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## **CHLAMYDIA TRACHOMATIS AND PLACENTAL INFLAMMATION IN EARLY PRETERM DELIVERY**

G. Ingrid J.G. ROURS, MD PhD<sup>1,2</sup>, Ronald R. DE KRIJGER, MD PhD<sup>3</sup>, Alewijn OTT, MD PhD<sup>4</sup>, Hendrina F.M. WILLEMSE, MSc<sup>2</sup>, Ronald DE GROOT, MD PhD<sup>5</sup>, Luc J.I. ZIMMERMANN, MD PhD<sup>6</sup>, Renee F. KORNELISSE, MD PhD<sup>7</sup>, Henri A. VERBRUGH, MD PhD<sup>2</sup>, Roel P. VERKOOIJEN PhD<sup>2</sup>

<sup>1</sup>Department of Paediatric Infectious Disease & Immunology, Erasmus MC, Rotterdam, The Netherlands

<sup>2</sup>Department of Medical Microbiology & Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands

<sup>3</sup>Department of Pathology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands

<sup>4</sup>Laboratory for Infectious Diseases, Groningen, the Netherlands

<sup>5</sup>Department of Pediatrics, UMC St Radboud, Nijmegen, The Netherlands

<sup>6</sup>Department of Paediatrics, Maastricht University Hospital, Maastricht, The Netherlands

<sup>7</sup>Department of Paediatrics, Erasmus MC, Rotterdam, The Netherlands

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### **Corresponding author:**

G. Ingrid J.G. Rours

Department of Paediatrics, Erasmus MC-Sophia

Dr. Molewaterplein 60

3015 GJ Rotterdam, The Netherlands

T: +31-10-7036104

F: +31-10-7036811

E-mail: [Rours@mac.com](mailto:Rours@mac.com)

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**Abstract**

*Chlamydia trachomatis* may infect the placenta and subsequently lead to preterm delivery. Our aim was to evaluate the relationship between the presence of *Chlamydia trachomatis* and signs of placental inflammation in women who delivered at 32 weeks gestation or less. Setting: placental histology and clinical data were prospectively obtained from 304 women and newborns at the Erasmus MC-Sophia, Rotterdam, the Netherlands. *C. trachomatis* testing of placentas was done retrospectively using PCR. *C. trachomatis* was detected in 76 (25%) placentas. Histological evidence of placental inflammation was present in 123 (40%) placentas: in 41/76 (54%) placentas with *C. trachomatis* versus 82/228 (36%) placentas without *C. trachomatis* infection (OR 2.1, 95% CI 1.2-3.5). *C. trachomatis* infection correlated with the progression ( $p=0.009$ ) and intensity ( $p = 0.007$ ) of materno-fetal placental inflammation. *C. trachomatis* DNA was frequently detected in the placenta of women with early preterm delivery, and was associated with histopathological signs of placental inflammation.

**Key words:** *Chlamydia trachomatis*, placental inflammation, pregnancy, prematurity

## Introduction

Preterm birth is the main cause of perinatal morbidity and mortality worldwide accounting for 60%-80% of deaths of infants born without congenital abnormalities [1]. Preterm labor is associated with placental inflammation, especially with acute chorioamnionitis [2, 3]. Many studies associated clinical and histological chorioamnionitis with acute neonatal morbidity and mortality and, at least to some degree, also with neurological impairments and chronic lung disease [4-8].

*Chlamydia trachomatis* is the most common bacterial sexually transmitted infection worldwide, including in the Netherlands [9]. Starting as cervicitis, chlamydial infection may ascend and infect the placenta or amniotic fluid, which may subsequently lead to preterm delivery. The literature regarding the association of maternal *C. trachomatis* infection with preterm delivery, however, is conflicting due to differences in study design, population and microbiological test methods [10-24].

The aims of this study were to detect *C. trachomatis* infection in placentas of women with early preterm delivery and to associate *C. trachomatis* infection with histological signs of placental inflammation. The literature is very limited regarding this association, but may be relevant to explain a component causal effect of *C. trachomatis* infection on preterm delivery. In addition, we studied the association of *C. trachomatis* infection with delivery characteristics and neonatal outcome in this selected cohort of women.

## Methods

### Patients and Design

The chorioamnionitis study, during which the placentas were collected that we used in the current *Chlamydia trachomatis* study, was prospective, observational, and non-interventional. Pregnant women, who delivered live newborns between May 2001 and February 2003 at the Erasmus MC-Sophia, Rotterdam, the Netherlands, at a gestational age of 32 weeks or less, were eligible for the study. Regular health care workers (residents, research-nursing staff, neonatologists) informed the women about the study. Enrolment took place immediately after delivery when the newborns were admitted to the neonatal intensive care unit. Women were excluded from the study if the placenta was not available for histological examination and, at a later stage, if no more placental tissue was available to test for *C. trachomatis*.

Antenatal, perinatal and neonatal data were obtained from maternal and neonatal medical records and prospectively stored in a database. Follow up of neonates was until a postnatal age of 28 days or postmenstrual age of 36 weeks. Neonates, who were transferred to another hospital, were followed in order to complete the data. Placentas were examined for evidence of infection or other pathology.

Retrospectively, the placentas were examined for the presence of *C. trachomatis*.

Maternal ethnicity was self-classified in this study. In total 32 different nationalities were registered. Most women were Dutch (70%), Turkish (7%), Surinamese (4%) or Moroccan (3%). Nationalities were clustered in six ethnic groups: Central European, Eastern European, Mediterranean, Caribbean/South-American, Asian and African. We used the following definitions. Clinical signs of chorioamnionitis were recorded and defined by the presence of two or more of the following signs: abdominal pain, cervical discharge, leucocytosis (white blood cell count  $\geq 15 \times 10^9/l$ ), fever (maternal temperature  $> 38^\circ C$ ), and raised CRP (C-reactive protein  $\geq 15\mu/L$ ). Pre-eclampsia was defined as new-onset hypertension (blood pressure  $> 140/90$  mmHg) with proteinuria [25]. Fetal distress was considered present when indicated by the obstetrician based on cardiotocographic criteria. Gestational age was estimated by ultrasonography or by using the last menstrual period when reliable. Small for gestational age (SGA): birth weight less than two standard deviations of the mean for gestational age. RDS: was defined according to Gideon [26]. Broncho-pulmonary dysplasia (BPD): dependency on oxygen supplementation at a postmenstrual age of 36 weeks. Intraventricular haemorrhage (IVH) and periventricular leucomalacia (PVL) were defined

according to Volpe [27]. Necrotising enterocolitis (NEC) was defined as stage two or higher according to Bell [28].

### **Histopathology**

Immediately after delivery placentas and membranes were fixed in formalin for at least 16 hours. Sampling was performed according to a standard protocol with two membrane rolls, two cross sections of the cord and three representative blocks of the placental disk as a minimum. The tissues were thereafter, according to routine standard methodology for pathological specimens, embedded in paraffin until histopathological examination. To prevent inter-examiner variation, the same pathologist who was specialized in perinatal pathology examined all placentas for histological evidence of inflammation. The pathologist was blinded to clinical information. Placental inflammation was categorized according to a maternal inflammatory response (MIR) and fetal inflammatory response (FIR) as suggested by the Amniotic Fluid Infection Nosology Committee [29], and was considered positive if there was any evidence of a MIR and/or FIR. The MIR was divided into three stages (acute subchorionitis or chorionitis, acute chorioamnionitis, necrotizing chorioamnionitis) representing progression of disease, and two grades representing intensity of disease; the FIR was similarly divided into three stages (chorionic vasculitis or umbilical phlebitis, umbilical vasculitis or umbilical panvasculitis, (subacute) necrotizing funisitis or concentric umbilical perivasculitis) and two grades [29].

After histopathological examination, the placentas were stored in paraffin blocks at room temperature. Before testing for *C. trachomatis*, the paraffin blocks were cut on different days using the same microtome with single use disposable blades. Care was taken to clean materials between cases to avoid contamination. The paraffin blocks were cut until the surface was straight after which five samples per paraffin block each of 10 µm thickness were cut for further microbiological analysis.

### **Microbiology**

For DNA Isolation, tissue samples were deparaffinized with xylene at room temperature and washed in ethanol. Bacterial DNA was isolated using the QIA Amp Tissue Kit (QIAGEN, Hilden, Germany). After a first lysis with proteinase K, DNA was bound to a QIAamp spin column, the column washed twice, the purified DNA eluded from the column in an elution buffer AE, and preheated at 70°C.

For DNA amplification and detection, a Real-time PCR was carried out using the Lightcycler 2.0 system and FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Almere, The Netherlands). Each PCR reaction contained 3 mM MgCl<sub>2</sub> and 0.5 μM of each of the two primers NLO 5'-ATGAAAAACTCTIGAAATCG-3' (position 1-21) and NRO 5'-CTAACTGTAAGTGCATTT-3' (position 1128-1108) to amplify *C. trachomatis* plasmid DNA [30], was denatured for 10 minutes at 95 °C, subjected to 40 cycles of 15 sec at 95 °C, 10 sec at 60 °C and 20 sec at 72 °C. To check the specificity of amplification products, a melting curve analysis was performed consisting of heating from 65 °C to 95 °C at a rate of 0.1 sec per step and holding for 20 sec at each step for data acquisition. The melting temperature for the specific amplicons was 82 °C. For each sample, the PCR was done using a separate internal validation (β-globine PCR). For each run a negative control (water) and three positive controls (dilutions of purified *C. trachomatis*) were included.

### **Ethical aspects**

The study was approved by the Medical Ethics Committee for Research on Human Subjects of the Erasmus MC, Rotterdam, the Netherlands.

### **Statistical analysis**

Statistical analysis was performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc. Chicago, Illinois, USA). Since pre-eclampsia and chorioamnionitis are two distinct potential pathways to preterm delivery, stratified analysis was done regarding the differences in maternal morbidity. For comparison of categorical variables between groups the Pearson's Chi-square test or Fisher's Exact test was used. For comparison of continuous variables the two-tailed Student's T-test was used. The Mantel-Haenszel test was used to assess trends. Statistical significance was considered P<0.05.

## Results

Three hundred and twenty three pregnant women were eligible for the study. Placental tissue and clinical data were available for 304 (94%) women and their newborns.

### ***C. trachomatis* in relation to clinical characteristics**

*C. trachomatis* was detected by PCR in placental tissue of 76/304 (25%) women. One woman had a record of *C. trachomatis* infection prior to the current pregnancy. Three women were tested and found to be positive during the current pregnancy. None of these women had *C. trachomatis* detected in their placenta. Eighty-four women received antibiotics for PPRM or signs of infection. Chlamydia-positive women received more often antibiotics than chlamydia-negative women: 32/76 (42%) versus 52/228 (23%); (OR 2.5, 95% CI 1.4-4.4), and received more often erythromycin: 14 (18%) versus 20 (9%); (OR 2.4, 95% CI 1.1-5.2). Amoxicillin-clavulanic acid and amoxicillin were prescribed more often to chlamydia-positive women, but did not differ significantly between the two groups: 19 (25%) versus 36 (16%); (OR 1.8; 95% CI 0.9-3.3).

Antenatal, clinical and delivery characteristics of women with and without *C. trachomatis* infection are compared in tabel 1. Maternal age, parity, and gravidity were similar for women in both groups and the distribution of chlamydial detection was homogenous among ethnic groups ( $P=0.8$ ). Presence of two or more clinical signs of chorioamnionitis was not significantly more often observed among chlamydia-positive women than among chlamydia-negative women. PROM did not differ between the two groups. Chlamydia-positive women had significantly less often pre-eclampsia ( $P=0.002$ ) and delivered significantly less often after fetal distress ( $P=0.03$ ) and by caesarean section ( $P=0.002$ ) than chlamydia-negative women, and their newborns were significantly less often small for their gestational age ( $P=0.02$ ). Neonatal outcomes such as RDS, BPD, IVH, PVL, and neonatal mortality rate were not significantly different between neonates born to chlamydia-positive and chlamydia-negative women (data not shown).

Since the above-mentioned differences could be due to the differences in maternal morbidity (women without and with pre-eclampsia), a stratified analysis was performed as shown in table 2. After stratification, the differences between chlamydia-positive and chlamydia-negative women became non-significant. Among the women with pre-eclampsia, neonates born to chlamydia-positive women were less often small for gestational age than those born to chlamydia-negative women ( $P=0.02$ ). Neonatal

outcomes as BPD, IVH, PVL and neonatal mortality rate were not significantly different between strata (data not shown), but RDS grade I-II was observed more often in neonates born to 9/17 (53%) chlamydia-positive women with pre-eclampsia than in 25/96 (26%) chlamydia-negative women with pre-eclampsia (OR 3.2; 95% CI 1.1-9.2; P=0.03).

### ***C. trachomatis* in relation to histological signs of placental inflammation**

Histological evidence of placental inflammation was present in 123/304 (40%) women of whom 64 (52%) had inflammation diagnosed in both maternal and fetal tissue, 50 (41%) in maternal tissue only, and four (3%) in fetal tissue only. Five (4%) placentas showed other specific features including peripheral funisitis, acute villitis, acute intervillitis with intervillous abscesses, or the presence of decidual plasma cells, but had no signs of a maternal or fetal inflammatory response. Chlamydia-positive women had significantly more often histopathological signs of placental inflammation than chlamydia-negative women: 41/76 (54%) versus 82/228 (36%); (OR 2.1, 95% CI 1.2-3.5).

The progression and intensity of inflammation of maternal tissue only and the combination of maternal and fetal tissues were assessed in relation to *C. trachomatis* infection (Table 3). For the latter combination, the highest stage or grade of inflammation in either maternal or neonatal tissue was assessed in trend analysis. Chlamydia-positive women had significantly more often signs of inflammation in both maternal and fetal tissue for progression stage 3 and intensity grade 2 in their placentas than chlamydia-negative women. In addition, a significantly increasing trend towards more frequent detection of *C. trachomatis* was observed with increasingly higher scores for intensity (P=0.04) involving maternal inflammation only, and for progression (P=0.009) and intensity (P=0.007) of inflammation involving both maternal and fetal tissues.

## Discussion

*Chlamydia trachomatis* DNA was detected in a high proportion (25%) of placentas from women who had early preterm delivery ( $\leq 32$  weeks) and was associated with histopathological signs of placental inflammation.

The strength of this study is the prospective, observational design during which the placentas and clinical data were obtained, the large number of well-described participants, a high follow-up rate, and the use of nucleic acid amplification technique (NAAT) for the detection of *C. trachomatis*. In order to prevent contamination of samples during the process of cutting, during DNA isolation or during the PCR test itself, precautions were taken. Paraffin blocks were cut in badges on different days by two different people using a similar microtome with single-use disposable blades, and care was taken to clean materials between cases. In addition, the order of cutting was recorded and no clustering was observed in the detection of *C. trachomatis* in the cuts. In addition, DNA isolation was done on different days by different laboratory technicians in different locations, and pipetting was performed using aerosol-resistant tips. Furthermore, the PCR was done using an internal validation ( $\beta$ -globine PCR) for each sample separately, and for each run using a negative control (water) and three positive controls (dilutions of purified Chlamydia). None of the negative controls became positive. Therefore, contamination seems unlikely to have influenced the results of the study.

A limitation of the study is that we did not examine placentas from full term pregnancies. Inclusion of term specimens would have enabled additional assessment of the contribution of *C. trachomatis* infection to placental inflammation and its relation to prematurity. Unfortunately, the chorioamnionitis study was initially not designed as such and retrospectively we were not able to trace term specimens from women in the same region and time period for additional comparisons. Another limitation is that although a large number of women participated in this study, the numbers in each category after stratification are small. Furthermore, residual confounding as a result of co-infection by other pathogens was not tested as part of the study. Indeed, the finding of many cases of placental inflammation in the absence of *C. trachomatis* infection would support the presence of other inflammation-inducing pathogens in this cohort of women. Another limitation was that *C. trachomatis* testing was done retrospectively and that prospectively no specimens were collected from infants, which made it impossible to study vertical transmission.

The PCR technique has been used previously to detect *C. trachomatis* in placentas [31-33], but reports focusing on the use of PCR to detect Chlamydia in placentas of early preterm deliveries are very limited and from much smaller cohorts. One case report described a stillborn at 36 weeks of gestation in whose placenta *C. trachomatis* was identified [31]. In a Chinese study 59 specimens of chorionic villi were examined that had been collected from women attending an antenatal clinic for artificial abortion within the first trimester of pregnancy, of which three cases (5%) were found to be *C. trachomatis* positive [32]. In a Croatian study of women with a miscarriage between four and 19 weeks gestation, *C. trachomatis* was detected in only one of 108 placental tissues examined [33].

A 25% prevalence of *C. trachomatis* in pregnant women is high. It is much higher than the *C. trachomatis* prevalence (4%) found in one of our previous population-based studies among 4,055 pregnant women in the same region and time period, using the same test method on urines instead of placentas, and for whom the referral centre is the hospital in which women and neonates were enrolled for the present study. However, our finding is similar to the prevalence (18%) found in the women of the latter study who delivered at 32 weeks gestation or less [34]. The difference between these studies may be due to the fact that the current study is hospital based instead of population based, and because not all women who delivered prematurely participated in the other study. Although we were not able to directly assess an association between *C. trachomatis* infection and preterm delivery from the current study, our findings support those found in the previous study.

We found no significant association of *C. trachomatis* infection with clinical signs in women, neither in the total group nor after stratification. This is not surprising since up to 80% of chlamydial infections in women are known to be asymptomatic. In addition, urogenital symptoms are often non-specific and likely to be regarded as part of the pregnancy instead of disease and not to be reported. However, we did find an association between *C. trachomatis* infection and histopathological signs of placental inflammation. Histologically proven chorioamnionitis is considered the gold standard against which other clinical predictors of inflammation should be measured [2, 35, 36]. Histological evidence of placental inflammation was present in 40% of the women and significantly more in chlamydia-positive women than in chlamydia-negative women: 54% versus 36%. Virtually all placentas had signs of maternal inflammation whereas only half the time fetal inflammation was present. Only maternal inflammation would be concordant with infection originating from the maternal side. Our findings, therefore, suggest that ascending *C. trachomatis* infection during pregnancy may extend into the

placental tissues. Such invasion of placental tissue is likely to produce an inflammatory response that may trigger preterm labor and delivery [37]. Hence, in case of unexplained preterm delivery additional information can be obtained from histopathological examination of placental tissue and from testing for *C. trachomatis* using highly sensitive nucleic acid amplification techniques.

*C. trachomatis* is transmitted from mother to newborn during passage through an infected birth canal. However, there are several, mostly anecdotal, reports of newborns delivered by caesarean section that were infected with *C. trachomatis* [38-41]. Ascending chlamydial infection in women undergoing caesarean section may be due to PROM, but the possibility of a transmembrane or transplacental route has also been suggested in the pathogenesis of neonatal chlamydial infection [38, 40]. In our study 43% of chlamydia-positive women gave birth by caesarean section of whom 27% had PROM. Since collection and testing of specimens from the newborns was not included in the design of the study, we are unable to confirm the latter hypothesis. Likewise, we cannot ascribe any neonatal respiratory disease, RDS stage I-II, to *C. trachomatis* infection.

The data presented are highly suggestive of *C. trachomatis* being a component cause of preterm delivery through a pathway that involves a transmembrane or transplacental route of transmission for chlamydial infection leading to chorioamnionitis. Further studies regarding the role of *C. trachomatis* in either pathway leading to preterm delivery are needed.

## **Conclusion**

*C. trachomatis* DNA was frequently detected in the placenta of women with early preterm delivery and was associated with signs of inflammation in the placenta.

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**Table 1** Clinical characteristics of pregnancies according to *Chlamydia trachomatis* status

	<i>C. trachomatis</i> negative n=228	<i>C. trachomatis</i> positive n=76	OR (95% CI)
<b>- antenatal characteristics</b>			
maternal age	30.9 ± 5.3	30.1 ± 5.0	NA
para	1.8 ± 1.3	1.9 ± 1.1	NA
gravida	2.2 ± 1.7	2.0 ± 1.2	NA
<b>- clinical characteristics</b>			
≥ 2 clinical signs CA	48 (21)	24 (32)	1.7 (0.97 - 3.1)
fetal distress	114 (50)	27 (36)	0.6 (0.3 - 1.0)
PROM	63 (28)	24 (32)	1.3 (0.7-2.2)
pre-eclampsia	96 (42)	17 (22)	0.4 (0.2 - 0.7)
<b>- delivery characteristics</b>			
caesarean section	146 (64)	33 (43)	0.4 (0.3 - 0.8)
gestational age	29.1 ± 1.9	29.1 ± 1.9	NA

birth weight (gram)	1124 ± 347	1207 ± 350	NA
small for gestational age	67 (29)	12 (16)	0.5 (0.2 - 0.9)

Numbers are mean ± SD or number (%), n: number, OR: odds ratio, CI: confidence interval, NA: not applicable, CA: chorioamnionitis, PROM: premature rupture of membranes

**Table 2** Clinical characteristics according to *Chlamydia trachomatis* status by strata of pre-eclampsia

	<i>C. trachomatis</i> negative	<i>C. trachomatis</i> positive	OR (95% CI)
<b>No pre-eclampsia</b>	n=132 (69)	n=59 (31)	
<b>- antenatal characteristics</b>			
maternal age	30.8 ± 5.1	30.1 ± 4.8	NA
para	1.9 ± 1.3	1.9 ± 1.1	NA
gravida	2.3 ± 1.7	2.0 ± 1.2	NA
<b>- clinical characteristics</b>			
≥ 2 clinical signs CA	47 (36)	24 (41)	1.2 (0.7-2.3)
fetal distress	41 (31)	16 (27)	0.8 (0.4 - 1.6)
PROM	60 (45)	24 (41)	0.8 (0.4-1.5)
<b>- delivery characteristics</b>			
caesarean section	59 (45)	20 (34)	0.6 (0.3 - 1.2)
gestational age	28.8 ± 2.0	29.1 ± 2.0	NA
birth weight (gram)	1194 ± 354	1223 ± 359	NA
SGA	18 (14)	9 (15)	1.1 (0.5 - 2.7)
<b>Pre-eclampsia</b>	n=96 (85)	n=17 (15)	
<b>- antenatal characteristics</b>			
maternal age	31.1 ± 5.6	30.0 ± 5.7	NA
para	1.7 ± 1.2	1.8 ± 1.1	NA
gravida	2.0 ± 1.7	1.9 ± 1.2	NA
<b>- clinical characteristics</b>			

≥ 2 clinical signs CA	1 (1)	0 (0)	NA
fetal distress	73 (76)	11 (65)	0.6 (0.2 - 1.7)
PROM	3 (3)	1 (6)	1.9 (0.2-19.8)
<b>- delivery characteristics</b>			
caesarean section	87 (91)	13 (77)	0.3 (0.1 - 1.3)
gestational age	29.5 ± 1.7	29.1 ± 1.8	
birth weight (gram)	1027 ± 314	1151 ± 321	
SGA*	49 (51)	3 (18)	0.2 (0.06 - 0.7)

Numbers are mean ± SD or number (%), n: number, OR: odds ratio, CI: confidence interval, NA: not applicable, CA: chorioamnionitis, PROM: premature rupture of membranes, SGA: small for gestational age. Analysis with Pearson's Chi-square test or Fisher's Exact test; \*P=0.02

**Table 3** Risk of *Chlamydia trachomatis* infection according to progression and intensity of placental tissue inflammation

Placenta tissue	Total, n	<i>C. trachomatis</i> positive, n (%)	OR (95% CI)#
no placenta inflammation	181	35 (19)	1.0 (reference)
any placenta inflammation##	123	41 (33)	2.1 (1.2 - 3.5)
<b>Maternal inflammation only</b>	50	16 (32)	2.0 (0.98 - 4.0)
<b>Progression*</b>			
stage 1	24	8 (33)	2.1 (0.8 - 5.3)
stage 2	20	6 (30)	1.8 (0.6 - 5.0)
stage 3	6	2 (33)	2.1 (0.4 - 11.8)
<b>Intensity**</b>			
grade 1	43	13 (30)	1.8 (0.9 - 3.8)
grade 2	7	3 (43)	3.1 (0.7 - 14.6)
<b>Maternal &amp; fetal inflammation</b>	64	22 (34)	2.2 (1.2 - 4.1)
<b>Progression***</b>			
stage 1	4	1 (25)	1.4 (0.1 - 13.7)

stage 2	20	6 (30)	1.8 (0.6 - 5.0)
stage 3	40	15 (38)	2.5 (1.2 - 5.2)
<b>Intensity***</b>			
grade 1	25	7 (28)	1.6 (0.6 - 4.1)
grade 2	39	15 (38)	2.6 (1.2 - 5.5)

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#OR compared to placenta with no inflammation, ##P=0.01

Mantel-Haenszel trend analysis: \*P=0.09, \*\* P=0.04, \*\*\*P=0.01