HCA there was increased expression of TLR 1 (32.7 fold increase; \( p = 0.002 \)), TLR 2 (12.0 fold increase; \( p = 0.02 \)) and TLR 4 (18.4 fold increase; \( p = 0.008 \)) in the amnion of PTL-CA (S4 Table). This observation was not replicated in the chorion, although there was a trend for higher expression in the PTL-CA group. LY96 (41.1 fold increase; \( p = 0.002 \)), MyD88 (2.5 fold increase; \( p = 0.009 \)), IRAK2 (13.7 fold increase; \( p = 0.006 \)) and SARM1 (8.8 fold increase; \( p = 0.003 \)) all showed increased expression in the amnion of PTL-CA.

Table 1. Maternal characteristics.

|                      | TSL-CA | PTL-CA | PTL+CA | PTL+CA |
|----------------------|--------|--------|--------|--------|
| Mean maternal age (yrs) | 30.9   | 0.0634 | 25.9   | 0.4010 |
| Mean BMI             | 22.84  | 0.6064 | 23.88  | 0.9567 |
| Mean birthweight (g)  | 3211.1 | 0.0001 | 1963.5 | 0.0289 |
| Mean gestational age (weeks + days) | 40+2 0.0001 | 30+5 0.5377 | 29+5 0.0001 |
| % Smoker             | 11     | 0.29   | 38     | 1.00   |
| % Previous Preterm birth | 0   0.2059 | 25 0.6557 | 38 0.0537 |
| % Antenatal Corticosteroids | 0 0.0004 | 88 1.0000 | 92 0.0001 |

Difference in means assessed using unpaired t-test (\( p < 0.05 \)). Categorical data assessed using Fishers exact test (\( p < 0.05 \)).

*Comparison between TSL-CA and PTL-CA
**Comparison between PTL-CA and PTL+CA
***Comparison between TSL-CA and PTL+CA

doi:10.1371/journal.pone.0124298.t001

Table 2. Fetal outcome data.

|                      | PTL-CA n(%) | PTL-CA n(%) | p    |
|----------------------|-------------|-------------|------|
| Early fetal complications | 5 (63)     | 8 (67)      | 1.0000 |
| Bronchopulmonary dysplasia | 1 (13)     | 4 (33)      | 0.6027 |
| Neonatal death        | 0 (0)       | 1 (8)       | 1.0000 |

Data on preterm infants assessed using fishers exact test (\( p < 0.05 \)).

doi:10.1371/journal.pone.0124298.t002
In both amnion and chorion lower gestational age was significantly correlated with increased expression of TLR 1 (Fig 2), LY96, IRAK2 and the negative regulator SIGIRR. TLR 4, SARM1, MyD88 and TIRAP showed a significant correlation of increased expression with lower gestational age in the amnion only. TLR 2, TLR 6 and IL8 showed the same pattern though just in the chorion (S5 Table).

The effect of chorioamnionitis on gene expression
To assess the effect of HCA, gene expression from PTL+CA was compared firstly to TSL-CA. In keeping with our findings comparing PTL-CA and TSL-CA, MyD88 and SARM1 showed increased expression in the amnion, suggesting that the increased expression of these genes is a feature of the membrane gestational age rather than inflammatory status. TLR 1 and 2 showed increased expression in both chorion and amnion (Table 3). IL8, IRAK2 and LY96 mirrored this pattern with increased expression in both tissues.

When comparing the preterm labour groups (PTL+CA and PTL-CA), TLR 1, TLR 2 and LY96 showed increased expression with HCA (Fig 3) in the chorion. Both IL8 and IRAK2 showed increased expression in the amnion. The majority of the inflammatory genes showed a trend towards increased expression with HCA (Table 4).

We used linear regression to assess the relationship between gene expression and histological staging[22] in all preterm samples (S6 and S7 Tables). An increased maternal response and fetal response correlated with increased expression of TLR 1, TLR 2, LY96, IL8 and IRAK2 (p<0.05) in both amnion and chorion and of TLR 4 in the amnion. The data for TLR 1 and TLR 2 in the chorion is shown in Fig 4.

Table 3. Gene expression: PTL+CA vs TSL-CA.

| Gene  | Amnion p | Chorion p |
|-------|----------|-----------|
| HMGB1 | -1.2303  | 0.763159  | 1.1146  | 0.695966 |
| IL8   | 319.8713 | 0.024156  | 54.6698 | 0.037295 |
| IRAK2 | 109.7893 | 0.000412  | 27.9439 | 0.029515 |
| LY96  | 105.4454 | 0.024194  | 25.9928 | 0.000296 |
| MyD88 | 2.9342   | 0.040499  | 1.0541  | 0.708289 |
| SARM1 | 4.1177   | 0.010122  | 1.025   | 0.455544 |
| SIGIRR| 3.3354   | 0.110863  | 2.142   | 0.722095 |
| TIRAP | 1.901    | 0.121438  | 1.0724  | 0.267937 |
| TLR1  | 82.5792  | 0.035394  | 18.8113 | 0.001473 |
| TLR2  | 80.3276  | 0.045811  | 9.3475  | 0.000451 |
| TLR4  | 38.0362  | 0.0512    | 1.1705  | 0.275484 |
| TLR6  | 2.3639   | 0.034343  | 1.6584  | 0.277442 |

In both amnion and chorion lower gestational age was significantly correlated with increased expression of TLR 1 (Fig 2), LY96, IRAK2 and the negative regulator SIGIRR. TLR 4, SARM1, MyD88 and TIRAP showed a significant correlation of increased expression with lower gestational age in the amnion only. TLR 2, TLR 6 and IL8 showed the same pattern though just in the chorion (S5 Table).

Discussion
This is the first study to describe the involvement of TLR 1 in preterm histological chorioamnionitis (pHCA) separately in both amnion and chorion. Alongside TLR 1; TLR 2, LY96, IL8 and IRAK2 expression increased in pHCA when controlling for inflammation and gestational age. Increased expression of these genes, together with TLR 4, was found with higher degree of inflammation. Overall this increase in expression of multiple genes shows a heterogeneous
inflammatory picture. It is important to put this picture into perspective clinically as the host response to pathogens is recognised as the primary event in the development of clinically significant chorioamnionitis[25]. The fetal inflammatory response (FIRS) is the pathway by which chorioamnionitis is associated with clinically significant sequelae[26]. Histologically, FIRS can be seen in the fetal inflammatory response. Our study shows this increased fetal response, via staging, correlates with increased expression of the above mentioned genes and therefore we infer their involvement in clinically significant pHCA.

TLR 1 is known to form heterodimers with TLR 2 on the surface of cell membranes allowing a far greater recognition of bacterial diversity. These heterodimers pre-exist and are not induced by the ligand. With relevance to this study TLR 1/TLR 2 heterodimers can be activated by bacterial triacylated lipoproteins [27] found in gram positive bacteria and genital mycoplasms. Expression of TLR 1 in pHCA has been examined in the membranes [28]. Reassuringly
the authors also found pHCA was associated with increased expression of TLR 1 and TLR 2 when compared to preterm without HCA. As the authors did not separate amnion from chorion the results could not comment on the difference between each tissue. Our work has established this is present in the chorion in this setting with only a trend towards it in the amnion. In keeping with this, Gillaux et al.[29] attempted to model the effect of stimulation of TLR 1 and TLR 2 in amniotic epithelial cells but did not demonstrate an increase in inflammatory cytokines. TLR 1 has also been studied in other conditions; genetic variants of TLR 1 have been shown to increase susceptibility to complications of sepsis, leprosy, pelvic inflammatory disease and placental malaria [30–33]. In support of a role for TLR 1 in HCA, levels of the soluble form of TLR 1 (along with the soluble form of TLR 2 and 6) were found to be raised in amniotic fluid in cases of PTB with microbial invasion of the amniotic cavity[34]. Moreover, in gastrointestinal and intratracheal lipopolysacaride exposure in fetal sheep, designed to investigate chorioamnionitis-induced fetal gut injury, Kacerovsky et al. [31] reported mRNA upregulation of TLR 1 as well as TLR 2, 4 and 6[35].

We found pHCA was associated with increased expression of TLR 2 but not TLR 4 mRNA consistent with the findings of Kim et al [18]. However our study provides other evidence of TLR 4 involvement in pHCA. Although we found a trend for increased TLR 4 expression with pHCA we did find increased expression of the key accessory protein for LPS-induced TLR 4 signalling, LY96. LY96 cooperates with both TLR2 and TLR4 in the innate immune response. TLR interacting protein MyD88 acts via IRAK2 to activate NFκB. SARM1 is a negative regulator of MyD88 dependant TLR signalling Further the correlation between the degree of histological inflammation and increased expression of TLR 4, whilst not as strong as TLR 1, 2 and LY96, would also point towards association. Therefore it would appear that TLR 1, TLR 2 and TLR 4 may all appear to play a role in signalling in pHCA.

The explanation for the heterogeneous inflammatory profile generated by pHCA is likely due to the activation of specific TLRs by their ligands (both exogenous and endogenous). The
Fig 3a. TLR1 and TLR2 Amnion

Figure 3b. TLR1 and TLR2 Chorion

Fig 3. Fig 3a TLR expression in the amnion: Preterm labour with and without chorioamnionitis is associated with increased expression of TLR 1 and TLR 2 compared to term labour. All individual samples represented on plot, black line represents mean of samples. A significantly increased mean expression of TLR1 and TLR 2 was noted in both the preterm birth groups when comparing to TSL-CA. (TLR1; PTL+CA mean expression 82.58 vs TSL-CA; p = 0.035, PTL-CA mean expression 32.73 vs TSL-CA; p = 0.002) (TLR2; PTL+CA mean expression 80.33 vs TSL-CA; p = 0.046, PTL-CA mean expression 32.73 vs TSL-CA; p = 0.002)
exogenous ligands from a number of gram positive and negative bacteria from the lower genital tract, pathogenic mycoplasmas and microorganisms from the oral tract have all been associated with sPTB. Microbiological studies in sPTB have found problems determining the relevance of culture-proven infection, given the polymicrobial nature of sPTB. Jones et al. reported 2 or more bacterial species in over 60% of placentas and membranes collected following preterm birth [36]. Different types of microorganisms have been shown to have markedly different and distinct effects upon fetal membrane TLR expression patterns; mycoplasma hominis (but not ureaplasma urealyticum, gardnerella vaginalis or Group B Streptococcus) has been associated with increased expression of TLR 4, TLR 6 and TLR 8 mRNA in term membranes [37]. Urea-plasma parvum has been reported to upregulate TLR 1/TLR 2 and TLR 6/TLR 2 heterodimers [38] although Triantafilou et al. demonstrated involvement of TLR 6/TLR 2 and TLR 9[39]. The heterogeneity of identified organisms and the inflammatory pattern generated makes tailoring potential interventions challenging. Variation may also be explained by TLRs reacting to endogenous 'danger signals' such as damage associated molecular patterns (DAMPs), released when cells are damaged, rather than simply by the presence of microorganisms, and both TLR 2 and TLR 4 have multiple known endogenous ligands [40].

In the setting of sPTB, it is the association with inflammation rather than culture-proven infection that correlates with both adverse outcomes and pathology[41]. Our study clearly shows that increased level of expression of TLR 1 and TLR 2 correlate with increased histological inflammation. Though the study numbers are too small to make any meaningful comment on the neonatal outcome data, the literature shows pHCA is clearly linked to the development of cerebral palsy and increased concentrations of proinflammatory cytokines in amniotic fluid and cord blood [42]. It stands to reason the prevention or amelioration of inflammation via TLR signalling could prove effective in both reduction in sPTB and poor neonatal outcomes.

Table 4. Gene expression: PTL+CA vs PTL-CA.

| Gene   | Amnion | p       | Chorion | p       |
|--------|--------|---------|---------|---------|
| HMGB1  | -1.4171| 0.351268| 1.195   | 0.928029|
| IL8    | 35.2146| 0.04856 | 29.7566 | 0.051431|
| IRAK2  | 8.0085 | 0.026099| 11.0543 | 0.050549|
| LY96   | 2.5618 | 0.10761 | 10.2697 | 0.002528|
| MyD88  | 1.1651 | 0.282318| 1.1852  | 0.691993|
| SARM1  | -2.1468| 0.164682| -1.7167 | 0.663633|
| SIGIRR | 1.7599 | 0.686741| -1.2182 | 0.3628  |
| TIRAP  | -1.1721| 0.846974| 1.0561  | 0.603179|
| TLR1   | 2.5228 | 0.199452| 6.9289  | 0.0395  |
| TLR2   | 6.6696 | 0.111477| 6.9289  | 0.00355 |
| TLR4   | 2.0698 | 0.189518| 1.1847  | 0.44966 |
| TLR6   | -2.5174| 0.198552| 1.3163  | 0.771412|

Mean expression values shown. Students t-test used to test for significance (p<0.05). Expression normalised to GapDH. Gene expression assessed by fold change (2^ΔΔCT).

doi:10.1371/journal.pone.0124298.t004
Fig 4. Severity of histological staging of chorioamnionitis correlates with expression of TLR 1 and TLR 2. This figure shows the relationship between expression of TLR 1 and TLR 2 in the chorion and staging of maternal (4a) and fetal (4b) inflammation. Least squares linear regression was used. All samples staged via Redline criteria included. Higher staging of maternal inflammation correlates with higher expression of TLR1 (R² = 0.332; p = 0.008) and TLR 2 (R² = 0.475; p = 0.001). Higher staging of fetal inflammation shows a similar pattern for TLR 1 (R² = 0.199; p = 0.049) and TLR 2 (R² = 0.433; p = 0.002). Expression normalised to GapDH. Gene expression assessed by fold change (2^ΔΔCT). 

doi:10.1371/journal.pone.0124298.g004
Indeed magnesium sulphate, a compound shown to be neuroprotective in the setting of preterm birth, has been shown to decrease TLR stimulated production of proinflammatory cytokines from neonatal monocytes [43].

Though TLR 1, TLR 2 and TLR 4 have distinct ligands, they activate a common pathway via the IRAK family and nuclear activation of transcription factor NFκB. Targeting downstream events in this pathway with immunomodulators has been shown to inhibit the proinflammatory cascade and resultant uterine activity in the non human primate model of sPTB [44–46]. This however may not protect the fetus from adverse sequelae associated with ascending infection. TLRs fulfill many of criteria regarded as essential to consider them therapeutic targets; they are over expressed in disease, knockout mice are resistant to disease, ligands exacerbate inflammation and genetic difference in TLRs correlate with risk of disease. Indeed outside of reproductive science, there are clinical phase trials using agonism/antagonism of TLR signalling in viral and bacterial infections, cancer and autoimmune disorders[13]. Further, in the setting of preterm birth TLR 4 antagonism inhibits LPS-induced uterine contractility and proinflammatory cascade in pregnant rhesus monkeys [47]. Antagonism of TLR 1 and TLR 2 in the setting of ascending infection in the fetal membranes has not yet been assessed. Our findings provide the first evidence that signalling events via these receptors are likely to be required for the resulting inflammation seen in pHCA. While the results need confirmation, our findings suggest an intervention focusing on a single TLR is unlikely to be successful in this setting.

Though we are confident that our results are robust there are some potential limitations to our study. The limitations relate to the difficulty of defining a preterm control group. The phenotyping of the preterm groups was based on the presence or absence of histological inflammation. Whilst samples from PTL CA did not display HCA there were other placental findings such as features of uteroplacental insufficiency, ischaemia and villitis. Given that sPTB is not a normal process we did anticipate that though PTL CA would not have HCA they also would not be ‘normal’ and we accepted this would be a limitation. We also considered lower stages of histological inflammation. The stage of maternal inflammation at which point HCA is stated to present is stage 2[22]. As you can see from Fig 4, study samples showing stage 1 inflammation (subchorionitis) and included in PTL CA, displayed higher levels of TLR 1 expression than those with a complete absence of histological inflammation. The inclusion of these samples in PTL CA can make conclusions based on comparisons of this group with TSL CA less reliable.

Our study was the first to consider the expression of TLR signalling in both the amnion and chorion separately in pHCA. The fetal membranes are composed of these distinct layers. The amnion comprises a single layer of cuboidal epithelial cells and a thin layer of connective tissue. The chorion is comprised of somatic mesoderm (in contact with the amnion) and extra villous trophoblast. In our study the pattern of expression was similar in both tissues regardless of the group. However, when comparing the 2 preterm groups it was in the chorion that expression of TLR 1, TLR 2 and LY96 was significantly increased with pHCA. There is debate in the literature about the precise order in which the series of events transpire in ascending infection. This relates the initial focus of infection. It has been argued that process of chorioamnionitis, as seen via the histological staging, is a response to infection in the amniotic fluid with associated amniotrophism (diffuse infiltration of maternal neutrophils from decidua towards the amniotic cavity). This view has been supported by work showing a higher 16sRNA gene copy number in the amnion when compared to the chorion for each stage of chorioamnionitis [48]. This would suggest that the organisms ascend, cross the membranes without causing an inflammatory response until the amniotic fluid is involved. Alternatively, it has also been argued that the inflammatory response is generated in response to bacterial invasion of the choriodecidual space secondary to colonisation of the uterus[49]. Colonisation of the uterus may precede pregnancy and only when the endometrial cavity is sealed by the expanding membranes in mid
pregnancy does this infection become symptomatic. This raises the question as which of the fetal membranes is initially involved in ascending infection and highlights the importance of considering both.

Supporting Information

**S1 Table. Signalling array analysis: PTL\textsuperscript{+CA} vs TSL\textsuperscript{-CA}.** Mean expression values shown. Students t-test used to test for significance (p<0.05). Expression normalised to GapDH. Gene expression assessed by fold change (2\textsuperscript{ΔΔCT}).

(DOCX)

**S2 Table. Signalling array analysis: PTL\textsuperscript{-CA} vs TSL\textsuperscript{-CA}.** Mean expression values shown. Students t-test used to test for significance (p<0.05). Expression normalised to GapDH. Gene expression assessed by fold change (2\textsuperscript{ΔΔCT}).

(DOCX)

**S3 Table. Signalling array analysis: PTL\textsuperscript{+CA} vs PTL\textsuperscript{-CA}.** Mean expression values shown. Students t-test used to test for significance (p<0.05). Expression normalised to GapDH. Gene expression assessed by fold change (2\textsuperscript{ΔΔCT}).

(DOCX)

**S4 Table. Gene expression: PTL\textsuperscript{-CA} vs TSL\textsuperscript{-CA}.** Mean expression values shown. Students t-test used to test for significance (p<0.05). Expression normalised to GapDH. Gene expression assessed by fold change (2\textsuperscript{ΔΔCT}).

(DOCX)

**S5 Table. The relationship between gene expression and gestational age without inflammation (PTL\textsuperscript{-CA} and TSL\textsuperscript{-CA}).** Least squares linear regression (p<0.05) was used. Expression normalised to GapDH. Gene expression assessed by fold change (2\textsuperscript{ΔΔCT}).

(DOCX)

**S6 Table. The relationship between gene expression and histological staging (maternal).** All samples from PTL\textsuperscript{+CA} and PTL\textsuperscript{-CA} were examined. Least squares linear regression (p<0.05) was used. Expression normalised to GapDH. Gene expression assessed by fold change (2\textsuperscript{ΔΔCT}).

(DOCX)

**S7 Table. The relationship between gene expression and histological staging (fetal).** All samples from PTL\textsuperscript{+CA} and PTL\textsuperscript{-CA} were examined. Least squares linear regression (p<0.05) was used. Expression normalised to GapDH. Gene expression assessed by fold change (2\textsuperscript{ΔΔCT}).

(DOCX)

Acknowledgments

We acknowledge and appreciate the work of Barbara Innes in preparing the samples for histological examination. We also acknowledge and thank Women’s Services at the Royal Victoria Infirmary, Newcastle Upon Tyne for allowing us access for tissue collection.

Author Contributions

Conceived and designed the experiments: GJW SCR JNB AJTC. Performed the experiments: GJW. Analyzed the data: GJW AJTC. Contributed reagents/materials/analysis tools: GJW JNB AJTC. Wrote the paper: GJW AJTC. Performed histological analysis: JNB.
References

1. Goldenberg RL, Culhane JF, Iams JD, Romero R (2008) Epidemiology and causes of preterm birth. The Lancet 371: 75–84.
2. Blanc WA (1961) Pathways of fetal and early neonatal infection. Viral placentitis, bacterial and fungal chorioamnionitis. J Pediatr 59: 473–496. PMID: 13869795
3. Blanc WA (1953) Amniotic and neonatal infection; quick cytdiagnosis]. Gynaecologia 136: 100–110. PMID: 13095841
4. Benirschke K (1960) Routes and Types of Infection in the Fetus and the Newborn. AMA J Dis Child 99: 714–721. PMID: 13798887
5. Zhang JM, Kraus FT, Aquino TI (1985) Chorioamnionitis: a comparative histologic, bacteriologic, and clinical study. Int J Gynecol Pathol 4: 1–10. PMID: 3880150
6. Hillier SL, Martius J, Krohn M, Kiviat N, Holmes KK, et al. (1988) A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. N Enrg J Med 319: 972–978. PMID: 3262199
7. Pankuch GA, Appelbaum PC, Lorenz RP, Botti JJ, Schachter J, et al. (1984) Placental microbiology and histology and the pathogenesis of chorioamnionitis. Obstet Gynecol 64: 802–806. PMID: 6390279
8. Romero R, Salafia CM, Athanassiadis AP, Hanaoka S, Mazor M, et al. (1992) The relationship between acute inflammatory lesions of the preterm placenta and amniotic fluid microbiology. Am J Obstet Gynecol 166: 1382–1388. PMID: 1595794
9. Wu YW, Colford JM Jr. (2000) Chorioamnionitis as a risk factor for cerebral palsy: A meta-analysis. JAMA 284: 1417–1424. PMID: 10989405
10. Bersani I, Thomas W, Speer CP Chorioamnionitis—the good or the evil for neonatal outcome? J Matern Fetal Neonatal Med 25 Suppl 1: 12–16. doi: 10.3109/14767058.2012.663161 PMID: 22309119
11. Sebire NJ, Carroll SG, Newbold M, Nicolaides KH (1996) Preterm prelabour amniorrhexis: relation to histological chorioamnionitis. J Matern Fetal Med 5: 227–231. PMID: 8930793
12. Romero R, Espinoza J, Gonçalves LF, Kusanovic JP, Friel LA, et al. (2006) Inflammation in preterm and term labour and delivery, Seminars in Fetal and Neonatal Medicine 11: 317–326. PMID: 16839830
13. Hennessy EJ, Parker AE, O’Neill LA. Targeting Toll-like receptors: emerging therapeutics? Nat Rev Drug Discov 9: 293–307. doi: 10.1038/nrd3203 PMID: 20380038
14. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, et al. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301: 640–643. PMID: 12855817
15. Klaffenbach D, Rascher W, Rollinghoff M, Dotsch J, Meissner U, et al. (2005) Regulation and signal transduction of toll-like receptors in human chorioncarcinoma cell lines. Am J Reprod Immunol 53: 77–84. PMID: 15790341
16. Youssef RE, Ledingham MA, Bollapragada SS, O’Gorman N, Jordan F, et al. (2009) The Role of Toll-Like Receptors (TLR-2 and-4) and Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1) in Human Term and Preterm Labor. Reproductive Sciences 16: 843–856. doi: 10.1177/1933719109336621 PMID: 19564644
17. Tyson-Capper AJ, Zhang Q, Robson SC. Anti-Inflammatory Action of Progesterone on TLR-4 and COX-2 Activation Is Not Mediated by Nuclear Progesterone Receptors. Reproductive Sciences 17: 420.
18. Kim YM, Romero R, Chaiworapongsa T, Kim GA, Kim MR, et al. (2004) Toll-like receptor-2 and-4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis. American Journal of Obstetrics and Gynecology 191: 1346–1355. PMID: 15507964
19. Adams KM, Lucas J, Kapur RP, Stevens AM (2007) LPS induces translocation of TLR4 in amniotic epithelium. Placenta 28: 477–481. PMID: 17055575
20. Nhan-Chang C-L, Romero R, Tarca AL, Mittal P, Kusanovic JP, et al. Characterization of the transcriptome of chorioamniotic membranes at the site of rupture in spontaneous labor at term. American Journal of Obstetrics and Gynecology 202: 462.e461–462.e441. doi: 10.1016/j.ajog.2010.02.045 PMID: 20452490
21. Marvin KW, Keelan JA, Eykholt RL, Sato TA, Mitchell MD (2002) Use of cDNA arrays to generate differential expression profiles for inflammatory genes in human gestational membranes delivered at term and preterm. Mol Hum Reprod 8: 399–408. PMID: 11912289
22. Redline RW, Faye-Petersen O, Heller D, Qureshi F, Savell V, et al. (2003) Amniotic Infection Syndrome: Nosology and Reproducibility of Placental Reaction Patterns. Pediatric and Developmental Pathology 6: 435–448. PMID: 14708737
23. Sabiosciences. Available: http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php
24. Nhan-Chang C-L, Romero R, Tarca AL, Mittal P, Kusanovic JP, et al. (2010) Characterization of the transcriptome of chorioamniotic membranes at the site of rupture in spontaneous labor at term. American Journal of Obstetrics and Gynecology 202: 462.e461–462.e441. doi: 10.1016/j.ajog.2010.02.045 PMID: 20452490

25. Menon R, Taylor RN, Fortunato SJ (2010) Chorioamnionitis—a complex pathophysiologic syndrome. Placenta 31: 113–120. doi: 10.1016/j.placenta.2009.11.012 PMID: 20031205

26. Lai J, Magee F, Qiu Z, Hoube J, Von Dadelszen P, et al. (2005) Chorioamnionitis with a fetal inflammatory response is associated with higher neonatal mortality, morbidity, and resource use than chorioamnionitis displaying a maternal inflammatory response only. Am J Obstet Gynecol 193: 708–713. PMID: 16150264

27. Triantafilou M, Gamper FG, Haston RM, Mouratis MA, Morath S, et al. (2006) Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. J Biol Chem 281: 31002–31011. PMID: 16880211

28. Moco NP, Martin LF, Pereira AC, Polettini J, Peracoli JC, et al. (2013) Gene expression and protein localization of TLR-1, -2, -4 and -6 in amniocchorion membranes of pregnancies complicated by histologic chorioamnionitis. Eur J Obstet Gynecol Reprod Biol 171: 12–17. doi: 10.1016/j.ejogrb.2013.07.036 PMID: 24125907

29. Gilliaux C, Mehats C, Vaiman D, Cabrol D, Breuiller-Fouche M, et al. Functional screening of TLRs in human amniotic epithelial cells. Journal of Immunology 187: 2766–2774. doi: 10.4049/jimmunol.1100217 PMID: 21775685

30. Pino-Yanes M, Corrales A, Casula M, Blanco J, Muriel A, et al. Common variants of TLR1 associate with organ dysfunction and sustained pro-inflammatory responses during sepsis. PLoS ONE [Electronic Resource] 5: e13759.

31. Wong SH, Gochhait S, Malhotra D, Pettersson FH, Teo YY, et al. Leprosy and the adaptation of human toll-like receptor 1. PLoS Pathogens 6: e1000979. doi:10.1371/journal.ppat.1000979 PMID: 20617178

32. Taylor BD, Darville T, Ferrell RE, Kammerer CM, Ness RB, et al. Variants in toll-like receptor 1 and 4 genes are associated with Chlamydia trachomatis among women with pelvic inflammatory disease. Journal of Infectious Diseases 205: 603–609. doi: 10.1093/infdis/jir822 PMID: 22238472

33. Harman L, Bedu-Addo G, Eggelte TA, Schumann RR, Mockenhaupt FP, et al. The toll-like receptor 1 variant S248N influences placental malaria. Infection, Genetics & Evolution 10: 785–789.

34. Kacerovsky M, Andrys C, Drahosova M, Musilova I, Hornychova H, et al. Soluble Toll-like receptor 1 family members in the amniotic fluid of women with preterm prelabor rupture of the membranes. Journal of Maternal-Fetal & Neonatal Medicine 25: 1699–1704.

35. Wolfs TG, Kramer BW, Thuijs G, Kemp MW, Saito M, et al. Chorioamnionitis-induced fetal gut injury is mediated by direct gut exposure of inflammatory mediators or by lung inflammation. American Journal of Physiology—Gastrointestinal & Liver Physiology 306: G382–393.

36. Jones HE, Harris KA, Azizia M, Bank L, Carpenter B, et al. (2009) Differing prevalence and diversity of bacterial species in fetal membranes from very preterm and term labor. PLoS One 4: e8205. doi: 10.1371/journal.pone.0008205 PMID: 19997613

37. Abrahams VM, Potter JA, Bhat G, Peltier MR, Saade G, et al. Bacterial modulation of human fetal membrane Toll-like receptor expression. American Journal of Reproductive Immunology 69: 33–40. doi: 10.1111/aji.12016 PMID: 22967004

38. Shimizu T, Kida Y, Kuvano K (2008) Ureaplasma parvum lipoproteins, including MB antigen, activate NF-(kappa)B through TLR1, TLR2 and TLR6. Microbiology 154: 1318–1325. doi: 10.1099/mic.0.2007/016212-0 PMID: 18451040

39. Triantafilou M, Gianvili V, Aboklaish AF, Spiller OB, Kotecha S, et al. Synergic activation of toll-like receptor (TLR) 2/6 and 9 in response to Ureaplasma parvum & urealyticum in human amniotic epithelial cells. PLoS ONE [Electronic Resource] 8: e61199. doi: 10.1371/journal.pone.0061199 PMID: 23593431

40. Yu L, Wang L, Chen S. Endogenous toll-like receptor ligands and their biological significance. J Cell Mol Med 14: 2592–2603. doi: 10.1111/j.1582-4934.2010.01127.x PMID: 20629986

41. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friell L, et al. (2007) The role of inflammation and infection in preterm birth. Semin Reprod Med 25: 21–39. PMID: 17205421

42. Yoon BH, Romero R, Park JS, Kim CJ, Kim SH, et al. (2000) Fetal exposure to an intra-amniotic inflammation and the development of cerebral palsy at the age of three years. Am J Obstet Gynecol 182: 675–681. PMID: 10739529
43. Sugimoto J, Romani AM, Valentin-Torres AM, Luciano AA, Ramirez Kitchen CM, et al. Magnesium decreases inflammatory cytokine production: a novel innate immunomodulatory mechanism. J Immunol 188: 6338–6346. doi:10.4049/jimmunol.1101765 PMID: 22611240

44. Sadowsky DW, Haluska GJ, Gravett MG, Witkin SS, Novy MJ (2000) Indomethacin blocks interleukin 1beta-induced myometrial contractions in pregnant rhesus monkeys. Am J Obstet Gynecol 183: 173–180. PMID: 10920327

45. Sadowsky DW, Novy MJ, Witkin SS, Gravett MG (2003) Dexamethasone or interleukin-10 blocks interleukin-1beta-induced uterine contractions in pregnant rhesus monkeys. Am J Obstet Gynecol 188: 252–263. PMID: 12548226

46. Gravett MG, Adams KM, Sadowsky DW, Grosvenor AR, Witkin SS, et al. (2007) Immunomodulators plus antibiotics delay preterm delivery after experimental intraamniotic infection in a nonhuman primate model. Am J Obstet Gynecol 197: 518 e511–518. PMID: 17980193

47. Adams Waldorf KM, Persing D, Novy MJ, Sadowsky DW, Gravett MG (2008) Pretreatment with toll-like receptor 4 antagonist inhibits lipopolysaccharide-induced preterm uterine contractility, cytokines, and prostaglandins in rhesus monkeys. Reprod Sci 15: 121–127. doi:10.1177/1933719107310992 PMID: 18187405

48. Kim MJ, Romero R, Gervasi MT, Kim JS, Yoo W, et al. (2009) Widespread microbial invasion of the chorioamniotic membranes is a consequence and not a cause of intra-amniotic infection. Lab Invest 89: 924–936. doi: 10.1038/labinvest.2009.49 PMID: 19506551

49. Goldenberg RL, Andrews WW, Hauth JC (2002) Choriodecidual infection and preterm birth. Nutr Rev 60: S19–25. PMID: 12035853