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## EXODUS 1 (CCL20): EVIDENCE FOR THE PARTICIPATION OF THIS CHEMOKINE IN SPONTANEOUS LABOR AT TERM, PRETERM LABOR, AND INTRAUTERINE INFECTION

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### Abstract

**Aim**—CCL20, also known as MIP-3 alpha, is a chemokine that participates in chemotaxis of immature dendritic cells, effector/memory T-cells, and B-lymphocytes. The objectives of this study were to determine whether CCL20 can be detected in amniotic fluid (AF) and if AF concentration of this chemokine changes with advancing gestational age, parturition (term and preterm), and intra-amniotic infection/inflammation (IAI).

**Methods**—A cross-sectional study was conducted including the following groups: 1) midtrimester of pregnancy (n=65); 2) term not in labor (TNL; n=22); 3) term in labor (TIL; n=47); 4) spontaneous preterm labor (PTL) who delivered at term (n=57); 5) spontaneous PTL without IAI who delivered preterm (n=71); and 6) spontaneous PTL with IAI (n=38). AF CCL20 concentrations were determined using ELISA.

**Results**—1) The median AF CCL20 concentration in TNL was higher than that of midtrimester patients; 2) Women in spontaneous labor at term had a higher median AF concentration of CCL20 than patients at term not in labor; 3) Patients with spontaneous PTL and IAI had a significantly higher median AF concentration of CCL20 than those without IAI who delivered preterm and those who delivered at term. Moreover, women with spontaneous PTL without IAI who delivered preterm had a significantly higher median AF concentration than those with PTL who subsequently delivered at term.

**Conclusions**—1) CCL20 is a physiologic constituent of AF and its concentration increases as term approaches; 2) spontaneous labor (term and preterm) in the absence of IAI is associated with an increased bioavailability of AF CCL20 suggesting that an increase in CCL20 is part of the common pathway of human parturition; 3) patients with IAI had dramatic elevations in the AF CCL20 concentrations suggesting that this chemokine participates in the host response to infection or other stimuli associated with intra-amniotic infection.

## Keywords

Amniotic fluid; cytokine; inflammation; LARC; macrophage inflammatory protein 3 alpha; parturition; pregnancy

## INTRODUCTION

Intra-amniotic infection/inflammation (IAI) is causally linked to spontaneous preterm labor and delivery [102] and it is associated with both maternal and neonatal morbidity and mortality [120]. Amniotic fluid is traditionally considered to be sterile [102]. The first line of defense against ascending infection is composed of the cervical mucus plug [27,47,48,103] and chorioamniotic membranes [72,128]; as well as cellular components of the innate immune system including neutrophils, macrophages, natural killer cells, and trophoblasts [42,102]. Microorganisms, their products, and other stimuli elicit an inflammatory response which leads to an increased production of pro-inflammatory cytokines by gestational tissues [25,71] amniotic fluid [12,14,15,24,71,100,101,104-106,136], maternal serum [90], and fetal cord blood [15,41,105,136]. It has been proposed that pro-inflammatory cytokines and chemokines participate in the mechanisms responsible for the initiation of labor in the setting of infection, but also in spontaneous labor at term [101]. One mechanism through which inflammation is produced involves increased leukocyte trafficking which is induced by chemokines [18,30,31,64,66,70].

Chemokines are a group of structurally related chemotactic proteins implicated in the recruitment of cells to sites of inflammation. They also participate in homeostatic cellular trafficking [118]. Exodus-1, also known as CC chemokine ligand 20 (CCL20), is the only chemokine known to interact with the CC chemokine receptor 6 (CCR6) [6], a property shared with some antimicrobial peptides: the  $\beta$ -defensins [123,135]. Together, CCL20 and CCR6 play a role in both homeostatic and inflammatory states. The evidence in support of this is: 1) CCL20 and CCR6 are involved in recruiting immature dendritic cells to sites where antigen entry may occur [10,17,21,22,40,62,93,114,133,135]; 2) mediate effector/memory T-cell interactions with endothelial cells [26,78,93,113,135]; and 3) participate in the migration of memory B-lymphocytes [9,11,23,73,79,118].

There is a paucity of information about CCL20 in human pregnancy. *In vitro*, CCL20 mRNA is expressed in fetal lung and liver [108]. *In vivo*, one study with a small sample size suggests no change in maternal serum CCL20 concentrations in women with preterm labor and delivery compared to either preterm controls or women at term in labor [77]. A second study demonstrates increased CCL20 protein levels in decidual biopsies in patients with preeclampsia compared to normal controls at the time of delivery [55]. So far, the concentration of CCL20 in amniotic fluid has not been reported. The objective of this study was to determine whether CCL20 can be detected in the amniotic fluid and if the amniotic fluid concentration of this chemokine changes with gestational age, in the presence of spontaneous labor (term and preterm), or with intra-amniotic infection/inflammation.

## METHODS

A cross-sectional study was designed by searching our clinical database and bank of biological samples, including women in the following groups: Group 1 consisted of women in the mid-trimester of pregnancy (14-18 weeks) who underwent amniocentesis for genetic indications and delivered normal infants at term (n=65). Group 2 included women with normal term gestations. This group was subdivided into women not in labor (TNL; n=22)

and women in spontaneous labor (TIL; n=47). Group 3 included women with spontaneous preterm labor (PTL) and intact membranes that were subdivided into the following categories: 3A) patients with PTL who delivered at term (n=57); 3B) patients with PTL who delivered preterm without IAI (n=71); 3C) patients with PTL who delivered preterm with IAI (n=38). Patients with preterm premature rupture of membranes, multi-fetal gestation, chromosomal abnormalities, and fetal congenital malformations were excluded.

All women provided written informed consent prior to the collection of amniotic fluid. The collection of amniotic fluid samples was approved by the institutional review boards of Wayne State University, Pennsylvania Hospital, Sotero del Rio Hospital and the National Institutes of Child Health and Human Development. Many of these samples have been used previously in studies of cytokines, chemokines, growth factors, arachidonic acid metabolites, and antimicrobial peptides in amniotic fluid.

## Definitions

Women were considered to have a normal pregnancy outcome if they did not have any obstetrical, medical, or surgical complications of pregnancy, and delivered a term neonate of appropriate birth weight for gestational age without congenital anomalies or complications. Preterm labor was diagnosed by the presence of at least two regular uterine contractions occurring every 10 minutes associated with cervical changes requiring hospitalization before 37 weeks of gestation. Preterm delivery was defined as delivery before 37 weeks of gestation. Intra-amniotic infection was defined as a positive amniotic fluid culture for microorganisms. Intra-amniotic inflammation was defined as an amniotic fluid white blood cell (WBC) count  $>100$  cells/mm<sup>3</sup>.

## Sample collection

Amniotic fluid was obtained by transabdominal amniocentesis under ultrasonographic guidance for genetic indications, to assess the microbial state of the amniotic cavity, and to determine fetal lung maturity in patients approaching term. Immediately upon retrieval, amniotic fluid was transported to the laboratory in a sterile capped syringe and cultured for aerobic and anaerobic bacteria as well as genital *mycoplasmas*. White blood cell count, glucose concentration and Gram-stain were also performed shortly after collection. The results of these tests were used for subsequent clinical management. Amniotic fluid not required for clinical purposes was centrifuged and stored frozen at  $-70^{\circ}\text{C}$  until analysis.

## CCL20 immunoassay

Amniotic fluid concentrations of CCL20 were determined by the use of specific and sensitive enzyme-linked immunoassays. CCL20 immunoassays were purchased from R&D Systems (Minneapolis, MN, USA) and were validated in our laboratory for use with human amniotic fluid before the conduction of this study. Validation included spike and recovery experiments, which produced parallel curves indicating that amniotic fluid constituents (matrix) did not interfere with antigen-antibody binding in this assay system. Amniotic fluid samples were incubated in duplicate wells of the micro titer plates, which have been pre-coated with a monoclonal antibody specific for CCL20. During this incubation any CCL20 present in the standards or amniotic fluid samples was bound by the immobilized antibodies in the assay plates. After repeated washing and aspiration to remove all unbound substances, an enzyme-linked polyclonal antibody specific for CCL20 was added to the wells. Unbound enzyme conjugate was removed by repeated washing and a substrate solution was added to the wells of the assay plates and color developed in proportion to the amount of CCL20 bound in the initial step. The color development was stopped with the addition of an acid solution and the intensity of color was read using a programmable spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). The concentrations of CCL20

in amniotic fluid samples were determined by interpolation from individual standard curves composed of recombinant human CCL20. The calculated inter- and intra-assay coefficients of variation for CCL20 immunoassays in our laboratory were 5.1% and 2.3%. The lower limit of detection (sensitivity) was calculated to be 3.33 pg/ml.

### Statistical analysis

The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to test for normal distribution of the data. Since a normal distribution was not achieved after logarithmic transformation, Kruskal-Wallis with post-hoc tests (Mann-Whitney U tests) were used to determine the differences of the median among groups. Spearman rank correlation was utilized to assess correlations. SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. A p-value <0.05 was considered statistically significant.

## RESULTS

Table 1 displays the demographic and clinical characteristics of patients with spontaneous preterm labor. There were no significant differences in the median gestational age at amniocentesis between groups.

### Detectable Immunoreactive CCL20 in Amniotic Fluid

CCL20 was detectable in 88.3% (265/300) of amniotic fluid samples. CCL20 was below the limit of detection for the following: midtrimester 41.5% (27/65), term not in labor 22.7% (5/22), term in spontaneous labor 2.1% (1/47), PTL without IAI 1.4% (1/71), and PTL with term delivery 1.8% (1/57).

### AF CCL20 Concentration is Higher at Term than in the Midtrimester

The median amniotic fluid concentration of CCL20 was higher in women at term not in labor than in women in the midtrimester of pregnancy (TNL: 34.6 pg/ml, range: 0.0 – 134.0 vs. midtrimester: 15.3 pg/ml, range: 0 – 158.3; p=0.04; Figure I).

### Spontaneous Labor at Term is Associated with Increased AF CCL20

Women at term in spontaneous labor had significantly higher median amniotic fluid concentrations of CCL20 than women at term not in labor (TIL: 76.4 pg/ml, range: 0-3933.6 vs. TNL: 34.6 pg/ml, range: 0-134; p=0.001; Figure II).

### Spontaneous Preterm Labor without Intra-Amniotic Infection/Inflammation is Associated with Increased AF CCL20 Concentration

Patients with spontaneous PTL without IAI had significantly higher concentrations of CCL20 than patients with PTL who delivered at term (Preterm labor without IAI: 119.9 pg/ml, range: 0-15905.4 vs. Preterm labor with term delivery: 45.6 pg/ml, range: 0-293.4; p<0.001; Figure III).

### Intra-amniotic Infection/Inflammation is Associated with Dramatic Elevations of AF CCL20

Among patients with spontaneous PTL and intact membranes, those with IAI had significantly higher concentrations of CCL20 than those without IAI (1250.3 pg/ml, range: 22.8-42049.8 vs. 119.9 pg/ml, range: 0-15905.4, respectively; p<0.001), and than those who delivered at term (45.6 pg/ml, range: 0-293.4 respectively; p<0.001; Figure III).

## DISCUSSION

### Principal findings of this study

1) CCL20 is frequently detectable in amniotic fluid; 2) The median amniotic fluid concentration of CCL20 is higher at term than in the midtrimester; 3) Spontaneous labor (term and preterm) is associated with high AF concentrations of CCL20; and 4) Intra-amniotic infection/inflammation is accompanied by dramatic elevations in AF CCL20.

### What is CCL20?

CCL20, also known as Exodus-1 [54] or macrophage inflammatory protein-3 $\alpha$  (MIP-3 $\alpha$ ) [108], or liver and activation-regulated chemokine (LARC) [6,50], is a chemokine known to interact exclusively with the CC chemokine receptor CCR6 [6,50,54,108,118]. Expression of CCL20 mRNA has been demonstrated in adult tissues such as skin [116], lung [50,97,124], liver [50], stomach [97], appendix [97,108], colon [97], thymus [97,108], and lymph nodes [108]. Several disease processes are associated with CCL20 production including atopic dermatitis [92], rheumatoid arthritis [81,110], psoriasis [22,52,116], tonsillitis [21,22], inflammatory bowel disease [115], cystic fibrosis [124], and cancers of the breast and cervix [7,114]. Some biological fluids are also known to contain CCL20; specifically, cerebrospinal fluid in patients with acute pneumococcal meningitis [69] and *E. coli* infected urine [98]. Additionally, mRNA expression of CCL20 has been described in vaginal epithelium [19], the uterine cervix [38], placenta [97], and fetal lung and liver [108].

The CCL20 gene is located on chromosome 2q33-37 [50]. Mapping studies demonstrate that gene transcription of CCL20 is modifiable through its promoter region which contains binding sites for several transcription factors including nuclear factor- $\kappa$ B (NF- $\kappa$ B) [45,50,74,93,126]. Mounting evidence suggests that NF- $\kappa$ B is a critical nuclear factor involved in the regulation of CCL20 transcription [19,34,39,45,53,59,60,63,68,74,94,109,126]: 1) deletion and mutation of the NF- $\kappa$ B promoter region results in abrogated luciferase reporter activity for CCL20 [34,45,68,126]; 2) transcription of CCL20 is decreased in vitro by specific NF- $\kappa$ B inhibitors including MG-132, a protease inhibitor that blocks NF- $\kappa$ B gene activation [53,63], helenalin, an NF- $\kappa$ B specific DNA-binding inhibitor [68], and Bay 11-7085 an intracellular NF- $\kappa$ B pathway inhibitor [19]; 3) mutations in the subunits of the NF- $\kappa$ B inhibitor I $\kappa$ B, abrogates the activation of NF- $\kappa$ B and results in decreased luciferase reporter activity and decreased CCL20 protein expression [60,63,94,126]; 4) CCL20 expression, both mRNA and protein, is markedly enhanced by exposing cells in-vitro to pro-inflammatory factors including LPS [53,54,114,117] and the cytokines TNF- $\alpha$  [34,52-54,60,63,68,114,124], IL-1 $\beta$  [19,34,52,53,60,68,74,117,124], IL-1 $\alpha$  [63,68], and IL-17 [52,68]; proteins known to act via NF- $\kappa$ B [5,46,68,76,88,89,119,134]; and 5) Immunohistochemical staining (Alexa Fluor 488) demonstrates translocation of the NF- $\kappa$ B subunit p65 from the cytoplasm to the nucleus after IL-17 stimulation [68].

Interestingly, NF- $\kappa$ B is produced after ligation of Toll-like receptors (TLRs) which, in turn, are involved in initiating the innate immune response [102]. This activation is followed by the production of cytokines, chemokines, and antimicrobial peptides. Together with its receptor CCR6, CCL20 participates in directing chemotaxis in the normal physiologic state and under inflammatory stress. The evidence in support of this is: 1) CCL20 expression, both mRNA and protein, is identifiable in normal tissues [19,38,50,97,108,116,124]; 2) CCL20 is chemotactic for immature dendritic cells [6,10,17,21,22,40,54,58,62,93,114,133,135], effector/memory T-cells [78,93,113,135], and memory B-lymphocytes [9,11,23,73,79] as measured by chemotaxis assays using microchemotaxis chambers; 3) calcium mobilization studies demonstrate increased cytosolic

calcium concentrations indicative of cellular activation [6,21,79,93]; 4) CCL20, both mRNA and protein, is inducible by inflammatory cytokines [19,34,52-54,60,63,68,74,114,117,124]; and 5) CCL20 is expressed in both acutely [21,22] and chronically [22,52,81,92,110,116] inflamed tissues.

### **CCL20 and pregnancy**

The study reported herein is the first report of CCL20 concentrations in amniotic fluid. The findings that the amniotic fluid concentration of CCL20 changes with advancing gestational age and in the presence of labor, both term and preterm, are novel. Only one study has reported CCL20 concentrations in relation to parturition. Laudanski et al [77]. examined maternal serum concentrations of nine chemokines, including CCL20, in three groups: women at 26-36 weeks in preterm labor who delivered preterm, (n=17) women not in labor with subsequent term delivery (n=13), and women in labor at term (n=8). No difference was found in the maternal serum concentration of CCL20 between groups; however, this study did not control for intra-amniotic infection/inflammation a process associated with preterm parturition [12,15,41,90,100,101,105,106,136]. Additionally, it is possible that the small sample size may account for the lack of difference in the serum concentration of CCL20 among these groups making a type II error possible. It remains to be determined if maternal serum concentrations of CCL20 change in the presence of intra-amniotic infection/inflammation.

Activation of innate immunity is a component of normal pregnancy [107]. Phenotypic changes occur in maternal monocytes and granulocytes in normal pregnant women [91]. The result of which is up-regulation of inflammatory mediators as evidenced by increased expression of CD11b, CD14, and CD64 as well as increased production of intracellular reactive oxygen species [91]. The finding that amniotic fluid concentration of CCL20 is increased in women at term in labor compared to women at term not in labor supports the hypothesis that spontaneous labor is an inflammatory state [43].

Intravascular inflammation is also present in preeclampsia [13,37,44,99,111]. Recently, Huang et al. [55] compared the expression of CCL20, using immunohistochemistry and ELISA, in decidual tissue obtained at the time of cesarean delivery in placental bed biopsies from normal (n=7) and preeclamptic patients (n=7) matched for gestational age. In preeclampsia, the expression of CCL20 was significantly increased.

### **Intra-Amniotic Infection/Inflammation and AF CCL20**

The finding that intra-amniotic infection/inflammation in patients with spontaneous preterm labor is associated with highly increased median amniotic fluid CCL20 concentrations is novel and suggests that this chemokine participates in the host response to microbial invasion as well as intra-amniotic inflammation in which microorganisms cannot be detected with standard cultivation techniques. CCL20 joins several chemokines known to exist in the amniotic fluid including IL-8 [28,33,51,51,70,83,85,131], IL-18 [51,65,84,95], monocyte chemotactic protein-1 (MCP-1) [16,30,31,51,66], MCP-2 [51,64], MCP-3 [51,64], GRO- $\alpha$  [18], and psoriasin [96].

The origin of CCL20 in the amniotic fluid is not known and may be produced from either maternal [77] and/or fetal compartments. The fetal membranes are known to secrete cytokines, including IL-1 $\alpha$  [33,56,75,83,85], IL-1 $\beta$  [28,83,85,86,138], and TNF- $\alpha$  [32,33,83,138], as well as the chemokine IL-8 [28,33,70,83,85,131]. Additionally, epithelial tissues appear to be the major producers of CCL20 [7,19,21,22,38,50,52,81,92,97,110,114,116,124]. Since the amnion and chorion are derived from epithelium, it is plausible that these tissues produce and release CCL20 into the



amniotic fluid. Fetal tissue may also be a source of CCL20 as in vitro evidence examining fetal lung and liver demonstrate expression of CCL20 mRNA [63,108].

Interestingly, CCL20 secreted from primary cultures of human airway epithelia demonstrates a spectrum of salt-sensitive antimicrobial activity specifically to *E. coli*, *Klebsiella pneumoniae*, *L. monocytogenes*, *M. catarrhalis*, and *P. aeruginosa* PA01 [124]; an effect that may be mediated by inducing increased bacterial membrane permeability [124]. Amniotic fluid is known to be antimicrobial [3,35,124,127,129,130], an activity that may partially be due to the presence of specific antimicrobial peptides [1,29,49,80,123,137]. Furthermore, B2-defensin, an antimicrobial peptide found in amniotic fluid, uses the CCL20 receptor: CCR6 [123,135]. It is possible that CCL20 retains its antimicrobial activity in amniotic fluid thereby accounting for the marked increase in CCL20 concentration in the setting of intra-amniotic infection.

CCL20 may also function as a chemoattractant in the reproductive organs. Dendritic cells, both mature and immature, have been identified in human decidua basalis and decidua parietalis from first trimester abortions [4,36,55,61,67], placental bed biopsies at the time of delivery [55], and cervical stroma [57,125]. Umbilical cord blood is also a source of dendritic cell populations [2,122,132]. Additionally, both mature and immature dendritic cells have been isolated in maternal serum between 20 and 35 weeks gestation [132] and at delivery [2,132]. Interestingly, the percentage of dendritic cells in maternal serum is significantly lower than non-pregnant controls [2]. This observation may represent cellular trafficking to the reproductive organs; no direct evidence exists at this time that uterine or cervical dendritic cells, mature or immature, participate in parturition. However, this is an interesting area of investigation.

Effector/memory T-cells (CD4+ and CD45RO+) are present in peripheral blood from normal pregnant patients and those with complications [13,20,82], as well as in human decidua [112,121]. Additionally, while B cells are found infrequently in the endometrium [87] and cervical stroma [8], memory B cells have not been described in human gestational tissues. Furthermore, neither immature dendritic cells, effector/memory T-cells, nor memory B-lymphocytes have been identified in amniotic fluid; the site from which CCL20 was measured in this report. Therefore, the role of CCL20 may be unrelated to previously recognized functions.

In conclusion, the amniotic fluid concentration of CCL20 increased as a function of gestational age and spontaneous labor (term and preterm), as well as intra-amniotic infection/inflammation, is associated with a significant increase in the amniotic fluid concentration of this chemokine. We propose that CCL20 is involved in the mechanisms of parturition as well as in the host response to microbial invasion and other pathologic processes.

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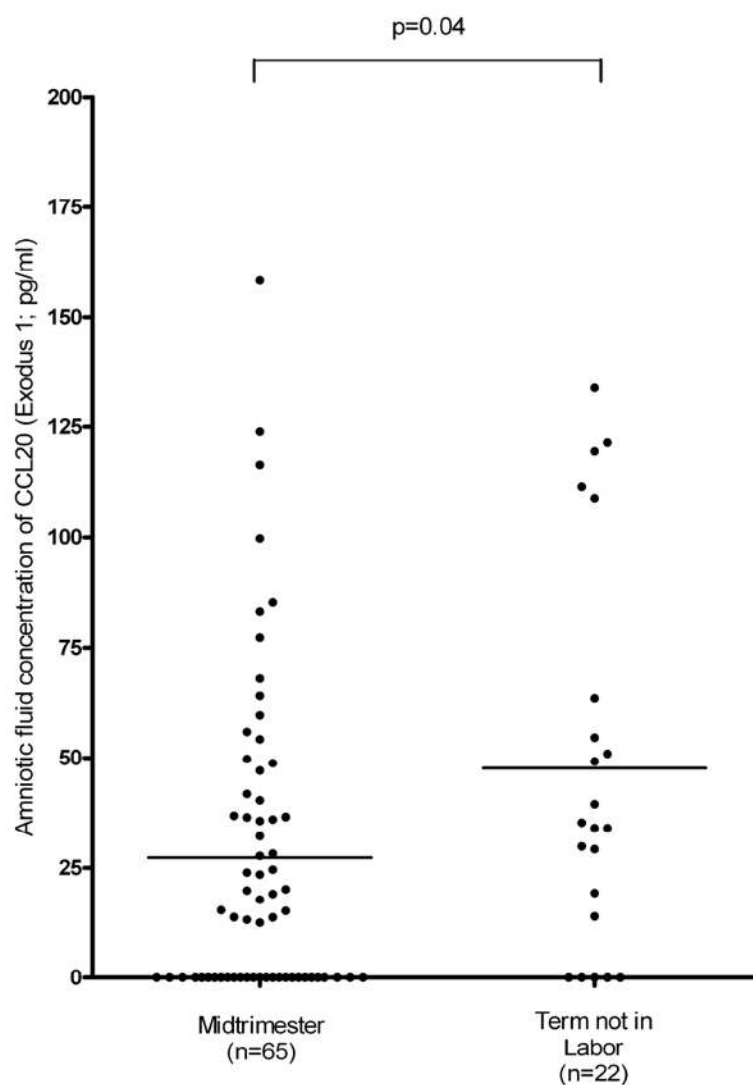
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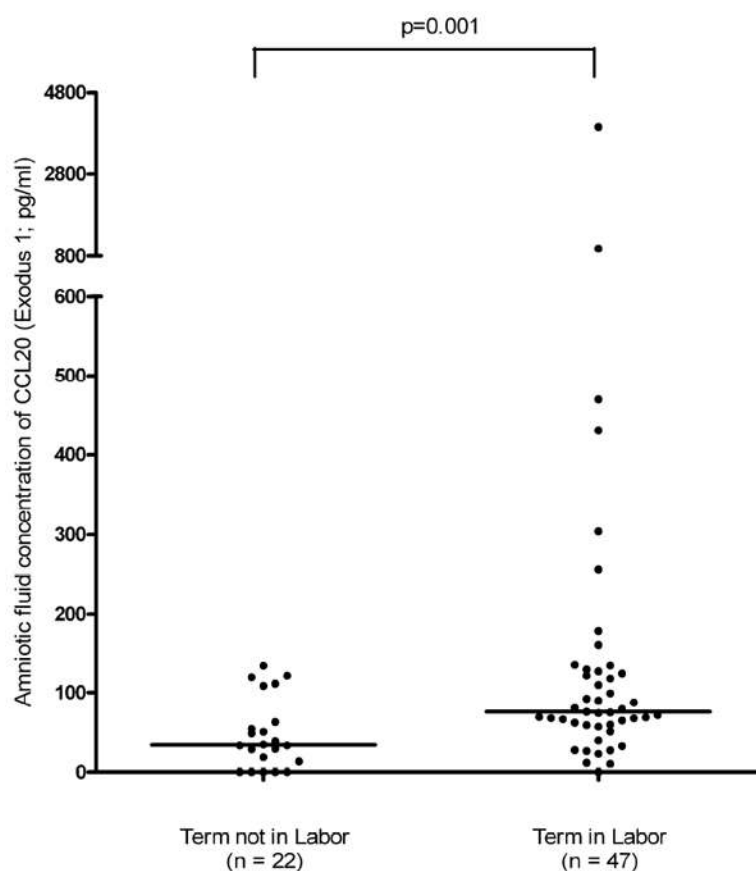


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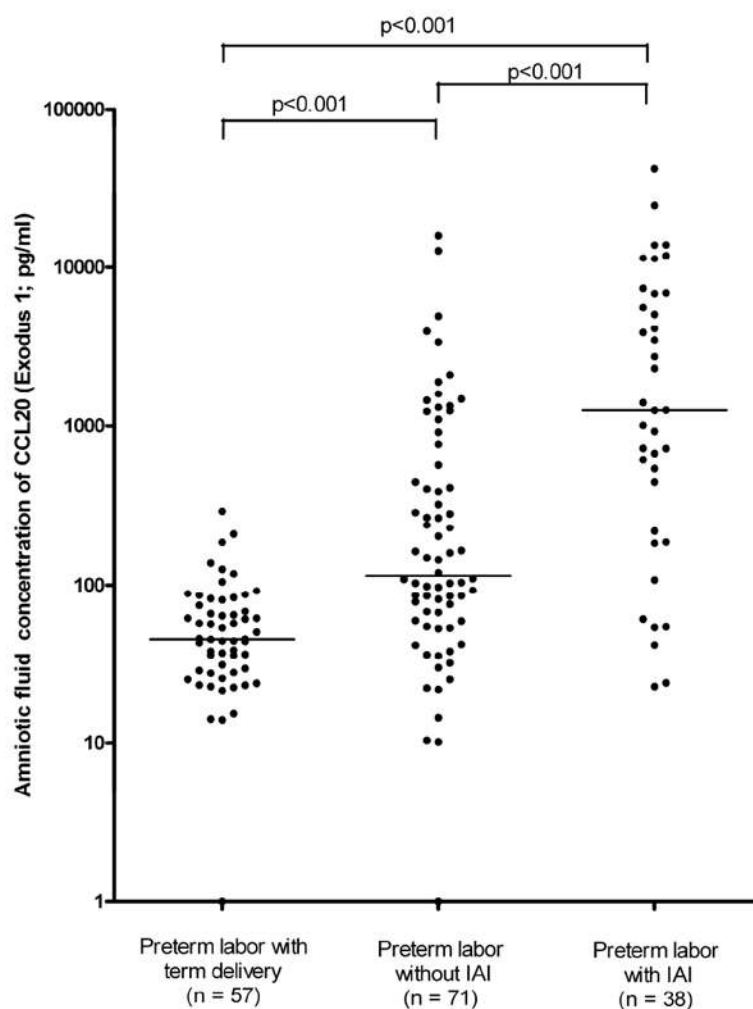
**Figure 1.**

The median amniotic fluid (AF) concentrations of CCL20 in women at term not in labor (TNL) and in the mid-trimester of pregnancy. Amniotic fluid concentration of CCL20 increased significantly with advancing gestational age (TNL: 34.6 pg/ml, range: 0.0 – 134.0 vs. mid-trimester: 15.3 pg/ml, range: 0 – 158.3;  $p=0.04$ ).



**Figure II.**

AF concentration of CCL20 in women with normal term gestations comparing women not in labor (TNL) and women in spontaneous labor (TIL). Women at term in spontaneous labor had significantly higher median amniotic fluid concentrations of CCL20 than women at term not in labor (TIL: 76.4 pg/ml, range: 0-3933.6 vs. TNL: 34.6 pg/ml, range: 0-134;  $p=0.001$ ).



**Figure III.**

AF concentration of CCL20 in women with spontaneous preterm labor (PTL) and intact membranes. The median AF concentration of CCL20 was significantly higher in women with PTL with intra-amniotic infection/inflammation (IAI) than in those with PTL without IAI (1250.3 pg/ml, range: 22.8-42049.8 vs. 119.9 pg/ml, range: 0-15905.4, respectively;  $p<0.001$ ), and than in patients with preterm labor who delivered at term (45.6 pg/ml, range: 0-293.4 respectively;  $p<0.001$ ). Additionally, patients with PTL without IAI had significantly higher concentrations of CCL20 than patients with PTL who delivered at term (119.9 pg/ml, range: 0-15905.4 vs. 45.6 pg/ml, range: 0-293.4, respectively;  $p<0.001$ ). Data are displayed on  $\text{Log}_{10}$  scale.

Table 1

Demographic and clinical characteristics of the study population

Characteristics	PTL with Term Delivery (n=57)	PTL and PTD without IAI (n=71)	PTL and PTD with IAI (n=38)	p-value
Maternal age (years) <sup>†</sup>	22 (15 - 39)	22 (16 - 44)	23 (15 - 41)	NS
Parity <sup>†</sup>	2 (1 - 6)	2 (1 - 9)	2 (1 - 9)	NS
Prior history of Preterm delivery	24.6% 14/57	38.0% 27/71	26.3% 10/38	NS
Gestational age at amniocentesis (weeks) <sup>†</sup>	30.6 (19 - 35)	29.5 (19 - 34)	26.1 (20 - 34)	NS
Gestational age at delivery (weeks)	38.6 (37 - 42)	32.9 (21 - 36)	28.2 (20 - 36.9)	<0.05 <sup>‡</sup>
Birth weight (gms)	2948 (2390 - 4080)	1765 (360 - 2920)	1085 (280 - 2950)	< 0.05 <sup>‡</sup>

The values are given as median (ranges) or percentages (number).

PTL; preterm labor, PTD; preterm delivery, IAI; intra-amniotic infection/inflammation.

<sup>†</sup>Kruskal – Wallis with posthoc analysis.

<sup>‡</sup>Kruskal – Wallis with posthoc analysis, significance only between PTL and PTD with IAI vs. PTL with Term Delivery