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Midtrimester amniotic fluid concentrations of interleukin-6 and interferon-gamma-inducible protein-10: evidence for heterogeneity of intra-amniotic inflammation and associations with spontaneous early (< 32 weeks) and late (> 32 weeks) preterm delivery

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Abstract

Introduction—Intra-amniotic inflammation is traditionally defined as an elevation of amniotic fluid interleukin (IL)-6. Previous case control studies have suggested an association between an elevated midtrimester amniotic fluid IL-6 and preterm delivery, although such an association has been recently challenged. Intra-amniotic inflammation can also be defined by an elevation of the T-cell chemokine, Interferongamma-inducible protein (IP)-10. An elevation in amniotic fluid IP-10 has been associated with chronic chorioamnionitis, a lesion frequently found in late spontaneous preterm birth and fetal death. In contrast, an elevation in amniotic fluid IL-6 is typically associated with acute chorioamnionitis and funisitis. This study was conducted to examine the relationship between an elevation in amniotic fluid IL-6 in the midtrimester and preterm delivery at or before 32 weeks of gestation, and the amniotic fluid concentration of IP-10 and preterm delivery after 32 weeks of gestation.

Materials and methods—This cohort study included 847 consecutive women undergoing genetic midtrimester amniocentesis; in 796 cases, amniotic fluid and pregnancy outcome was available for study after exclusion of abnormal karyotype and/or fetal congenital anomalies. Spontaneous preterm delivery was defined as early (≤32 weeks) or late (after 32 completed weeks of pregnancy). The amniotic fluid and maternal blood concentrations of IL-6 and IP-10 were measured by specific immunoassays.

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Results—1) The prevalence of preterm delivery was 8.3% (66/796), while those of early and late spontaneous preterm delivery were 1.5% (n = 12), and 4.5% (n = 36), respectively; 2) patients who had a spontaneous preterm delivery after 32 weeks of gestation had a higher median amniotic fluid IP-10 concentration than those who delivered at term [median 713 pg/mL, inter-quartile range (IQR) 509 – 1427 pg/mL vs. median 589 pg/mL, IQR 402 – 953 pg/mL; P = 0.006] and an elevation of amniotic fluid IP-10 concentration above 502 pg/mL (derived from an ROC curve) was associated with late spontaneous preterm delivery [odds ratio 3.9 (95% CI 1.6 – 9.9)]; 3) patients who had a spontaneous preterm delivery ≤32 weeks of gestation had a higher median amniotic fluid IL-6 concentration than those who delivered at term [median 2052 pg/mL, IQR 435 – 3015 pg/mL vs. median 414 pg/mL, IQR 209 – 930 pg/mL; P = 0.006], and an elevated amniotic fluid IL-6 concentration above 1740 pg/mL (derived from an ROC curve) was associated with early spontaneous preterm delivery [odds ratio 9.5 (95% CI 2.9 – 31.1)]; 4) subclinical intra-amniotic inflammation, defined as an elevation of IL-6 (≥2.9 ng/mL) or IP-10 (≥2.2 ng/mL) concentration above the 95th percentile of patients who had uncomplicated term delivery (n = 652 for IL-6 and n = 633 for IP-10), was observed in 6.3% (50/796) and 5.8% (45/770) of cases, respectively. Although each type of inflammation is a risk factor for spontaneous preterm delivery, many patients had a term delivery without complication; 5) the amniotic fluid in the midtrimester did not contain microorganisms detectable with cultivation techniques.

Conclusions—Intra-amniotic inflammation is heterogeneous. Some patients have elevated amniotic fluid concentrations of IL-6, and are at risk for spontaneous preterm delivery before 32 weeks of gestation, while others have an elevated IP-10 (a chemotactic T-cell chemokine) and such patients are at risk for spontaneous preterm delivery after 32 weeks of gestation. A fraction of patients have subclinical intra-amniotic inflammation and deliver at term. The clinical significance of this condition remains to be determined.

Keywords

Chemokines; chorioamnionitis; chronic chorioamnionitis; cytokines; early preterm labor; late preterm labor; maternal anti-fetal rejection; midtrimester amniocentesis; pregnancy

INTRODUCTION

The amniotic cavity is considered sterile for bacteria during normal pregnancy [15, 147, 150]. The presence of bacteria, or microbial invasion of the amniotic cavity (MIAC), is generally regarded as a pathologic state [16, 50, 61, 70, 94, 119, 124, 154, 159, 172, 176, 190]. MIAC has been reported in approximately 10% of cases with preterm labor and intact membranes [61, 154, 175], 33% of patients with preterm premature rupture of membranes (PROM) [60, 61, 151, 159], approximately 50% of patients with cervical insufficiency [162], 9% of patients with an asymptomatic short cervix [71], 26% of patients with preterm labor and a short cervix (< 15 mm) [59], 18% of patients during term labor [169], 34% of patients with premature rupture of membranes at term [165], 14% of patients with idiopathic vaginal bleeding [58] and 5% of patients with placenta previa [101], using standard cultivation techniques. However, the frequency of MIAC may be even higher if broad primers are used to detect microbial footprints [2, 31, 32, 35, 36, 44, 69, 73, 78, 106, 120, 129, 141, 143], and also if specific primers [80, 131] are used to detect the most common microorganisms, such as genital Mycoplasmas [47, 76, 107, 128, 144, 206]. Moreover, these techniques have detected microbial footprints in the amniotic fluid of patients who developed preeclampsia [34] or delivered a small-for-gestational-age (SGA) neonate [33].

Few studies have examined the microbial state of the amniotic cavity in the midtrimester [13, 19, 53, 65, 74, 104] and the results have varied. Some have reported sterile amniotic fluid [26, 42] while others have found the presence of microorganisms in an unexpectedly high number of cases [13, 65, 104]. The presence of microorganisms in the amniotic cavity

elicits, in some cases, an inflammatory response that can be detected by demonstration of an elevation of the amniotic fluid white blood cell count [18, 43, 161, 171], a decrease in amniotic fluid glucose [28, 45, 46, 66, 90, 157, 171], or an elevation in proinflammatory cytokines, such as interleukin-6 (IL-6) [9, 29, 79, 82, 136, 156, 167, 170, 171, 177, 178, 200, 201], MCP-1 [20, 27, 40, 75], and matrix metalloproteinases (MMPs) such as MMP-8 [40, 85, 88, 89, 95, 96, 112, 123, 126, 132, 134, 135].

Such inflammatory response has been extensively studied in preterm labor with intact membranes [6, 10, 11, 21, 25, 50 – 52, 63, 64, 72, 82, 95, 117, 105, 160, 164, 166, 194, 198], preterm PROM [109 – 111, 114, 115, 133, 183], cervical insufficiency [96], and asymptomatic women with a short cervix [81, 187, 188]. In general, the presence of an inflammatory response is a poor prognostic sign and is associated with preterm delivery and neonatal complications [23, 57, 67, 182, 190, 197 – 205, 207, 208, 210]. However, there have been cases in which there is a positive amniotic fluid culture for microorganisms, but no evidence of an inflammatory response. In a few cases we have been successful in eradicating intra-amniotic infection with systemic antibiotic administration to the mother [71, 116, 163]. We have observed many cases in which there is evidence of an intra-amniotic inflammatory response, as determined by an elevation in amniotic fluid cytokines (i.e., IL-6) while there is no evidence of microbial invasion with culture techniques or even broad range polymerase chain reaction (PCR) [95 – 97, 187, 210]. The etiology of these cases of intra-amniotic inflammation remains unknown.

In 1995, we reported a case-control study in which women who had a pregnancy loss after a midtrimester amniocentesis had a higher amniotic fluid IL-6 concentration than patients who delivered at term [173]. Subsequently, several casecontrol studies have demonstrated an association between the amniotic fluid IL-6 concentration at the time of mid-trimester amniocentesis and preterm delivery [186, 191, 193]. The same has been reported for other cytokines [4, 20, 30, 102, 103, 139, 140, 186, 210]. A limitation of case control studies is that they are prone to biases. In order to determine the clinical significance of intra-amniotic inflammation, a cohort study is necessary.

Until recently, the standard definition of intra-amniotic inflammation relied on the demonstration of an elevation of amniotic fluid white blood cell count (largely neutrophils) [161, 171], or proinflammatory cytokines, such as IL-6 [9, 29, 79, 82, 102, 136, 156, 167, 170, 171, 177, 178, 201], IL-1 beta [10, 72, 122, 148, 152, 155, 158, 160, 164, 168, 203], TNF [108, 153, 166, 174, 177, 200 – 202], chemokines [21, 40, 68, 121, 125, 201], or MMP-8 [79, 84, 85, 88, 89, 95, 96, 112, 113, 123, 126, 132, 134, 135]. We have recently identified a different form of intra-amniotic inflammation characterized by an elevation of T-cell chemokines [87, 99, 100, 127, 196], and demonstrated that such inflammatory process is associated with chronic chorioamnionitis, a lesion characterized by the infiltration of maternal lymphocytes into the amnion and chorion [48, 49, 77, 87].

The proposed mechanism for this lesion is that an increased concentration of amniotic fluid T-cell chemokines generates a gradient whereby maternal T-cells infiltrate the fetal tissue (chorion and amnion). Therefore, this lesion appears to represent a manifestation of maternal anti-fetal rejection because autoreactive maternal T-cells infiltrate the graft (fetal membranes) and cause apoptosis [87]. This would be equivalent to transplantation rejection, in which there is lymphocyte infiltration and graft dysfunction. Of interest is that this lesion is observed frequently in cases of late preterm delivery and in a very high number of cases of fetal death [98]. CXCL-10 (or IP-10) is a T-cell chemokine which is elevated in the amniotic fluid of patients with chronic chorioamnionitis [87]; however, it is unknown when this inflammatory process begins. It is possible that elevated IP-10 concentrations associated

with intra-amniotic inflammation are present in the midtrimester: if so, they could predispose to late preterm delivery.

The purpose of this study was to determine if an elevation of amniotic fluid IL-6 is associated with early preterm delivery (≤32 weeks of gestation), and if an elevation of amniotic fluid concentration of IP-10 is associated with preterm delivery after 32 weeks of gestation.

MATERIALS AND METHODS

Study design and participants

This was a prospective cohort study that included 847 women with singleton gestations undergoing midtrimester amniocentesis for clinical indications (advanced maternal age, abnormal quad/triple test, family history of chromosomal abnormalities, suspected fetal anomalies or viral infection and maternal request). Patients were invited to donate amniotic fluid for research purposes, as well as a sample of maternal blood obtained at the time of amniocentesis. The clinical outcome was obtained by chart review or by contacting the referring physician. Exclusion criteria were patients who were lost to follow-up and did not have information about the outcome of pregnancy (n = 25), chromosomal abnormalities, and confirmed fetal anomalies (n = 26). These patients were excluded from the study because the purpose was to determine the relationship between amniotic fluid concentrations of cytokines and microbiologic studies and pregnancy outcome in patients without fetal congenital anomalies. A total of 796 patients with a sample of amniotic fluid were left for analysis. Of these patients, 647 (81.3%) had a sample of maternal plasma. The collection of samples as well as clinical data was approved by the Institutional Review Boards of the participating Institutions (Padova, Azienda Ospedaliera, Treviso Azienda Ospedaliera, Veneto Region, Italy). All patients provided written informed consent. The hospital has a Federal Wide Assurance.

Clinical definitions

Although preterm delivery is traditionally defined as birth before 37 weeks of gestation, we used two different gestational age points to define subgroups of preterm birth. The first was preterm birth at or before 32 weeks of gestation. This gestational age was selected because in these patients, bacterial intra-amniotic infection/inflammation is a frequent mechanism of disease, in which the amniotic fluid concentrations of chemokines (such as IL-8 and MCP-1) [25, 40], cytokines (e.g., IL-6, TNF-α, IL-1) [37, 50, 54 – 56, 152, 179], damage-associated molecular patterns (HMGB-1) [180], heat shock proteins [22], and antimicrobial peptides [17, 38, 39, 184], are elevated at the time of diagnosis of preterm labor. Intra-amniotic infection and acute chorioamnionitis is less frequent in patients who deliver preterm after 32 weeks of gestation [87]. The second subgroup of preterm birth was preterm delivery after 32 weeks of gestation. The rationale for this definition is that after 32 weeks of gestation, chronic chorioamnionitis and vascular lesions emerge as frequent placental lesions. The mechanism of disease responsible for chronic chorioamnionitis is thought to be maternal antifetal rejection. There is also evidence for antibody-mediated fetal rejection; maternal antibodies against paternal human leukocyte antigens (HLA) have been demonstrated in patients with chronic chorioamnionitis [93, 99, 100, 127]. The control group consisted of uncomplicated term deliveries.

Biological samples and analysis

Amniotic fluid was obtained by transabdominal amniocentesis and 4 to 5 mL was collected for research purposes. Amniotic fluid samples were centrifuged at 1300 × *g* for 10 min and stored at −80 ° C until use. The amniotic fluid underwent Gram stain examination [149],

amniotic fluid white blood cell count [161], amniotic fluid glucose [157], culture for aerobic and anaerobic bacteria as well as *Mycoplasma* species. Plasma was collected immediately in ethylenediaminetetraacetic acid (EDTA) evacuated tubes, centrifuged in a refrigerated centrifuge (2500 *g*) and stored at −80 ° C until use. Concentrations of IL-6 and IP-10 in amniotic fluid and maternal plasma were measured by a specific immunoassay according to the manufacturers' instructions (R&D Systems, Minneapolis, MN, USA). For amniotic fluid, the sensitivity of the assay was 2 pg/mL and 4.9 pg/mL; intra-assay coefficient of variation was 4.2% and 2.3%; inter-assay coefficient of variation was 5.2% and 3.9%, respectively. For maternal plasma, the sensitivity of the assay was 0.1 pg/mL and 4.9 pg/mL; intra-assay coefficient of variation was 1.2% and 3.7%; inter-assay coefficient of variation was 3.7% and 3.8%, respectively.

Statistical analysis

Data distribution was tested with Kolmogorov-Smirnov and Shapiro-Wilk tests. Comparisons in the median concentrations of IL-6 and IP-10 among and between groups were tested with Kruskal-Wallis analysis of variance followed by post-hoc Mann-Whitney *U*-test (adjusted for multiple comparisons with the Bonferroni correction). Comparisons between proportions were performed using contingency table, $\chi 2$ or Fisher's exact tests. Receiver operating characteristic (ROC) curve analysis was employed to determine optimal amniotic fluid concentrations of IL-6 and IP-10 that identified patients who subsequently had an early (≤32 weeks) or late (32 weeks) spontaneous preterm birth, respectively. Logistic regression was used to examine the association between elevated concentrations of amniotic fluid IL-6 or IP-10 and spontaneous preterm delivery, adjusting for history of spontaneous preterm delivery (yes/no), parity (nulliparous vs. multiparous), gestational age at amniocentesis (weeks) and duration of sample storage (years). A P-value of < 0.05 was considered significant. The analysis was performed with SPSS, version 15 (SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical characteristics of the study population

Table 1 reports the clinical and obstetrical characteristics of the study population. The prevalence of preterm delivery was 8.3% (66/796), while that of early and late spontaneous preterm births were 1.5% (n = 12) and 4.5% (n = 36), respectively. The remaining preterm deliveries were indicated preterm births (2.3%; n = 18): placenta previa (n = 4), preeclampsia (n = 4), gestational hypertension (n = 3), hemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome (n = 2), preeclampsia with SGA (n = 1), gestational diabetes (n = 1), placental abruption (n = 1), Rh isoimmunization (n = 1) and a psychiatric disorder (n = 1). The median gestational age at delivery was 23.2, 36.0 and 35.5 weeks for early spontaneous, late spontaneous and indicated preterm birth, respectively.

There was no significant difference in the median maternal age or gestational age at amniocentesis among groups (Table 1). As expected, patients who subsequently had either early or late spontaneous preterm delivery more frequently had a history of spontaneous preterm birth than those who had a term delivery or indicated preterm birth (P < 0.001). The median duration of sample storage ranged from 3.3 to 3.7 years and there was no significant difference among groups (P = 0.7).

The indications for amniocentesis were: 1) advanced maternal age (n = 475; 59.7%); 2) abnormal serum screening (n = 230; 28.9%); 3) suspected congenital anomaly (n = 47; 5.9%); 4) family history (n = 14; 1.8%); 5) suspected viral infection (n = 11; 1.4%); 6) maternal request (n = 11; 1.4%); and 7) other (n = 8; 1%).

Amniotic fluid Gram stain, white blood cell count, glucose, and culture results

The amniotic fluid Gram stain was negative in all cases; however, two patients had a positive amniotic fluid culture for bacteria (the organisms isolated were *Pseudomonas aeruginosa* and *Staphylococcus auricularis*). In both cases, the indication for performing amniocentesis was advanced maternal age (> 35 years) and the amniotic fluid concentrations of glucose, IL-6 and IP-10 were within normal range. Both patients delivered a term neonate with a normal karyotype and appropriate weight for gestational age [41]. Newborns had no evidence of infection at discharge.

Amniotic fluid IL-6 and IP-10

The median amniotic fluid IL-6 concentration was significantly higher in patients who had a preterm delivery ≤32 weeks than in those who had a term delivery (median 2052 pg/mL, IQR 435 – 3015 pg/mL vs. median 414 pg/mL, IQR 209 – 930 pg/mL; P = 0.006). In contrast, the median amniotic fluid IL-6 concentration in patients who had a preterm delivery >32 weeks was not significantly different from that of the patients who delivered at term (median 414, IQR 259 – 2348 pg/mL vs. median 414 pg/mL, IQR 209 – 930 pg/mL; P = 0.5) (Table 2, Figure 1).

The median amniotic fluid IP-10 concentration was significantly higher in patients who had a preterm delivery > 32 weeks than in those who had a term delivery (median 713.7, IQR 509 – 1427 pg/mL vs. median 583, IQR 403 – 954 pg/mL, P = 0.006). In contrast, the median amniotic fluid IP-10 concentration was not significantly different in patients with a preterm delivery ≤32 weeks of gestation from that of patients who delivered at term (median 823, IQR 342 – 2253 pg/mL vs. median 583, IQR 403 – 954 pg/mL, P = 0.3) (Table 2, Figure 2).

ROC curve analysis indicated that an amniotic fluid concentration of IL-6 ≥1740 pg/mL had an area under the curve of 0.73 (95% CI 0.6 – 0.9; P = 0.007), a sensitivity of 58% (7/12), a specificity of 88% (688/784), a positive predictive value of 7% (7/103), and a negative predictive value of 99% (688/693) for the identification of patients who subsequently delivered ≤32 weeks of gestation. The corresponding indices for amniotic fluid concentration of IP-10 > 502 pg/mL were 0.64 (95% CI 0.5 – 0.7; P = 0.007), 83% (30/36), 40% (295/734), 6% (30/469) and 98% (295/301), respectively, for the identification of patients who subsequently delivered > 32 weeks of gestation.

An elevation of amniotic fluid IL-6 concentration above 1740 pg/mL was associated with early (≤32 weeks) spontaneous preterm delivery with an odds ratio of 9.5 (95% CI 2.9 – 31.1) after adjusting for history of spontaneous preterm delivery, parity, gestational age at amniocentesis and duration of sample storage. Similarly, an elevation of amniotic fluid IP-10 concentration above 502 pg/mL was associated with late (> 32 weeks) spontaneous preterm delivery with an odds ratio of 3.9 (95% CI 1.6 – 9.9) after adjusting for similar potential confounders.

There was no significant difference in the median amniotic fluid concentration of either IL-6 or IP-10 between patients who delivered because of maternal or fetal indications and women who delivered at term [IL-6; unadjusted P = 0.03, adjusted P = 0.09 (Bonferroni correction) and IP-10; P = 0.9].

When the analysis in the control group was restricted to women who delivered at term (n = 652 and n = 633 for IL-6 and IP-10, respectively) without obstetrical complications (i.e., abruption, SGA, fetal death, gestational hypertension and preeclampsia), the 95th percentile cut-off for amniotic fluid IL-6 and IP-10 were 2935 pg/mL and 2200 pg/mL, respectively.

There were 6.3% (50/796) and 5.8% (45/770) of patients who had amniotic fluid IL-6 and IP-10 above these cut-offs.

Among patients (n = 50) who had amniotic fluid IL-6 above this extremely high concentration, only 8% (n = 4) delivered before 32 weeks of gestation and the majority delivered at term (84%; 42/50). Similarly, among patients who had amniotic fluid IP-10 above this extremely high concentration (n = 45), only 11% (n = 5) delivered after 32 weeks of gestation and the majority delivered at term (82%; 37/45). Among the patients who delivered at term, women with extremely high IL-6 concentrations had a significantly higher rate of pregnancy complications (gestational hypertension, preeclampsia, SGA, placental abruption and placental previa) than those with lower IL-6 concentrations [extremely high IL-6 23.8% (10/42) vs. normal/low IL-6 9.9% (68/688), P = 0.009]. In contrast, this difference was not significant in women with extremely high IP-10 who delivered at term [extremely high IP – 10 13.2% (6/37) vs. normal/low IP-10 10.3% (69/671), P = 0.27].

Maternal plasma IL-6 and IP-10

Neither the maternal plasma IL-6 or IP-10 concentrations in the midtrimester were associated with the occurrence of spontaneous preterm delivery ≤32 weeks or > 32 weeks of gestation. There was no association between maternal plasma IL-6 or IP-10 concentrations and the occurrence of an indicated preterm birth (Table 2, Figures 3 and 4).

DISCUSSION

Principal findings of the study

Among patients who underwent a mid-trimester amniocentesis for clinical indications, the following observations were made: 1) patients who subsequently had a spontaneous preterm delivery at or before 32 weeks had a higher median amniotic fluid IL-6 concentration than those who delivered at term or > 32 weeks of gestation; 2) patients who had a spontaneous preterm delivery > 32 weeks had a higher median amniotic fluid IP-10 than those who delivered at term; 3) there were no significant differences in median midtrimester amniotic fluid concentrations of IL-6 and IP-10 between women who had an indicated preterm birth and those who delivered at term; and 4) standard cultivation techniques for aerobic and anaerobic bacteria as well as *Mycoplasma* species yielded negative results for the majority (99.8%) of patients. A positive amniotic fluid culture was found in two cases (2/796; 0.2%). However, these patients had a term delivery and they had no evidence of intra-amniotic inflammation (by amniotic fluid white blood cell count, glucose concentration, or the amniotic fluid levels of IL-6 or IP-10). Therefore, these cases are likely to represent contamination of the specimens.

Amniotic fluid IP-10 and preterm delivery after 32 weeks of gestation

This study is the first to demonstrate an association between amniotic fluid IP-10 concentrations and the risk for spontaneous preterm delivery after 32 weeks of gestation. Chemokines of the CXC family share the expression of their specific receptors on both leukocytes and endothelial cells [181], and have a role in the control of inflammation and angiogenesis [12, 14, 145, 181]. IP-10 has been implicated in preeclampsia because of its proinflammatory and anti-angiogenic properties. Indeed, Gotsch et al. [62] reported a higher median maternal plasma IP-10 concentration in patients with preeclampsia than in controls.

ROC curve analysis indicated that an amniotic fluid concentration of IP-10 > 502 pg/mL in the mid-trimester had a sensitivity of 83%, a specificity of 40%, a positive predictive value of 6% (7/103), and a negative predictive value of 98% for the identification of patients who subsequently delivered after 32 weeks of gestation. IP-10 has been implicated in the

mechanism responsible for organ rejection [130, 138, 185, 195]. We have previously demonstrated an association between an elevation of IP-10 in the amniotic fluid, maternal blood, and umbilical cord blood and the diagnosis of chronic chorioamnionitis and villitis of unknown etiology in patients with preterm labor, and in particular, late preterm labor [86]. We have proposed that a fraction of late preterm labor is a result of maternal-fetal rejection [86, 87], and that IP-10 elevations in amniotic fluid act as a T-cell chemotactic cytokine responsible for the recruitment of T-cells from the maternal decidua into the chorioamniotic membranes.

When intra-amniotic inflammation was defined as an amniotic fluid concentration of IP-10 above 2200 pg/mL (which corresponds to the 95th percentile of patients who delivered at term without any complications), the prevalence of intra-amniotic inflammation among patients who underwent midtrimester amniocentesis was 5.8% (45/770).

In this study, placental pathology was not available in the majority of cases, because ours is a referral center for prenatal diagnosis, so patients often delivered at other institutions. It would be important to determine if there is an association between an elevation of amniotic fluid IP-10 in the midtrimester and the subsequent diagnosis of chronic chorioamnionitis and/or villitis of unknown etiology.

Indicated preterm deliveries

A subset of patients in our study was delivered preterm due to maternal or fetal indications. There were no significant differences in the median midtrimester amniotic fluid and maternal plasma concentrations of IL-6 and IP-10 between women who had an indicated preterm delivery and those who delivered at term. Therefore, biomarkers used in this study do not seem to be associated with the subsequent development of indicated preterm delivery, which is largely due to SGA and preeclampsia.

Amniotic fluid IL-6 and pregnancy outcomes

We reported IL-6 in amniotic fluid in patients with preterm labor and subsequently in preterm PROM, and demonstrated an association with microbiologically proven intra-amniotic infection using cultivation techniques [9, 29, 79, 82, 136, 156, 167, 170, 171, 177, 178, 200, 201]. However, an observation that has been consistent across studies was that a subgroup of patients with a high concentration of IL-6 in amniotic fluid but negative amniotic fluid culture for bacteria, are at high risk for adverse pregnancy outcome including preterm delivery, a short amniocentesis-to-delivery interval, and neonatal complications [3, 37, 96, 158, 191, 193, 210]. We reported that among patients undergoing midtrimester amniocentesis, those who lost their pregnancy had a higher median amniotic fluid concentration of IL-6 than of those patients who delivered at term [173].

Subsequently, a large number of studies have confirmed these observations. Indeed, amniotic fluid IL-6 is widely considered a sensitive and specific parameter for the detection of intra-amniotic infection [9, 29, 79, 82, 136, 156, 167, 170, 171, 177, 178, 200, 201]. Consequently, an elevated IL-6 has become synonymous with the presence of intra-amniotic inflammation [9, 29, 79, 82, 136, 156, 167, 170, 171, 177, 178, 200, 201, 210].

Most previous studies examining the relationship between amniotic fluid IL-6 and pregnancy outcome have been case control studies. Such studies have been largely consistent in demonstrating a higher median concentration of IL-6 in patients who delivered preterm [9, 29, 79, 82, 136, 156, 167, 170, 171, 177, 178, 200, 201]; however, some investigators have not found this association [7]. The advantages of a cohort study over case-control studies are well known.

The main finding of this study was that, among patients who had a preterm delivery (< 37 weeks of gestation), only those with early preterm birth (≤32 weeks of gestation) had a significantly higher median midtrimester IL-6 amniotic fluid concentration than women delivering at term. The cut-off for analysis ≤32 weeks and between 32 (1/7) and 36 (6/7) weeks of gestation were selected pre-hoc. We did so because of previous observations that patients with an elevated amniotic fluid IL-6 tended to have spontaneous rupture of membranes, spontaneous abortion after a midtrimester amniocentesis, or preterm delivery shortly after the procedure or early preterm deliveries (defined as ≤32 weeks of gestation) [191, 193]. We also made a similar observation using other biological markers of intra-amniotic inflammation, such as matrix metalloproteinase (MMP)-8 [209], and chemokines, such as monocyte chemotactic protein (MCP)-1 [40]. Therefore, we reasoned that an intra-amniotic inflammatory process characterized by an elevation of amniotic fluid IL-6 would be associated with early preterm delivery, but not necessarily with a late preterm delivery. Our data suggest that this is indeed the case. The most frequent cause of intra-amniotic inflammation is microbial invasion of the amniotic cavity with genital Mycoplasmas [61, 147, 154, 159, 206, 210]. However, in this cohort study that included 847 patients, most amniotic fluid specimens were sterile using cultivation techniques. Therefore, the cause of intra-amniotic inflammation has not been identified. It is possible that a fraction of these patients had subclinical bacterial infections which escaped detection with cultivation techniques and which may have been identified using molecular microbiologic techniques. Future studies are necessary to address this question. Another possibility is that the inflammatory process is caused by viruses [5, 24, 92]. Such microorganisms have been found in the amniotic cavity in other studies [8, 118, 142, 189, 192]. It is also possible that the inflammatory process does not have an infectious origin. Indeed, inflammation is a non-specific host response to tissue injury, and can be induced under sterile conditions. Recently, we have reported the presence of damage-associated molecular patterns (DAMPs) in amniotic fluid [180]. These molecules can recognize “danger signals” that may lead to inflammation by mechanisms which involve a different set of pattern recognition receptors from those involved in the detection of microorganisms, although there may be some overlap [91, 137]. For example, some Toll-like receptors can recognize endogenous ligands released during tissue damage [1, 83, 84].

This is the first study to allow a rigorous calculation of the diagnostic indices and predictive values of elevated amniotic fluid IL-6, because previous studies were of a case-control nature, and therefore, have limitations in the generation of positive and negative predictive values. ROC curve analysis indicated that an amniotic fluid concentration of IL-6 ≥1740 pg/mL. This cutoff had a sensitivity of 58%, a specificity of 88%, a positive predictive value of 7% (7/103), and a negative predictive value of 99% for the identification of patients who subsequently delivered ≤32 weeks of gestation.

When intra-amniotic inflammation was defined as an amniotic fluid concentration of IL-6 above 2935 pg/mL (which corresponds to the 95th percentile of patients who delivered at term without any complications), the prevalence of intraamniotic inflammation was 6% (50/796) among patients who underwent mid-trimester amniocentesis. These observations are noteworthy for several reasons. First, they indicate that an inflammatory process defined as an elevation of amniotic fluid IL-6 in the midtrimester would only account for approximately half of all preterm deliveries before 32 weeks of gestation. Second, our findings indicate that many patients have an elevation of amniotic fluid IL-6 in the midtrimester, and this is not followed by early spontaneous preterm delivery or even preterm delivery (< 37 weeks). Most patients with an elevated amniotic fluid IL-6 had an uncomplicated delivery at term. It is possible that a combination of cytokines, chemokines, and growth factors may identify patients at risk for the subsequent development of different

obstetrical syndromes (e.g., preterm labor, preterm prelabor rupture of membrane, fetal death, SGA, preeclampsia, etc.) [146].

Maternal plasma concentrations of IP-10 and IL-6

Of interest, the median maternal plasma IP-10 concentration was not significantly different between patients who delivered preterm and those who delivered at term. This indicates that the inflammatory process is confined to the amniotic cavity and cannot be detected by measuring IP-10 in the maternal circulation. This was also the case for IL-6.

Clinical significance of the observations

This study confirms that there are at least two distinct types of intra-amniotic inflammatory processes in the midtrimester of pregnancy; one that is characterized by an elevation of amniotic fluid IL-6 and a second one, by elevations of the T-cell chemokine, IP-10. The former is a risk factor for early preterm delivery (< 32 weeks), while the latter is a risk factor for late preterm delivery (defined for the purposes of this study as > 32 weeks). Of interest is that we have identified a subgroup of patients that have either form of intra-amniotic inflammation but have an apparently normal pregnancy outcome. The significance, etiology, natural history, and long-term consequences of these inflammatory processes remain to be established. Moreover, we have defined these two types of inflammation by using one cytokine (IL-6) and one chemokine (IP-10). These two types of intra-amniotic inflammation may represent two extreme phenotypes, and some patients have an elevation (above the 95th percentile for both) of both IL-6 and IP-10, which may constitute the evolution of one type of inflammation into a different type. It is noteworthy that the cytokine and chemokine network in the amniotic cavity is complex, and we anticipate that there would be changes in the profile of other cytokines and chemokines, which have not been studied yet. Such studies are needed to characterize not only the intra-amniotic inflammatory response, but also what such profile will tell us about the consequences of the inflammatory process on the fetus and pregnancy.

Strengths and limitations of the study

The major strength of this study is the cohort design and the ascertainment of a relatively large number of patients. Also, the definition of preterm delivery at < 32 weeks of gestation and after 32 weeks of gestation was decided pre-hoc, based upon previous studies [87, 147, 150, 154]. Future studies are required to examine whether microbial invasion of the amniotic cavity can be detected in cases with the different types of inflammation using molecular techniques, such as broad range PCR and specific primers. It would also be interesting to determine if viral invasion of the amniotic cavity can account for some cases of intra-amniotic inflammation and if so, what type.

Another limitation is that placental pathology was not available for examination. Unfortunately the majority of the study population did not deliver in the same institution where the amniotic fluid had been collected and, as a consequence, it was not possible to study an adequate number of placentas for a correlation with acute and chronic chorioamnionitis.

CONCLUSION

This is the first cohort study to report the relationship between midtrimester amniotic fluid IP-10 and IL-6 concentrations and pregnancy outcome. We report an association between elevated amniotic fluid IP-10 concentrations and the subsequent development of spontaneous preterm delivery after 32 weeks of gestation. We also provide evidence for an association between an elevated amniotic fluid IL-6 concentration and spontaneous preterm

delivery before 32 weeks of gestation. Importantly, we have identified that a fraction of patients who undergo a midtrimester amniocentesis have a subclinical inflammatory process, defined either as an elevated IL-6 or IP-10. Further studies are required to determine the short- and long-term clinical significance for mother and infant of such subclinical processes.

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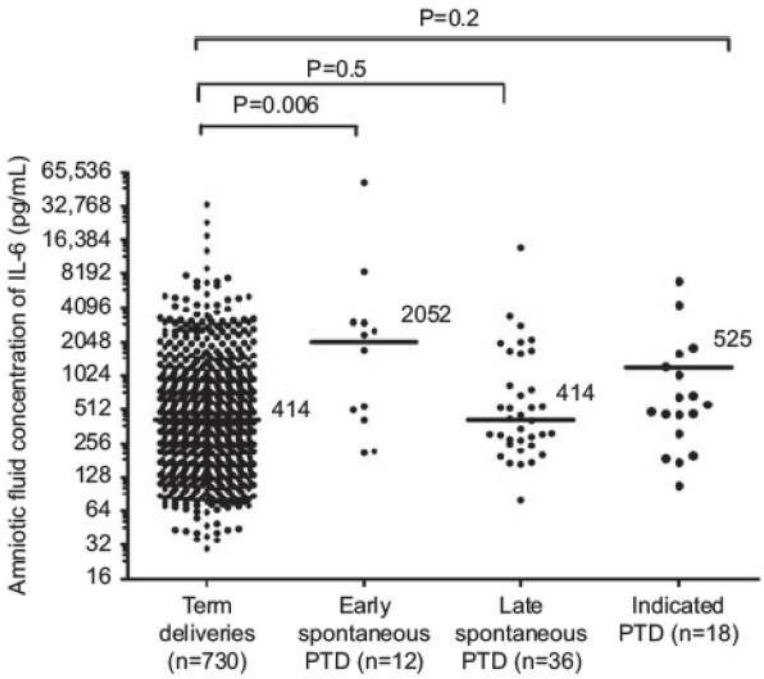

Figure 1.

Median amniotic fluid IL-6 concentration in the controls, spontaneous preterm delivery ≤32 weeks, spontaneous preterm delivery > 32 weeks and indicated preterm group (preterm ≤32 weeks vs. controls P = 0.006, preterm > 32 weeks vs. controls P = 0.5).

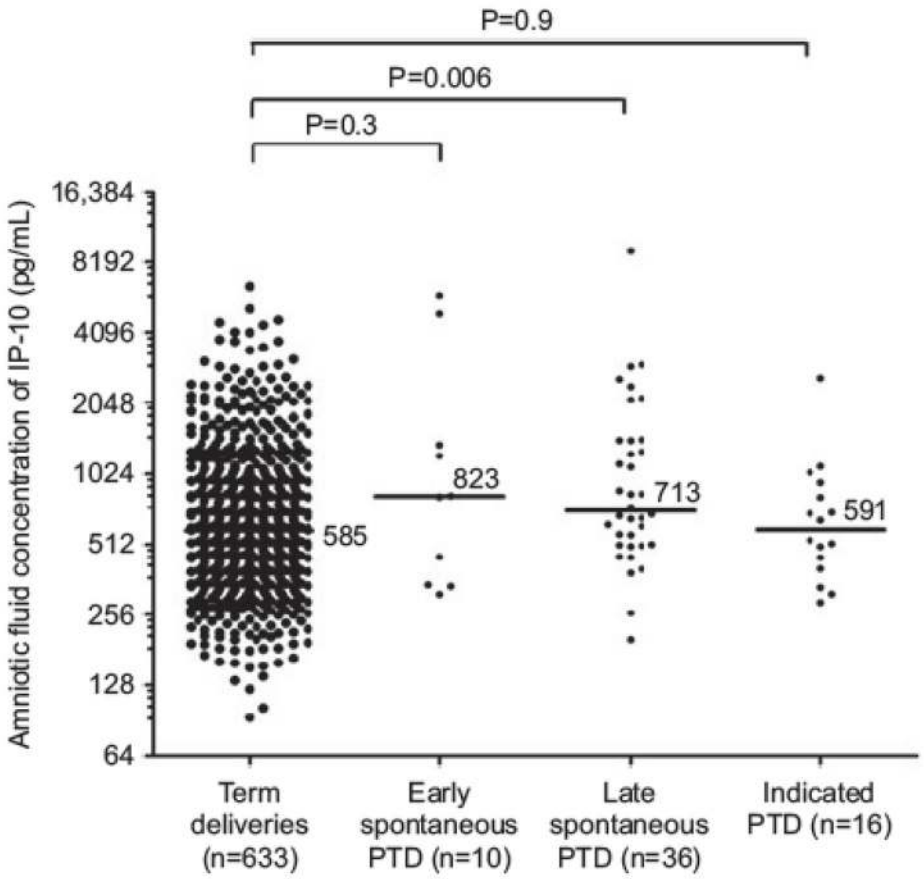

Figure 2.

Median amniotic fluid IP-10 concentration in the controls, spontaneous preterm deliveries ≤ 32 weeks, spontaneous preterm deliveries > 32 weeks and indicated preterm group (preterm group > 32 weeks vs. controls P = 0.006, preterm group ≤32 weeks vs. controls P = 0.3).

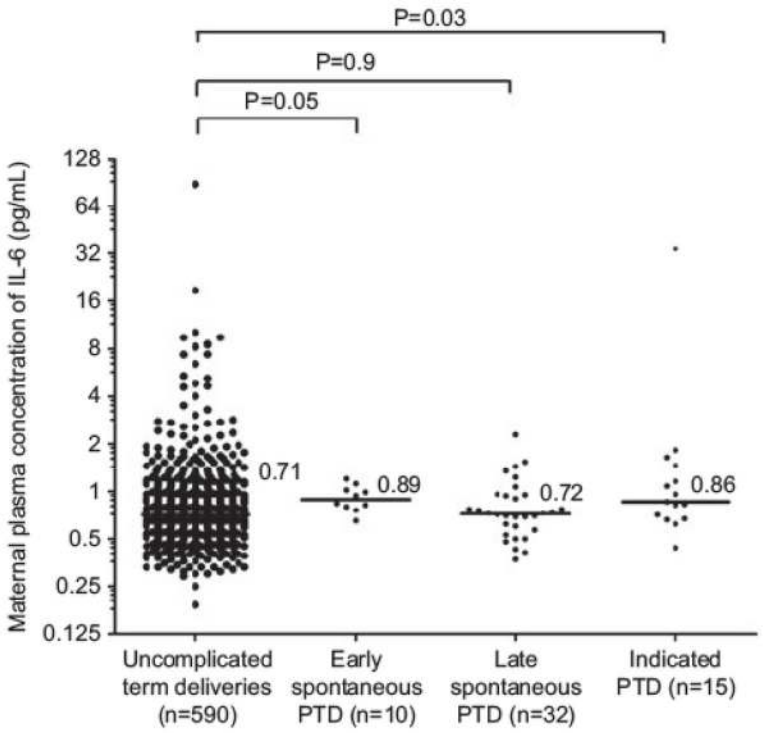

Figure 3.

Median maternal plasma IL-6 concentration in the controls, spontaneous delivery ≤32 weeks, spontaneous preterm delivery > 32 weeks and indicated preterm group (preterm group ≤32 weeks vs. controls P = 0.05, preterm group > 32 weeks vs. controls P = 0.9).

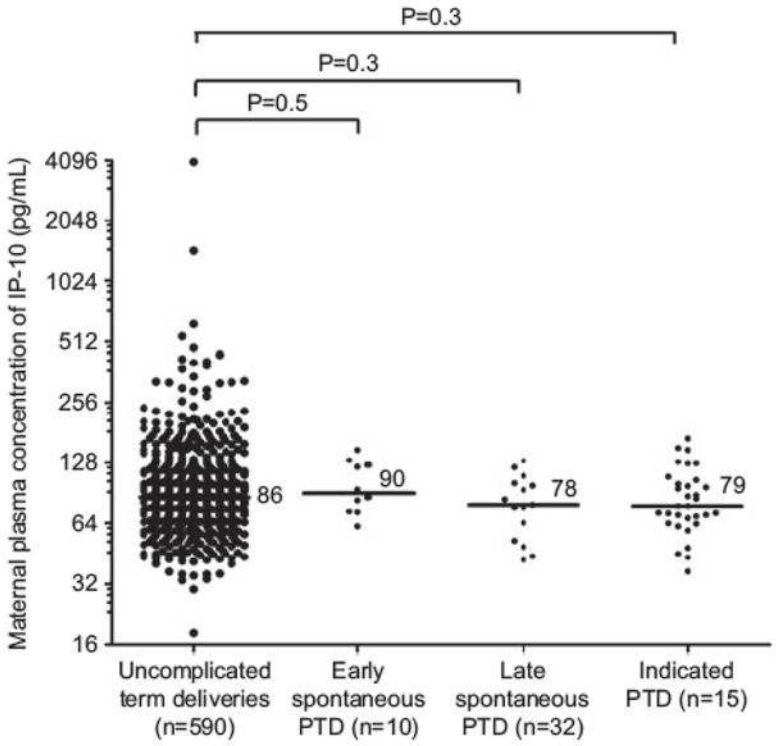

Figure 4.

Median maternal plasma IP-10 concentration in the controls (uncomplicated term deliveries), spontaneous preterm deliveries ≤32 weeks, spontaneous preterm deliveries > 32 weeks and indicated preterm group (preterm group > 32 weeks vs. controls P = 0.3, preterm group ≤32 weeks vs. controls P = 0.5).

Table 1

Clinical characteristics of the study population (n=796)

	Term deliveries	Early SPTD ≤32 weeks (n=12)	Late SPTD >32 weeks (n=36)	Indicated preterm delivery (n=18)	P-value
Age (years)	36 (35–38)	38(33–39)	36 (34–38)	38 (35–40)	0.2
Ethnicity					
Caucasian	719 (98.5%)	10 (83.3%)	35 (97.2%)	18 (100%)	0.004
African American	4 (0.5%)	1 (8.3%)	1 (2.8%)	-	
Asian	3 (0.4%)	-	-	-	
Others	4 (0.5%)	1 (8.3%)	-	-	
Nulliparous	285 (39.0%)	6 (50%)	20 (55.6%)	8 (44.4%)	0.2
Previous SPTD (among multiparous)	21/445 (4.7%)	2/6 (33.3%)	4/16 (25.0%)	0/10	<0.001
GA at amniocentesis (weeks)	16.3 (15.9–16.9)	16.5 (15.7–16.9)	16.3 (15.9–17.1)	16.1 (15.7–16.8)	0.9
AF WBC count (cells/mL)	3 (2–6)	3 (1–10)	5 (3–9)	5 (3–8)	0.002
AF glucose (mg/dL)	48 (43–52)	40 (36–46)	48 (39–51)	45 (41–50)	0.05
Amniocentesis-to-delivery interval (weeks)	23.2 (22–24)	5.7 (1.3–13.1)	19 (17–20)	17.9 (14.5–19.8)	<0.001
GA at delivery (weeks)	39.7 (38.9–40.7)	23.2 (18.1–28.9)	36.0 (34.5–36.3)	35.5 (34.5–36.3)	<0.001
Birth weight (g)	3380 (3120–3620)	1035 (493–1341)	2577 (2300–2800)	2170 (1275–2582)	<0.001

Values expressed as median (inter-quartile) or number (percent).

SPTD = spontaneous preterm delivery, GA = gestational age, AF = amniotic fluid.

P = Kruskal-Wallis or Pearson $\chi 2$.

Table 2

Amniotic fluid and maternal blood IL-6 and IP-10 concentrations by types of preterm delivery.

	Term deliveries (n=730)	Early SPTD ≤32 weeks (n=12)	Late SPTD >32 weeks (n=36)	Indicated preterm delivery (n=18)	P-value
AF interleukin-6 (pg/mL)	414 (209–930)	2052.5 (435–3015)	414.9 (259–2348)	525.1 (283–1316)	0.02
AF interferon-gamma-inducible protein-10 (pg/mL)	583 (403–954)	823.6 (342–2253)	713.7 (509–1427)	591.5 (414–911)	0.04
Duration of sample storage (years)	3.47 (3.0–3.8)	3.76 (2.5–4.3)	3.36 (2.4–3.9)	3.46 (2.6–3.8)	0.7
MB interleukin-6 (pg/mL)	0.71 (0.55–0.93)	0.89 (0.79–1.04)	0.72 (0.54–0.95)	0.86 (0.67–1.46)	0.04
MB interferon-gamma-inducible protein-10 (pg/mL)	86.6 (68.0–113.1)	90.8 (72.9–126.8)	78.4 (64.9–104.3)	79.5 (52.4–101.7)	0.4

P = Kruskal-Wallis test, AF = amniotic fluid, MB = maternal blood.

AF IL-6: early spontaneous preterm delivery (SPTD) vs. uncomplicated term deliveries: P = 0.006; late SPTD vs. uncomplicated term deliveries: P = 0.5; indicated preterm deliveries vs. uncomplicated term deliveries: P = 0.2.

AF IP-10: early SPTD vs. uncomplicated term deliveries: P = 0.3; late SPTD vs. uncomplicated term deliveries: P = 0.006; indicated preterm deliveries vs. uncomplicated term deliveries: P = 0.9.

MB IL-6: early SPTD vs. uncomplicated term deliveries: P = 0.05; late SPTD vs. uncomplicated term deliveries: P = 0.9; indicated preterm deliveries vs. uncomplicated term deliveries: P = 0.03 (P = 0.09 after adjusting for multiple comparisons).

MB IP-10: early SPTD vs. uncomplicated term deliveries: P = 0.5; late SPTD vs. uncomplicated term deliveries: P = 0.3; indicated preterm deliveries vs. uncomplicated term deliveries: P = 0.3.