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Clinical chorioamnionitis at term II: the intra-amniotic inflammatory response

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Abstract

Objective—Recent studies indicate that clinical chorioamnionitis is a heterogeneous condition and only approximately one-half of the patients have bacteria in the amniotic cavity, which is often associated with intra-amniotic inflammation. The objective of this study is to characterize the nature of the inflammatory response within the amniotic cavity in patients with clinical chorioamnionitis at term according to the presence or absence of 1) bacteria in the amniotic cavity and 2) intra-amniotic inflammation.

Materials and methods—A retrospective cross-sectional case-control study was conducted to examine cytokine and chemokine concentrations in the amniotic fluid (AF). Cases consisted of women with clinical chorioamnionitis at term (n = 45). Controls were women with uncomplicated pregnancies at term who did not have intra-amniotic inflammation and were in labor (n = 24). Women with clinical chorioamnionitis were classified according to the results of AF cultures, broad-range polymerase chain reaction coupled with electrospray ionization mass spectrometry,

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and AF concentration of interleukin-6 (IL-6) into those: 1) without intra-amniotic inflammation, 2) with microbial-associated intra-amniotic inflammation, and 3) with intra-amniotic inflammation without detectable bacteria. The AF concentrations of 29 cytokines/chemokines were determined using sensitive and specific V-PLEX immunoassays.

Results—1) The AF concentrations of pro- and anti-inflammatory cytokines/chemokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), macrophage inflammatory protein-1 beta (MIP-1β), and interleukin-8 (IL-8) (except Eotaxin-3) were significantly higher in women with clinical chorioamnionitis at term than in controls (term labor without intra-amniotic inflammation); 2) patients with microbial-associated intra-amniotic inflammation, and those with intra-amniotic inflammation without detectable bacteria, had a substantially different expression of cytokines and chemokines in AF compared to patients with spontaneous labor without intra-amniotic inflammation. However, no difference could be detected in the pattern of the intra-amniotic inflammatory response between patients with intra-amniotic inflammation with and without detectable bacteria; and 3) in patients with clinical chorioamnionitis at term but without intra-amniotic inflammation, the behavior of cytokines and chemokines in the AF was similar to those in spontaneous labor at term.

Conclusions—Patients with clinical chorioamnionitis who had microbial-associated intra-amniotic inflammation or intra-amniotic inflammation without detectable bacteria had a substantial upregulation of the intra-amniotic inflammatory response assessed by amniotic fluid concentrations of cytokines. A subset of patients with term clinical chorioamnionitis does not have intra-amniotic infection/inflammation, as demonstrated by elevated AF concentrations of inflammation-related proteins, when compared to women in term labor with uncomplicated pregnancies, suggesting over-diagnosis. These observations constitute the first characterization of the cytokine/chemokine network in the amniotic cavity of patients with clinical chorioamnionitis at term.

Keywords

amniocentesis; chemokines; cytokines; funisitis; histologic chorioamnionitis; interleukin-6 (IL-6); intra-amniotic infection/inflammation; microbial invasion of the amniotic cavity (MIAC); polymerase chain reaction coupled with electrospray ionization mass spectrometry (PCR/ESI-MS); sterile inflammation

Introduction

Clinical chorioamnionitis is defined by the presence of maternal fever and at least two or more of the following clinical criteria: maternal or fetal tachycardia; maternal leukocytosis; uterine tenderness; or foul-smelling amniotic fluid (AF) [1–12]. The standard clinical treatment of this condition is the administration of antibiotics because the diagnosis is considered to represent evidence of intra-amniotic bacterial infection [8, 13–19]. This intervention is expected to reduce the rate of complications in both mother and neonate [8, 13–16]. A role for bacterial infection in clinical chorioamnionitis is based on previous microbiologic studies [3], which have been largely based on cultivation techniques [3]. Recently, we have reported, using both cultivation and molecular microbiologic techniques, that 54% of patients with clinical chorioamnionitis had microbial-associated intra-amniotic

inflammation [presence of bacteria in AF and AF interleukin (IL)-6 concentration ≥ 2.6 ng/mL], and 24% had intra-amniotic inflammation without demonstrable bacteria (absence of bacteria in AF and AF IL-6 concentration ≥ 2.6 ng/mL), whereas 22% had no intra-amniotic inflammation (AF IL-6 concentration < 2.6 ng/mL) [11].

The objective of this study was to characterize the nature of the intra-amniotic inflammatory response in patients with a diagnosis of clinical chorioamnionitis at term by analyzing the behavior of the concentrations of cytokines and chemokines in the AF, according to the presence or absence of intra-amniotic inflammation and the presence of bacteria.

Material and Methods

Study population

A retrospective cross-sectional case-control study was conducted by searching the clinical database and bank of biologic samples of Wayne State University, the Detroit Medical Center, and the Perinatology Research Branch (NICHD/NIH). The inclusion criteria were: 1) singleton gestations; 2) gestational age ≥ 37 weeks; 3) sufficient AF obtained by transabdominal amniocenteses for molecular microbiologic studies; and 4) absence of fetal malformations.

Cases were women with clinical chorioamnionitis at term. These women were included in a prior study which contains a detailed description of sample collection, microbiological studies, and determination of AF IL-6 concentrations using a sensitive and specific enzyme-linked immunosorbent assay [11].

In brief, controls were women presenting with an episode of suspected preterm labor and with an uncertain gestational age. Sonographic fetal biometry had not been performed during pregnancy, as it was largely unavailable as part of the routine prenatal care. Patients were offered an amniocentesis to evaluate the status of fetal lung maturity, to determine whether tocolysis and steroids were required, and to determine the microbial status of the amniotic cavity. The lung maturity tests included a “shake” test (or Clements’ test), or counting the number of orange cells [20–23]. Lecithin/sphingomyelin ratio and other fetal lung maturity tests were not available at the institutions where this study was conducted. These women did not have intra-amniotic inflammation (AF IL-6 < 2.6 ng/mL) and were considered to be at term because they met the following criteria: 1) analysis of AF consistent with fetal lung maturity; 2) birthweight > 2500 g; 3) absence of respiratory distress syndrome or other complications of prematurity; and 4) physical examination by a pediatrician consistent with a term neonate.

All patients provided written informed consent and the use of biological specimens as well as clinical and ultrasound data for research purposes were approved by the Institutional Review Boards of NICHD, Wayne State University, and the Sótero del Río Hospital, Santiago, Chile. All patients were enrolled at the Sótero del Río Hospital in Santiago, Chile.

Clinical definitions

Clinical chorioamnionitis was diagnosed by the presence of maternal fever (temperature > 37.8°C) accompanied by two or more of the following criteria: 1) maternal tachycardia (heart rate > 100 beats/min); 2) uterine tenderness; 3) foul-smelling odor of the AF; 4) fetal tachycardia (heart rate > 160 beats/min); and 5) maternal leukocytosis (leukocyte count > 15,000 cells/mm^3) [3, 14].

Microbial invasion of the amniotic cavity (MIAC) was defined according to the results of AF culture and polymerase chain reaction coupled with electrospray ionization mass spectrometry (PCR/ESI-MS) (Ibis® Technology – Athogen, Carlsbad, CA) [24–27]. Intra-amniotic inflammation was diagnosed when the AF IL-6 concentration was ≥ 2.6 ng/mL [28–38]. Based on the results of AF cultures, PCR/ESI-MS and AF concentrations of IL-6, patients with clinical chorioamnionitis at term were classified as having: 1) no intra-amniotic inflammation, or 2) microbial-associated intraamniotic inflammation (combination of MIAC and intra-amniotic inflammation), or 3) intra-amniotic inflammation without detectable bacteria (an elevated AF IL-6 concentration without evidence of bacteria using both cultivation and molecular methods). Acute histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or in the chorioamniotic membranes [28, 39–44], and acute funisitis was diagnosed by the presence of neutrophils in the wall of the umbilical vessels and/or in the Wharton's jelly, using criteria previously described [37, 41–43, 45–49]. Fetal inflammatory response syndrome (FIRS) was defined as an umbilical cord IL-6 concentration > 11 pg/mL [25, 45, 50–59]. Acute inflammatory lesions of the placenta, or placental lesions consistent with AF infection, were defined by the presence of acute histologic chorioamnionitis and/or acute funisitis.

Multiplex determination of cytokines and chemokines

The AF concentrations of the following 29 cytokines/chemokines were determined with sensitive and specific V-PLEX immunoassays (Meso Scale Discovery, Gaithersburg, MD, USA) [Pro-inflammatory cytokines: interferon gamma (IFN-γ), IL-1α, IL-1β, IL-2, IL-6, IL-7, IL-12p70, IL-12/IL-23p40, IL-15, IL-16, IL-17a, tumor necrosis factor alpha (TNF-α), TNF-β, vascular endothelial growth factor (VEGF), granulocyte macrophage colony-stimulating factor; anti-inflammatory cytokines: IL-4, IL-5, IL-10, IL-13; and chemokines: IL-8, thymus and activation-regulated chemokine (TARC), Eotaxin, Eotaxin-3, macrophage-derived chemokine (MDC), macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemoattractant protein (MCP)-1, MCP-4, C-X-C motif chemokine 10 (CXCL-10), or interferon gamma-induced protein 10 (IP-10)]. Briefly, 50 μL of AF or a calibrator was dispensed into separate wells of the plates and incubated for 2 h with vigorous shaking at room temperature. The samples and calibrators were discarded and the plates were washed three times with phosphate buffered saline and 0.05% Tween 20, (Meso Scale Discovery) followed by an addition of 25 μL of the 1 × Detection Antibody Solution (Meso Scale Discovery) into each well. Plates were then incubated for 2 h with vigorous shaking at room temperature. The detection antibody was removed, and the plates were washed three times. One hundred and fifty microliters of 2X Read Buffer T (Meso Scale Discovery) were added to each well and the signals were read by the SECTOR® Imager 2400 (Meso Scale Discovery). Standard curves were generated and the assay values of the samples were

interpolated from the curves. The assay characteristics are described in the Supplementary Table. The coefficient of variation was less than 15% for 17 of the 29 analytes. For samples with concentrations below the limit of detection, missing values were replaced with 99% of the lowest detectable concentration. VEGF was removed from the analysis, as 90% (62/69) of participants had VEGF concentrations below the limit of detection of the assay.

Statistical analysis

Demographics data analysis: The Kolmogorov-Smirnov test was used to test whether the distribution of continuous variables was normal. Chi-square and Fisher's exact tests were used for comparisons of proportions. Kruskal-Wallis and the Mann-Whitney U tests were used to compare median concentrations of analytes between and among groups. Statistical analysis of demographics data was performed using SPSS 19 (IBM Corp, Armonk, NY, USA). A P value <0.05 was considered statistically significant. Comparison of analyte concentrations determined by multiplex assay was restricted to the analytes that were detected in a number of samples larger than half of the size of the smallest group. Statistical analysis was performed using Wilcoxon rank-sum test and R statistical environment [60]. Nominal P values were adjusted using the Benjamini and Hochberg method [61], controlling the false discovery rate at 5%.

Results

Characteristics of the study population

A total of 45 cases with clinical chorioamnionitis diagnosed at term (between 37 and 42 weeks of gestation) and 24 controls with uncomplicated pregnancies at term that were in labor were included in this study. Descriptive characteristics of the study population are displayed in Table 1. There were no significant differences in the median maternal age, frequency of nulliparity, median AF glucose, and median birthweight between term pregnancy in labor and clinical chorioamnionitis at term (all P-values > 0.05).

Upon admission, 66.7% (30/45) of patients had ruptured membranes at the time of amniocentesis. Only 20% (9/45) of these women were admitted with fever; the remainder (80%, 36/45) developed fever after hospital admission. In addition to maternal fever, the most frequent criteria that configured the diagnosis of clinical chorioamnionitis were maternal [91.1% (41/45)] and fetal tachycardia [75.6% (34/45)], which were followed by maternal leukocytosis [73.3% (33/45)]. Most women had a vaginal delivery [75.6% (34/45)], and half had placental lesions consistent with acute AF infection (23/45) (Table 2). All patients had epidural anesthesia during labor. Amniocenteses were performed before the administration of epidural analgesia in 78% (35/45) of these patients. Among those who received antibiotics (n = 42), 88% (37/42) received them after the amniocentesis. Five patients received antibiotics before the amniocentesis (in 3 and 2 patients the amniocentesis was performed 5 and 45 min after the administration of antibiotics, respectively).

When classified according to the presence or absence of both intra-amniotic inflammation and bacteria (by AF cultures and PCR/ESI-MS), 56% (25/45) of the patients had microbial-associated intra-amniotic inflammation in the amniotic cavity, 22% (10/45) had intra-

amniotic inflammation without demonstrable bacteria, and 22% (10/45) had no evidence of intra-amniotic inflammation (Table 2). A description of the identified bacteria in AF was reported previously [11]. Polymicrobial intra-amniotic infection was diagnosed in 27% (12/45) of patients with clinical chorioamnionitis at term.

Amniotic fluid cytokine and chemokine concentrations in cases and controls

Clinical chorioamnionitis vs. normal spontaneous labor at term—The median (interquartile range: IQR) cytokine and chemokine concentrations in AF between cases and controls are described in Table 3. Women with clinical chorioamnionitis had significantly higher AF concentrations for 8 of the 14 pro-inflammatory cytokines (after correcting for a false discovery rate of 5%) than controls. The fold-change difference in median concentrations of IL-1β, IFN-γ, TNF-α, TNF-β, IL-2, IL-12/IL-23p40, IL-6, and IL-12p70 ranged from 1.2 to 9 (Table 3). Cases also had significantly higher median AF concentrations of each of the four anti-inflammatory cytokines (IL-4, IL-10, IL-13, and IL-5), with a fold-change difference in median concentrations that ranged from 1.3 to 4. In this study, 6 of the 10 chemokines had significantly higher concentrations in cases than in controls (MIP-1β, IL-8, MIP-1α, TARC, MDC, and MCP-1), with fold changes ranging from 1.4 to 6 fold. Eotaxin-3 was the only chemokine with significant lower concentrations in clinical chorioamnionitis than in controls (Table 3).

Clinical chorioamnionitis without intra-amniotic inflammation, inflammation without detectable bacteria, or microbial-associated intra-amniotic inflammation vs. normal spontaneous labor at term

The median (IQR) concentration of the inflammation-related proteins in AF from women with clinical chorioamnionitis was classified by the presence or absence of both intra-amniotic inflammation and bacteria in the amniotic cavity (Table 4). There were no significant differences in the median AF concentration of any of the 14 pro-inflammatory cytokines between cases without intra-amniotic inflammation and controls (adjusted P values > 0.2). In contrast, the median of seven of these analytes differed significantly between cases with intraamniotic inflammation without demonstrable bacteria and controls. The median AF concentration of IFN-γ, TNF-α, IL-1β, IL-12/IL-23p40, IL-2, IL-6 and IL-12p70 was 2–15 times higher in cases with intra-amniotic inflammation without demonstrable microorganisms than in those who were in labor at term with uncomplicated pregnancies (Figure 1 and Table 4). The median concentration of these analytes, and TNF-β, IL-16 as well as IL-1α, was also significantly higher in cases with microbial-associated intra-amniotic inflammation than in controls, with a median fold difference that ranged from 1.7 to nearly 15 (Figure 1 and Table 4).

Of the four anti-inflammatory cytokines, only the median concentration of IL-10 was significantly lower in patients with clinical chorioamnionitis without intra-amniotic inflammation than in controls (adjusted P-value = 0.003; Table 4). In contrast, two of these four analytes were significantly higher in women with intra-amniotic inflammation without demonstrable bacteria, and all of them were significantly higher in patients with microbial-associated intra-amniotic inflammation, when the median concentration of each one of them was compared to that of controls. Women with clinical chorioamnionitis with intra-amniotic

inflammation without detectable bacteria had a median AF concentration of IL-10 and IL-13 2–3 fold higher than controls, whereas the median concentration of the four anti-inflammatory cytokines was 1.6–10 fold higher in patients with microbial-associated intra-amniotic inflammation than in controls (Table 4).

Of the 10 chemokines analyzed in this study, only the median AF concentration of Eotaxin-3 was significantly lower in cases without intra-amniotic inflammation than in those with uncomplicated pregnancies at term who were in labor (Table 4). Four of these chemokines were significantly higher in cases with intra-amniotic inflammation without detectable bacteria, whereas seven differed significantly between cases with microbial-associated intraamniotic inflammation, when each one was compared to controls. Women with clinical chorioamnionitis who had intra-amniotic inflammation without demonstrable bacteria had median concentrations of MIP-1β, CXCL-10 (IP-10), IL-8, (Figure 2) and TARC that were 3–7-fold higher than in controls. Women with clinical chorioamnionitis and microbial associated intra-amniotic inflammation had median concentrations of MIP-1β, MIP-1α, IL-8, (Figure 2) TARC, MDC, and MCP-1 that were 2–13-fold higher than those of controls, whereas the median concentration of Eotaxin-3 was 4 fold higher in controls than in these cases. CXCL-10 (IP-10) was the only analyte that had a significantly higher median AF concentration in cases with intra-amniotic inflammation without demonstrable bacteria, but not in cases with microbial-associated intraamniotic inflammation, compared to controls (Figure 2). IL-12p70 had the highest median fold change when comparing cases with intra-amniotic inflammation without demonstrable bacteria to controls (Figure 1). However, no difference could be detected in the pattern of the intraamniotic inflammatory response between patients with microbial and without bacterial intra-amniotic inflammation (Figure 1).

The remaining cytokines/chemokines scatterplots are displayed in Supplementary Figure 1 (pro-inflammatory cytokines), Supplementary Figure 2 (anti-inflammatory cytokines), and Supplementary Figure 3 (chemokines).

Discussion

Principal findings of the study

The principal findings of the study are as follows: 1) AF concentrations of most of the pro- and anti-inflammatory cytokines and chemokines (except Eotaxin-3) were significantly higher in women with clinical chorioamnionitis at term than in controls (women at term in labor without intraamniotic inflammation); 2) Patients with microbial-associated intra-amniotic inflammation (also known as intra-amniotic infection) and those with intra-amniotic inflammation without detectable bacteria had substantially different expression of cytokines and chemokines in the AF compared to patients with spontaneous labor without intra-amniotic inflammation; 3) Among patients with clinical chorioamnionitis and intra-amniotic inflammation, no differences could be detected in the profile of the intra-amniotic inflammatory response between those with and without bacteria in the AF; and 4) Interestingly, in patients diagnosed with clinical chorioamnionitis at term who had no evidence of intraamniotic inflammation, the profile of AF cytokines and chemokines was similar to that of patients in spontaneous labor at term without intra-amniotic inflammation.

Cytokine and chemokine profile in amniotic fluid of women with clinical chorioamnionitis at term

This is the first study to characterize the behavior of the chemokine and cytokine network in the amniotic cavity of women with clinical chorioamnionitis at term. A major finding is that an intra-amniotic inflammatory response is readily detectable in women with this diagnosis. Higher concentrations of pro-inflammatory cytokines and chemokines, as well as a select group of anti-inflammatory cytokines in the AF, were found in patients with clinical chorioamnionitis compared to those with spontaneous labor at term without intraamniotic inflammation.

As clinical chorioamnionitis is a heterogeneous condition, we examined the profile of cytokines and chemokines in the AF in different subsets of patients with this diagnosis. Specifically, we have shown that women with clinical chorioamnionitis can be subdivided into three subgroups based on the results of AF analysis for microorganisms and IL-6 concentrations: 1) microbial-associated intra-amniotic inflammation (microorganisms detected by culture or molecular microbiologic techniques and an elevated AF IL-6 concentration); 2) intra-amniotic inflammation without detectable microorganisms (an elevated AF IL-6 concentration without microorganisms detectable by culture or PCR); and 3) absent intra-amniotic inflammation (neither bacteria detectable and an AF IL-6 < 2.6 ng/mL). The key observations of this study are that patients with intra-amniotic inflammation (regardless of whether this was associated with microorganisms) had considerable elevations in the concentrations of pro- and anti-inflammatory cytokines, as well as chemokines in the AF compared to women with clinical chorioamnionitis without intra-amniotic inflammation. In contrast, there was no difference in the cytokine and chemokine profiles between women with clinical chorioamnionitis without intra-amniotic inflammation and those with spontaneous term labor without clinical chorioamnionitis except for IL-10 and Eotaxin-3.

Chemokines and cytokines differentially expressed in cases of microbial-associated clinical chorioamnionitis

The major cytokines overexpressed in patients with microbial-associated clinical chorioamnionitis are IL-1β, TNF-α, IFN-γ, IL-2, and the chemokines IL-8, MIP-1α, and MIP-1β. In contrast, we found that Eotaxin-3 concentrations were significantly lower in the same patients. These observations are consistent with previous studies reporting the intra-amniotic inflammatory response in patients with preterm labor and preterm prelabor rupture of the membranes (PROM) with MIAC have overexpression of the same cytokines and chemokines (i.e., IL-1β [62–76], TNF-α [63, 66, 68, 71–74, 77–79], IFN-γ [63, 68, 74, 80, 81], IL-8 [68, 71, 72, 74, 82–89], MIP-1α [68, 72, 74, 89–91], MIP-1β [63, 68, 72, 74], and IL-6 [33, 39, 50, 66, 68, 70–72, 74, 75, 80, 86, 88, 89, 92–98]). An elevation of IL-2 concentrations in the AF [80] and maternal circulation [99, 100] has been reported in patients with preterm delivery accompanied by histologic or clinical chorioamnionitis, respectively. The nature and intensity of the inflammatory response may be a function of a microbial type [101], virulence [101], or genetic factor that controls inflammation [102].

IL-1β [103–105] and TNF-α [106–109] are potent proinflammatory cytokines upregulated by microbial and nonmicrobial danger signals. These cytokines are produced by a wide

variety of host cells [104, 105, 110, 111], and can induce the production of prostaglandins (universal mediators of the onset of labor) [65, 112–129], and stimulate the production of matrix-degrading enzymes, which have been implicated in the mechanisms of membrane rupture [130–139] and cervical remodeling [138, 140–146]. These cytokines can also stimulate the production of antimicrobial peptides, which play an important role in host defense against microorganisms [147–164]. The observations reported herein, coupled with those described in studies of patients with preterm labor and preterm PROM, suggest that the elevation of these cytokines is a consistent feature of the intra-amniotic inflammatory response to microorganisms and their products.

Chemokines and cytokines differentially expressed in intra-amniotic inflammation without detectable bacteria

Cytokines and chemokines substantially over-expressed in the AF of patients with intra-amniotic inflammation without detectable bacteria are very similar to those upregulated in women with microbial-associated intra-amniotic inflammation – namely, TNF-α, IFN-γ, IL-1β, and IL-2, MIP-1β, MIP-1α, and IL-8. Of interest, the concentrations of CXCL-10 (or IP-10) and IL-12p70 were much higher in patients with intra-amniotic inflammation without detectable bacteria than in the control group (spontaneous labor at term). It is noteworthy that the AF concentration of CXCL-10 was not significantly higher in patients with microbial-associated inflammation than in controls, suggesting that an elevation of CXCL-10 might be a feature of sterile inflammation. Further work is required to confirm these findings; however, we previously reported that the AF concentration of CXCL-10 is elevated in patients who have chronic chorioamnionitis [31, 165–167] (in which there is infiltration of lymphocytes of the chorioamniotic membranes) in the absence of microorganisms in the amniotic cavity. We have proposed that an isolated elevation of CXCL-10 may represent a novel form of intra-amniotic inflammation associated with maternal anti-fetal rejection [167]. Previous reports have addressed the changes in the alarmin high mobility group box-1 in clinical chorioamnionitis at term [10].

IL-12 is a pro-inflammatory cytokine which induces the production of IFN-γ [168–170]. A previous study has reported that patients with preterm labor and acute histologic chorioamnionitis have higher concentrations of maternal-circulating IL-12 than those without this placental lesion [100]. Herein is the first report that the IL-12p70 subunit is elevated in the amniotic cavity of patients with clinical chorioamnionitis at term.

Clinical chorioamnionitis in the absence of an intra-amniotic inflammatory response

One of the findings in the current study is that a subset of patients with clinical chorioamnionitis does not have evidence of changes in the majority of AF cytokine and chemokine concentrations. Such patients can be readily identified because they do not have microorganisms in the amniotic cavity and the AF IL-6 concentration is consistently low and similar to that of patients in spontaneous labor at term (IL-6 < 2.6 ng/mL). AF IL-10 and Eotaxin-3 concentrations were significantly lower in patients with clinical chorioamnionitis without intra-amniotic inflammation than in those with uncomplicated pregnancy in labor at term. The coefficient of variation (CV) for the determination of Eotaxin-3 is high (> 15%) and, therefore, this finding should be interpreted with caution, and is one for which

replication is desired. Importantly, most of these patients do not have placental lesions associated with acute AF infection (i.e., acute histologic chorioamnionitis and/or funisitis). Why these patients have a fever is unclear; however, our results suggest that the inflammatory stimuli do not seem to arise from the amniotic cavity. Whether fever is the result of epidural analgesia or any other extra-amniotic inflammatory processes remains to be established [171–187]. Further studies are required to determine whether patients with or without intra-amniotic inflammation can be differentiated by assessing maternal plasma concentrations of cytokines and chemokines.

Strengths and limitations

The strengths of this study are that AF concentrations of multiple inflammation-related proteins were studied in women with clinical chorioamnionitis at term and in controls in labor at term. Advanced techniques were used to identify bacteria in the amniotic cavity of patients with clinical chorioamnionitis. We also leveraged AF specimens from women with uncertain gestational age who presented with suspected preterm labor without ultrasound dating, but subsequently were considered to have a term pregnancy based on the following characteristics: 1) spontaneous labor; 2) delivery within 48 h of amniocentesis; 3) analysis of AF consistent with fetal lung maturity; 4) birthweight > 2500 g; 5) absence of respiratory distress syndrome or other complications of prematurity; and 6) physical examination by a pediatrician which was consistent with the diagnosis of a term neonate.

Potentially limiting circumstances include the use of banked rather than fresh specimen; the coefficient of variation was > 15% for 12 analytes (Supplementary Table). Further studies with a larger number of observations are desirable because the lack of difference in the concentrations of cytokines and chemokines between patients with intra-amniotic infection and intra-amniotic inflammation with microorganisms may represent a type II error.

Conclusion

Microbial-associated intra-amniotic inflammation and intra-amniotic inflammation without detectable bacteria are associated with a dramatic upregulation of the intraamniotic inflammatory response. A subset of patients with term clinical chorioamnionitis without intra-amniotic inflammation has an expression of cytokines/chemokines in the AF similar to that of patients with labor at term without intra-amniotic inflammation. The observations reported herein have implications, as they shed light on the biology of a common complication of pregnancy, clinical chorioamnionitis at term.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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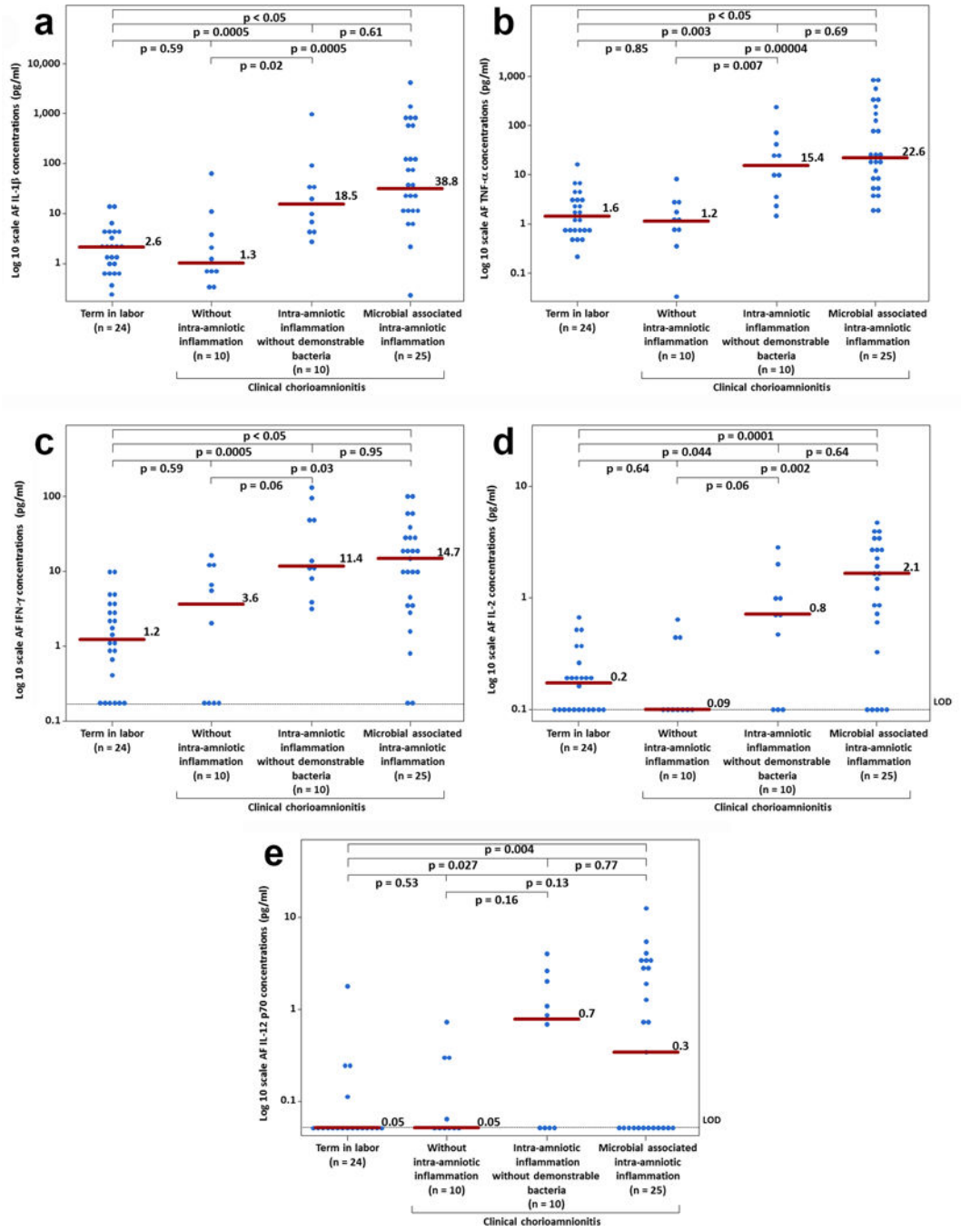

Figure 1. The amniotic fluid (AF) concentrations of pro-inflammatory cytokine in patients with term in labor (control) (n = 24), clinical chorioamnionitis without intra-amniotic inflammation (n = 10), clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria (n = 10), and clinical chorioamnionitis with microbial-associated intra-amniotic inflammation (n = 25

(A) The median AF concentrations of interleukin (IL)-1β are 2.6 pg/mL (term in labor), 1.3 pg/mL (clinical chorioamnionitis without intra-amniotic inflammation), 18.5 pg/mL (clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria), and 38.8 pg/mL (clinical chorioamnionitis with microbial-associated intra-amniotic inflammation).

(B) The median AF concentrations of tumor necrosis factor-alpha (TNF-α) are 1.6 pg/mL (term in labor), 1.2 pg/mL (clinical chorioamnionitis without intra-amniotic inflammation), 15.4 pg/mL (clinical chorioamnionitis with intraamniotic inflammation without demonstrable bacteria), and 22.6 pg/mL (clinical chorioamnionitis with microbial-associated intra-amniotic inflammation).

(C) The median AF concentrations of interferon-gamma (IFN-γ) are 1.2 pg/mL (term in labor), 3.6 pg/mL (clinical chorioamnionitis without intra-amniotic inflammation), 11.4 pg/mL (clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria), and 14.7 pg/mL (clinical chorioamnionitis with microbial-associated intra-amniotic inflammation). LOD = limit of detection.

(D) The median AF concentrations of interleukin-2 (IL-2) are 0.2 pg/mL (term in labor), 0.09 pg/mL (clinical chorioamnionitis without intra-amniotic inflammation), 0.8 pg/mL (clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria), and 2.1 pg/mL (clinical chorioamnionitis with microbial-associated intra-amniotic inflammation). LOD = limit of detection.

(E) The median AF concentrations of interleukin-12p70 (IL-12p70) are 0.05 pg/mL (term in labor), 0.05 pg/mL (clinical chorioamnionitis without intra-amniotic inflammation), 0.7 pg/mL (clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria), and 0.3 pg/mL (clinical chorioamnionitis with microbial-associated intra-amniotic inflammation). LOD: limit of detection.

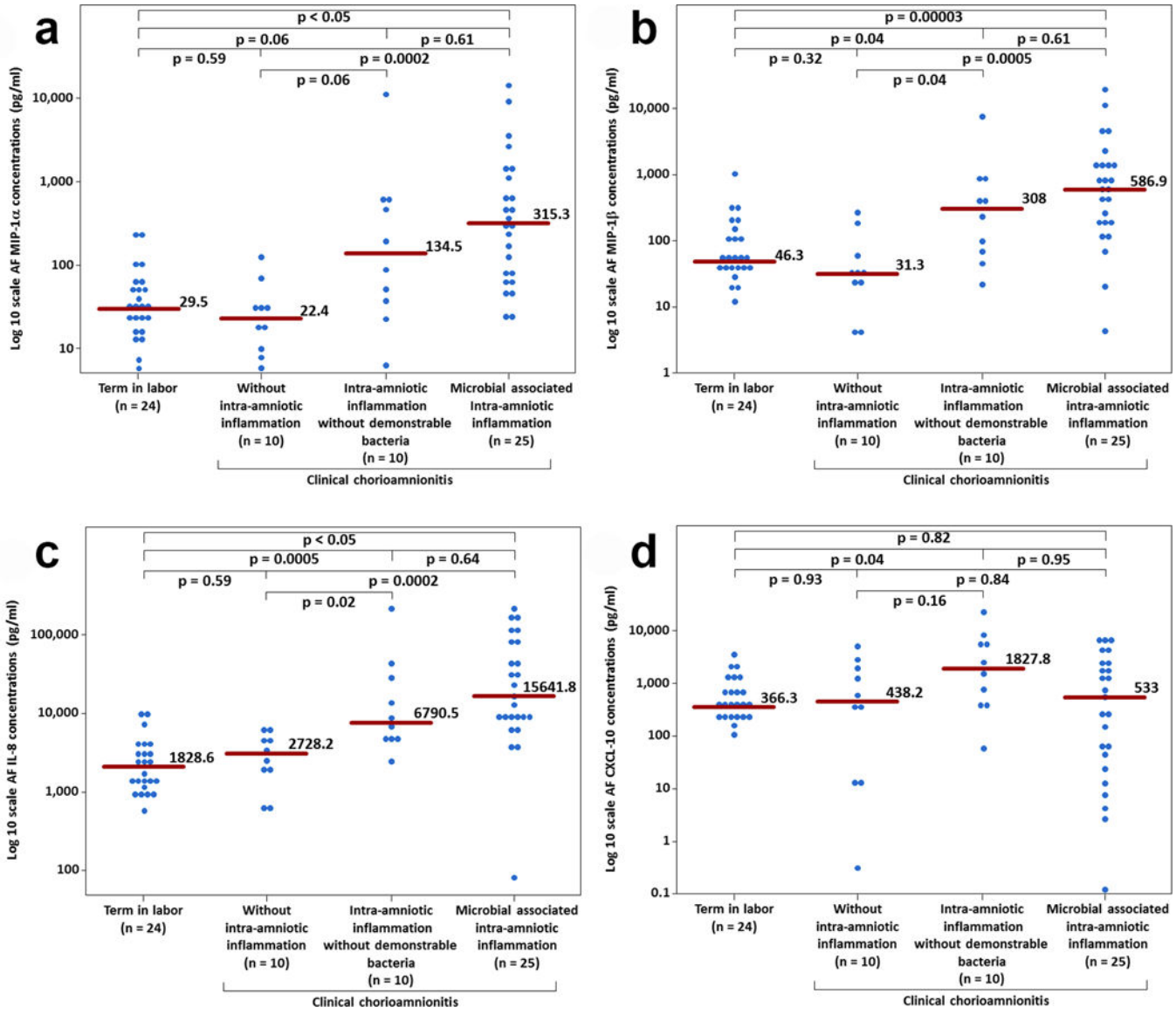

Figure 2. The AF concentrations of chemokine in patients with term in labor (control) (n = 24), clinical chorioamnionitis without intraamniotic inflammation (n = 10), clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria (n = 10), and clinical chorioamnionitis with microbial-associated intra-amniotic inflammation (n = 25

(A) The median AF concentrations of macrophage inflammatory protein-1alpha (MIP-1α) are 29.5 pg/mL (term in labor), 22.4 pg/mL (clinical chorioamnionitis without intra-amniotic inflammation), 134.5 pg/mL (clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria), and 315.3 pg/mL (clinical chorioamnionitis with microbial-associated intra-amniotic inflammation).

(B) The median AF concentrations of macrophage inflammatory protein-1 beta (MIP-1β) are 46.3 pg/mL (term in labor), 31.3 pg/mL (clinical chorioamnionitis without intra-amniotic inflammation), 308 pg/mL (clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria), and 586.9 pg/mL (clinical chorioamnionitis with microbial-associated intra-amniotic inflammation).

(C) The median AF concentrations of interleukin-8 (IL-8) are 1828.6 pg/mL (term in labor), 2728.2 pg/mL (clinical chorioamnionitis without intra-amniotic inflammation), 6790.5 pg/mL (clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria), and 15,641.8 pg/mL (clinical chorioamnionitis with microbial-associated intra-amniotic inflammation).

(D) The median AF concentrations of C-X-C motif chemokine 10 (CXCL-10) or interferon gamma (IFN-γ)-inducible protein 10 or IP-10 are 366.3 pg/mL (term in labor), 438.2 pg/mL (clinical chorioamnionitis without intra-amniotic inflammation), 1827.8 pg/mL (clinical chorioamnionitis with intra-amniotic inflammation without demonstrable bacteria), and 533 pg/mL (clinical chorioamnionitis with microbial-associated intra-amniotic inflammation).

Table 1

Clinical characteristics of the study population

	Term in labor (n=24)	Clinical chorioamnionitis at term (n=45)	P value
Maternal age (years)	21 (18.5–23.8)	21 (18–25)	0.6
Nulliparity	50% (12/24)	64.4% (29/45)	0.25
Body mass index (kg/m^2)	NA	23.7 (21.7–24.9)	NA
Amniotic fluid glucose (mg/dL)	8.7 (7.1–10)	9 (9–9)	0.5
Amniotic fluid white blood cells (cell/mm^3)	7 (2–24.8)	58 (5–695)	0.002
Gestational age at amniocentesis and delivery (weeks)	39 (38.1–39.9)	39.6 (38.9–40.7)	0.008
Birthweight (grams)	3380 (3032.5–3615)	3550 (3220–3790)	0.99

Data presented as median (interquartile range) or % (n); NA: not applicable.

Table 2

Clinical characteristics of the patients with clinical chorioamnionitis at term

	Median (IQR) or % (n/N)
Rupture of the membranes at the time of amniocentesis	66.7% (30/45)
Gestational age at amniocentesis (weeks)	39 (37.9–40)
Fever before admission	20% (9/45)
Maternal tachycardia (>100 beats/min)	91.1% (41/45)
Fetal tachycardia (>160 beats/min)	75.6% (34/45)
Uterine tenderness	8.9% (4/45)
Foul-smelling amniotic fluid	6.7% (3/45)
Maternal leukocytosis	73.3% (33/45)
Labor • **Spontaneous** • **Induced** • **C-section**	82.2% (37/45) 15.6% (7/45) 24.4% (11/45)
Epidural analgesia • **Before amniocentesis**	77.8% (35/45)
AF white blood cells (cells/mm^3)	58 (5–695)
AF glucose (mg/dL)	9 (9–9)
AF Gram stain positive	13 (6/46)
AF interleukin-6 (ng/mL)	6.4 (2.9–18.7)
Subgroups of clinical chorioamnionitis • **No intra-amniotic inflammation** • **Intra-amniotic inflammation without demonstrable microorganisms in the amniotic cavity** • **Microbial-associated intra-amniotic inflammation**	22.2% (10/45) 22.2% (10/45) 56% (25/45)
Polymicrobial intra-amniotic infection	27% (12/45)
Placental lesions consistent with amniotic fluid infection	51.1% (23/45)
Fetal inflammatory response syndrome	22% (10/44) *
Suspected neonatal sepsis	33.3% (15/45)

Data presented as median (IQR) or % (n), IQR: interquartile range; AF: amniotic fluid; GA: gestational age; PCR: polymerase chain reaction; ESI-MS: electrospray ionization mass spectrometry. Placental lesions consistent with AF infection: acute histological chorioamnionitis or acute funisitis; no intra-amniotic inflammation/infection: negative AF culture or PCR/ESI-MS and AF IL-6 < 2.6 ng/mL; MIAC: positive microorganisms by either AF cultures or PCR/ESI-MS without intra-amniotic inflammation); microbial-associated intra-amniotic inflammation (combination of MIAC and intra-amniotic inflammation); intra-amniotic inflammation without detectable microorganisms (an elevated AF IL-6 concentration without evidence of microorganisms using cultivation or molecular methods).

* Umbilical cord blood IL-6 concentration was not available for one case.

Table 3

Amniotic fluid cytokines and chemokines concentrations in term in labor vs. clinical chorioamnionitis at term

Analytes	Term in labor Median (IQR) (n=24)	Clinical Chorioamnionitis at term Median (IQR) (n=45)	Fold change	Adjusted P value
Pro-inflammatory cytokines				
IL-1β	**2.61 (1.07–5.5)**	**24.17 (4.93–119.8)**	**9.26**	**0.0001**
IFN-γ	**1.23 (0.35–3.08)**	**10.36 (3.46–26.08)**	**8.42**	**0.0001**
TNF-α	**1.59 (0.79–3.61)**	**10.38 (2.77–65.09)**	**6.51**	**0.0001**
TNF-β	**0.01 (0.009–0.04)**	**0.06 (0.009–0.09)**	**6.00**	**0.002**
IL-2	**0.17 (0.09–0.23)**	**0.82 (0.09–2.68)**	**4.82**	**0.004**
IL12IL23p40	**11.58 (8.87–17.42)**	**30.09 (24.07–45.94)**	**2.60**	**0.000005**
IL-6	**672.39 (385.41–964.89)**	**1668.03 (731.68–5589.26)**	**2.48**	**0.001**
IL-1α	**121.21 (80.6–164.305)**	**161.75 (98.93–243.84)**	**1.33**	**0.06**
IL-12p70	**0.049 (0.04–0.04)**	**0.06 (0.04–1.95)**	**1.21**	**0.005**
GM-CSF	52.35 (30.11–72.83)	59.02 (35.85–96.97)	1.13	0.39
IL-16 *	66.24 (40.45–117.83)	103.86 (41.17–323.71)	1.57	0.23
IL-15	16.46 (12.64–21.89)	18.43 (13.15–29.02)	1.12	0.41
IL-7	2.97 (2.41–3.53)	2.73 (2.05–3.43)	0.92	0.31
IL-17a	0.25 (0.21–0.38)	0.19 (0.04–0.39)	0.75	0.30
Anti-inflammatory cytokines				
IL-4	**0.12 (0.07–0.28)**	**0.53 (0.16–3.36)**	**4.42**	**0.004**
IL-10	**0.64 (0.31–1.23)**	**1.83 (0.45–9.71)**	**2.86**	**0.031**
IL-13	**5.83 (3.93–8.7)**	**15.76 (9.27–29.03)**	**2.70**	**0.0001**
IL-5	**0.24 (0.16–0.29)**	**0.32 (0.23–0.45)**	**1.33**	**0.004**
Chemokines				
MIP-1β	**46.33 (37.21–123.08)**	**276.07 (62.18–892.27)**	**5.96**	**0.007**
IL-8	**1828.6 (1069.005–2771.78)**	**7873.58 (3985.27–32,499.2)**	**4.31**	**0.000005**
MIP1- α	**29.48 (20.23–54.4)**	**118.13 (30.18–551.1)**	**4.01**	**0.002**
TARC	**6.31 (4.34–9.51)**	**13.47 (6.48–25.3)**	**2.13**	**0.037**
MDC	**74.37 (54.35–135.14)**	**151.74 (85.55–275.31)**	**2.04**	**0.021**
MCP-1	**1205.21 (828.22–1618.69)**	**1654.03 (1243.3–3887.23)**	**1.37**	**0.03**
Eotaxin-3	**5.49 (3.56–10.79)**	**1.23 (1.24 -4.15)**	**0.23**	**0.001**
CXCL-10 or IP-10	366.3 (280.9–834.81)	729.56 (61.93–2692.43)	1.99	0.49
MCP-4	59.66 (26.12–100.63)	66.57 (28.39–170.1)	1.12	0.28
Eotaxin	12.29 (9.23–16.35)	10.22 (3.21–19.24)	0.83	0.22

* IL16 has pro- and anti-inflammatory properties; The units of all analytes are pg/ml.

IQR: interqurtile, IFN-γ: interferon gamma, IL: interleukin, TNF: tumor necrosis factor, GM-CSF: granulocyte macrophage cology-stimulating factor, TARC: thymus and activation-regulated chemokine, MDC: macrophage-derived chemokine, MIP: macrophage inflammatory protein, CXCL-10: C-X-C motif chemokine 10, IP-10: interferon gamma-induced protein 10, MCP: monocyte chemoattractant protein-1

Table 4

Amniotic fluid cytokine and chemokine concentrations in the subgroups of patients with clinical chorioamnionitis at term

Analytes pg/ml	Term in labor (Controls) (n=24)	Clinical Chorioamnnionitis at Term (n=45)										
		Without intra-amniotic inflammation (n=10)		With intra-amniotic inflammation without demonstrable microorganisms (n=10)				With microbial-associated intra-amniotic inflammation (n=25)				
	Median (IQR)	Median (IQR)	Adjusted P-value (compared to term in labor)	Median (IQR)	Fold change (compared to term in labor)	Adjusted P-value (compared to term in labor)	Adjusted P-value (compared to without intra-amniotic inflammation)	Median (IQR)	Fold change (compared to term in labor)	Adjusted P-value (compared to term in labor)	Adjusted P-value (compared to without intra-amniotic inflammation)	Adjusted P-value (compared to intra-amniotic inflammation without demonstrable microorganisms)
Pro-inflammatory Cytokines												
TNF-α	1.6 (0.8–3.6)	1.21 (0.8–2.6)	0.85	15.4 (5.0–35.2)	9.63	0.003	0.007	22.6 (8.1–178.0)	14.14	0.0000002	0.00004	0.61
IFN-γ	1.2 (0.4–3.1)	3.56 (0.18–9.28)	0.59	11.4 (8.0–48)	9.24	0.0005	0.06	14.7 (3.7–27.8)	11.97	0.00008	0.03	0.70
IL-1β	2.6 (1.1–5.5)	1.3 (0.8–4.1)	0.59	18.5 (6.4–42.5)	7.08	0.0005	0.02	38.8 (15–597.9)	14.87	0.0000002	0.0005	0.61
IL-2	0.2 (0.1–0.2)	0.09 (0.1–0.4)	0.64	0.8 (0.2–1.2)	4.88	0.044	0.06	2.1 (0.7–4.0)	12.59	0.0001	0.002	0.61
TNF-β	0.01 (0.01–0.04)	0.05 (0.01–0.07)	0.51	0.04 (0.01–0.05)	3.5	0.25	0.94	0.08 (0.05–0.1)	8.00	0.0002	0.05	0.61
IL-12/IL-23p40	11.6 (8.9–17.4	18.7 (13.7–28.6)	0.31	35.8 (20.04–52.05)	3.09	0.001	0.08	37.1 (26.7–52.4)	3.20	0.0000004	0.001	0.64
IL-6	672.4 (385.4–964.9)	304.3 (155.3–637.3)	0.21	1425.5 (1224.7–5424.9)	2.1	0.0005	0.0003	3290.4 (1668–7660.7)	4.9	0.000001	0.00004	0.64
IL-16	66.24 (40.5–117.8)	31.4 (20.2–67.6)	0.21	113.7 (54.3–352.5)	1.72	0.22	0.06	152.8 (83.0–624.3)	2.31	0.02	0.005	0.77
IL-1α	121.21 (80.6–164.3)	117.7 (95.1–126.0)	0.93	192.2 (121.8–238)	1.59	0.13	0.13	207.03 (112.1–560.7)	1.71	0.02	0.08	0.89
IL-12p70	0.05 (0.05–0.05)	0.05 (0.05–0.2)	0.53	0.76 (0.05–1.7)	15.35	0.027	0.16	0.3 (0.05–3.02)	6.67	0.004	0.13	0.95
IL-15	16.5 (12.6–21.9)	15.5 (11.9–21.4)	0.88	19.6 (15.5–28.7)	1.19	0.42	0.38	20.6 (14.8–29.02)	1.25	0.33	0.26	0.95
GM-CSF	52.4 (30.1–72.8)	38 (27.5–69.3)	0.59	57.0 (32.0–82.7)	1.09	0.95	0.64	74.78 (51.5–115.2)	1.43	0.08	0.05	0.61
IL-7	2.97 (2.4–3.5)	2.53 (2.1–3.0)	0.53	2.5 (2.1–2.8)	0.84	0.15	1	3.1 (2.1–3.9)	1.05	0.82	0.36	0.61

Analytes pg/ml	Term in labor (Controls) (n=24)	Clinical Chorioamnnionitis at Term (n=45)										
		Without intra-amniotic inflammation (n=10)		With intra-amniotic inflammation without demonstrable microorganisms (n=10)				With microbial-associated intra-amniotic inflammation (n=25)				
	Median (IQR)	Median (IQR)	Adjusted P-value (compared to term in labor)	Median (IQR)	Fold change (compared to term in labor)	Adjusted P-value (compared to term in labor)	Adjusted P-value (compared to without intra-amniotic inflammation)	Median (IQR)	Fold change (compared to term in labor)	Adjusted P-value (compared to term in labor)	Adjusted P-value (compared to without intra-amniotic inflammation)	Adjusted P-value (compared to intra-amniotic inflammation without demonstrable microorganisms)
IL-17α	0.25 (0.21–0.38)	0.08 (0.04–0.26)	0.21	0.05 (0.04–0.2)	0.2	0.08	0.94	0.3 (0.04–0.5)	1.22	0.82	0.11	0.61
Anti-inflammatory Cytokines												
IL-10 *	0.6 (0.3–1.2)	0.04 (0.04–0.04)	0.003	2.1 (1.0–9.1)	3.2	0.04	0.007	2.61 (1.53–16.91)	4.08	0.00004	0.0001	0.64
IL-4	0.1 (0.07–0.3)	0.045 (0.01–0.17)	0.51	0.4 (0.2–3.1)	3.17	0.12	0.08	1.2 (0.5–4.1)	10	0.000008	0.0005	0.61
IL-13	5.8 (3.9–8.7)	6.54 (3.51–9.88)	1.0	14.2 (11.1–28.0)	2.44	0.0005	0.01	23.8 (13.9–34.2)	4.08	0.000008	0.001	0.64
IL-5	0.2 (0.2–0.3)	0.26 (0.22–0.35)	0.59	0.3 (0.2–0.4)	1.25	0.14	0.82	0.39 (0.3–0.5)	1.63	0.0008	0.09	0.61
Chemokines												
MIP-1β	46.3 (37.2–123.1)	31.3 (23.1–54.9)	0.32	308.0 (76.4–705.1)	6.65	0.04	0.04	586.9 (179.9–1451.3)	12.67	0.00003	0.0005	0.61
CXCL-10 or IP-10	366.3 (280.9–834.8)	438.2 (106.5–1752.7)	0.93	1827.8 (570.0–6297.5)	4.99	0.04	0.16	533.01 (41.4–2335.8)	1.46	0.82	0.84	0.61
MIP-1α	29.5 (20.2–54.4)	22.4 (10.9–29.8)	0.59	134.5 (39.3–533.8)	4.56	0.06	0.06	315.3 (71.6–1112.9)	10.69	0.000002	0.0002	0.61
IL-8	1828.6 (1069.0–2771.8)	2728.2 (1674.6–3943.2)	0.59	6790.5 (4516.7–23825.8)	3.71	0.0005	0.02	15641.8 (7873.6–73123.1)	8.55	0.0000002	0.0002	0.61
TARC	6.31 (4.3–9.5)	9.5 (2.7–14.9)	0.93	19.3 (13.2–25.2)	3.06	0.04	0.08	13.03 (6.3–35.2)	2.06	0.04	0.15	0.80
MDC	74.4 (54.4–135.1)	85.7 (53.8–142.1)	1.0	153.9 (80.7–211.7)	2.07	0.13	0.4	168.5 (109.0–347.7)	2.26	0.003	0.03	0.64
MCP-4	59.7 (26.1–100.6)	37.0 (13.5–119.0)	0.72	112.1 (57.7–140.6)	1.88	0.09	0.21	68.8 (31.2–254.5)	1.15	0.27	0.1	0.95
MCP-1	1205.2 (828.2–1618.7)	1170.0 (379.4–1506.6)	0.59	1817.31 (1314.73–2490.14)	1.51	0.1	0.07	2392.8 (1539.5–6830.8)	1.99	0.001	0.002	0.64
Eotaxin	12.3 (9.2–16.4)	6.9 (3.2–12.7)	0.21	6.1 (3.2–13.5)	0.5	0.14	0.84	11.7 (3.2–22.6)	0.95	0.82	0.12	0.61

Analytes pg/ml	Term in labor (Controls) (n=24)	Clinical Chorioamnionitis at Term (n=45)										
		Without intra-amniotic inflammation (n=10)		With intra-amniotic inflammation without demonstrable microorganisms (n=10)				With microbial-associated intra-amniotic inflammation (n=25)				
	Median (IQR)	Median (IQR)	Adjusted P-value (compared to term in labor)	Median (IQR)	Fold change (compared to term in labor)	Adjusted P-value (compared to term in labor)	Adjusted P-value (compared to without intra-amniotic inflammation)	Median (IQR)	Fold change (compared to term in labor)	Adjusted P-value (compared to term in labor)	Adjusted P-value (compared to without intra-amniotic inflammation)	Adjusted P-value (compared to intra-amniotic inflammation without demonstrable microorganisms)
Eotaxin-3*	5.49 (3.56–10.79)	1.2 (1.2–1.2)	0.003	1.2 (1.2–6.7)	0.23	0.09	0.38	1.2 (1.2–6.2)	0.23	0.01	0.12	0.95

IQR: interqurtile, IFN-γ: interferon gamma, IL: interleukin, TNF: tumor necrosis factor, GM-CSF: granulocyte macrophage cology-stimulating factor, TARC: thymus and activation-regulated chemokine, MDC: macrophage-derived chemokine, MIP: macrophage inflammatory protein, CXCL-10: C-X-C motif chemokine 10, IP-10: interferon gamma-induced protein 10, MCP-1: monocyte chemoattractant protein-1. The units of all analytes are pg/mL.

*Eight of 10 patients in this group had the concentrations of these proteins below the limit of detection.