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Fetal death: an extreme manifestation of maternal anti-fetal rejection

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Abstract

Objective—The aim of this study was to determine the association between chronic placental inflammation and amniotic fluid (AF) markers of maternal anti-fetal rejection as well as the presence of microorganisms in the AF fluid of patients with fetal death.

Study Design—This cohort study included 40 patients with fetal death whose placentas were examined for chronic inflammatory lesions and whose AF chemokine ligand (CXCL)10 and interleukin (IL)-6 concentrations were determined by immunoassays. AF was processed for

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bacteria, mycoplasmas and viruses using cultivation and molecular microbiologic techniques (i.e. PCR-ESI/MS).

Results—(1) The most prevalent placental findings were maternal vascular underperfusion (63.2%, 24/38), followed by chronic inflammatory lesions (57.9%, 22/38); (2) chronic chorioamnionitis (18/38) was three times more frequent than villitis of unknown etiology (6/38); (3) an elevated AF CXCL10 concentration (above the 95th centile) was present in 60% of the cases, and a receiver operating characteristics (ROC)-derived cut-off of 2.9 ng/mL had a sensitivity of 73% and a specificity of 75% in the identification of chronic placental inflammatory lesions; (4) only five cases had microbial invasion of the amniotic cavity, and the presence of microorganisms did not correlate with chronic placental inflammation.

Conclusion—In women with unexplained fetal death, there is an association between elevated AF CXCL10 and chronic placental inflammatory lesions. Therefore, we conclude that a subset of patients with fetal death may have endured a breakdown of maternal-fetal tolerance, which cannot be attributed to microorganisms in the amniotic cavity.

Keywords

amniotic fluid; bacteria; chorioamnionitis; chronic deciduitis; chronic inflammation; CXCL10/IP-10 interleukin-6 (IL-6); placenta; villitis of unknown etiology (VUE); viruses

Introduction

Fetal death, characterized by the cessation of heart activity after 20 weeks of gestation [1], is considered one of the “great obstetrical syndromes” [2–4] caused by multiple etiologies, including failure of the placenta to provide oxygen/nutrients to support fetal growth [5–14]; infections such as syphilis [15–19], malaria [16, 20–22], Zika virus [23–25] and Listeria monocytogenes [26–30]; fetal cardiac arrhythmias [31, 32]; congenital anomalies [6, 33–36]; umbilical cord accidents (i.e. “true” knots of the umbilical cord) [10, 35–39] and complications of monochorial placentation in multiple gestation pregnancies [40–42].

Affecting more than three million pregnancies per year, fetal death is a clinical challenge; at present, there are no good methods to assess the magnitude of risk or to prevent this devastating complication of pregnancy [43–45]. Although several classification systems have been proposed and are used to attempt to identify co-morbid events associated with fetal death [36, 46–63], a firm causal link between the events labeled in these classifications as “causes” of fetal death and the actual cause of death is extremely difficult to prove [39, 49, 58, 64–66]. Therefore, these classification systems provide a general idea of the context in which fetal death occurred, rather than providing unequivocal proof of the cause of death (e.g. total placental abruption [67, 68] or maternal trauma [69–71]).

The fetus and placenta are semi-allografts to the maternal host, as 50% of their genomes are paternally derived [72–76]. The placenta is widely considered to be the most successful transplant in nature, a biological adaptation required for viviparity, and accomplished by immune tolerance [72, 76–86]. Sir Peter Medawar, the founder of the field of Reproductive Immunology, crafted the fundamental question of the field [87, 88]: “The immunological problem of pregnancy may be formulated thus: how does the pregnant mother contrive to

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nourish within itself, for many weeks or months, a foetus that is an antigenically foreign body?" In other words, why are the fetus and placenta not rejected?

Tolerance, "the active state of antigen-specific nonresponsiveness," leading to diminished reactivity to paternal antigens expressed by the placenta and/or fetus, is considered key for a successful pregnancy [75–78, 83–86, 89–104]. The mechanisms responsible for tolerance in pregnancy have been reviewed by Elizabeth Bonney [90, 91, 93, 104–106], Sing-Sing Way [107, 108], and Adrian Erlebacher [72, 86, 109–112].

A fundamental question, largely overlooked, is whether semi-allograft rejection mediated by a breakdown of tolerance is a mechanism of disease responsible for adverse pregnancy outcome. Transplant rejection is characterized by the infiltration of donor CD8 + (cytotoxic) T cells into the graft and by organ dysfunction or failure [113–115]. Renal transplant rejection is diagnosed in patients who have a drop in the glomerular filtration rate (i.e. creatinine clearance) and an infiltration of lymphocytes from the donor into the transplanted organ [116, 117]. The presence of maternal lymphocytes at the maternofetal interfaces, such as the chorioamniotic membranes and villous tree, as seen in chronic chorioamnionitis and villitis of unknown etiology (VUE), respectively, demonstrates a breakdown in maternal (host) tolerance of the fetus (graft) [118–123]. In parallel fashion, chemokine markers of chronic inflammation, such as chemokine ligand 10 (CXCL10) were elevated in the amniotic fluid (AF) when signs of rejection were identified by placental histology [118, 121, 123, 124].

We have provided evidence that maternal anti-fetal rejection is operative in a subset of patients with spontaneous preterm labor [118–120, 122, 124–126], preterm prelabor rupture of the membranes (PPROM) [118], maternal floor infarction [127], and other obstetrical syndromes [121, 122, 126]. Three placental pathology lesions have been considered manifestations of maternal anti-fetal rejection: (1) chronic chorioamnionitis [118–122, 124, 126, 128, 129]; (2) VUE [122, 123, 126, 130, 131]; and (3) chronic deciduitis [122, 126, 129]. In these lesions, maternal lymphocytes infiltrate the fetal tissue (the chorioamniotic membranes in chronic chorioamnionitis, the villous tree in VUE, and the basal plate of the placenta in chronic deciduitis) [118, 123, 126, 130–134].

The ultimate form of transplant rejection is a total failure of the transplanted organ, which manifests itself as renal failure [117], cardiac failure [135], etc. Fetal death in the context of maternal anti-fetal rejection can be considered as the most severe expression of such a rejection. We previously reported that chronic chorioamnionitis is frequent in patients with fetal death [121, 126]; however, a pervasive question is whether some cases of chronic chorioamnionitis may be due to subclinical infection rather than to maternal anti-fetal rejection. Therefore, we conducted this study to determine whether fetal death associated with placental lesions consistent with maternal anti-fetal rejection is related to intra-amniotic infection. This clinical condition was diagnosed by the presence of bacteria and/or viruses using cultivation and molecular microbiologic techniques.

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Materials and methods

Patient population and study materials

This cohort study was performed at Hutzel Women's Hospital, Detroit, MI, USA. Subjects included patients who presented with unexplained fetal death (n = 40) defined as spontaneous death of a fetus at or after 20 weeks of gestation and not attributed to an identifiable cause. Patients with a multifetal gestation, pre-gestational diabetes, and prenatal diagnoses of fetal anomalies as well as chromosomal abnormalities were excluded from the study.

Patients diagnosed with fetal death by ultrasound were offered amniocentesis for karyotype determination and assessment for the presence of intra-amniotic infection. We selected cases in which AF samples were available from the Bank of Biological Materials of the Perinatology Research Branch, Wayne State University, and the Detroit Medical Center. Each participant provided written informed consent for the use of their information and biological specimens for research. The Institutional Review Boards of Wayne State University and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services (NICHD/NIH/DHHS), approved the collection of clinical information and use of biological materials for research purposes.

Small for gestational age was defined as birthweight less than the 10th percentile for gestational age [136]. Chronic maternal condition was defined as a chronic medical illness affecting an organ system that may require medication (e.g. asthma, lupus). Prior preterm birth was defined as a pregnancy delivering at or after 20+0 and before 37+0 weeks of gestation. Preeclampsia was defined as the presence of new-onset hypertension and proteinuria at or beyond 20+0 weeks of gestation [137]. Mean arterial blood pressure [138] was defined as diastolic blood pressure + (1/3*pulse pressure). The use of illicit drugs was determined by urine drug screening. Microbial invasion of the amniotic cavity was defined as a positive culture or positive result from molecular microbiologic techniques [139]. Clinical chorioamnionitis and placental abruption were diagnosed by physicians in the clinical setting.

Histopathologic examination

Histopathologic assessment of placental lesions was performed by examination of hematoxylin and eosin (H&E)-stained sections of the chorioamniotic membranes, placental disc and umbilical cord in each case. Diagnoses of findings were made by perinatal pathologists, according to previously published criteria [118, 126, 140–144]. The following categories of placental findings were characterized: findings consistent with amniotic fluid infection (AFI), fetal vascular thrombo-occlusive disease, maternal vascular underperfusion, and chronic inflammatory lesions. Sub-classes of chronic inflammatory lesions included in this study were chronic chorioamnionitis, VUE and chronic deciduitis. Chronic chorioamnionitis was diagnosed when lymphocytic infiltrates were observed in the chorionic trophoblast layer or chorioamniotic connective tissue [118, 126] (Figure 1). The severity of inflammation was scored on grade and stage. The extent of inflammation was graded as 0

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(no inflammation), 1 (more than two foci or patchy inflammation), or 2 (diffuse inflammation). The stage of inflammation was scored as stage 1 if amniotropic lymphocytic infiltration was limited to the chorionic trophoblast layer sparing the chorioamniotic connective tissue, and stage 2 if lymphocytic infiltration into the chorioamniotic connective tissue was noted. Villitis of unknown etiology was defined as the presence of lymphohistiocytic infiltrates in the chorionic villi [126, 144, 145]. Grading of VUE is based on the number of affected villi and the extent of involvement. Low-grade lesions affect less than 10 villi; high-grade lesions affect more than 10 villi. VUE can be characterized by whether the pattern of involvement is distal (terminal and intermediate villi), proximal (stem villi), or basal (anchoring villi). In this study, the pattern of VUE was characterized as either proximal or basal, according to the protocol at our institution. Chronic deciduitis was diagnosed by the presence of both lymphocytes and plasma cells in the basal plate of the placenta [133].

Enzyme-linked immunosorbent assay for CXCL10 and IL-6

Amniotic fluid concentrations of CXCL10 and interleukin (IL)-6 were measured by specific ELISA assays (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The sensitivity of the assay for AF CXCL10 and IL-6 was 6.82 pg/mL and 2.24 pg/mL, respectively. The inter-assay coefficient of variation was 2.75% for CXCL10 and 2.84% for IL-6; the intra-assay coefficient of variation was 3.87% and 3.68%, respectively. Gervasi et al. [146] reported reference ranges for AF CXCL10 sampled in the mid-trimester for pregnancies later delivered at term without complications; the 95th percentile for CXCL10 was defined as 2.2 ng/mL and this cut-off was used in our study [146]. IL-6 is an inflammation-related cytokine associated with systemic inflammation in the maternal [147], fetal [148, 149], and placental compartments [150] as well as with intra-amniotic infection and intra-amniotic inflammation [146, 151–167]. We used a cut-off of 2.6 ng/mL for the identification of intra-amniotic inflammation [157,168, 169].

Microbiologic evaluation

Amniotic fluid was transported in a capped sterile syringe to the clinical laboratory where it was cultured for aerobic and anaerobic bacteria, including genital Mycoplasmas and viruses. Determinations of the AF white blood cell count [170], glucose concentration [171] and Gram stain [172] were performed first. The AF was then centrifuged at 1300 × g for 10 min at 4°C and then stored at −80°C until analyzed by immunoassay. The analysis for the presence of bacteria, genital mycoplasmas and/or viruses was performed by cultivation and broad-range polymerase chain reaction (PCR) coupled with electrospray ionization mass spectrometry (PCR/ESI-MS) (Ibis® Technology, Athogen, Carlsbad, CA, USA) [139, 173–181].

Statistical analysis

Distributions of continuous variables were examined for skewness and normality. The Shapiro-Wilk test was performed to assess deviations of arithmetic data from normality. Bivariate analysis was performed with the Mann-Whitney U and Kruskal-Wallis tests for the assessment of non-parametric continuous variables (e.g. CXCL10, IL-6). Spearman's rho was used to assess correlations. Odds ratios and 95% confidence intervals (CI) were

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calculated to assess magnitudes of association. Statistical analysis was performed using SPSS Version 19 (SPSS Inc., Chicago, IL, USA). A two-tailed P value of <0.05 was considered significant.

Results

Study population

Forty patients with a fetal death met the inclusion criteria. Table 1 provides the clinical characteristics, obstetrical histories and laboratory results of the AF samples. Patients of African-American origin comprised 87.5% (35/40) of the study population. Only one patient had a prior fetal demise. The median gestational age at presentation was 30.9 weeks, indicating that most fetal deaths occurred in the third trimester, and 27.5% of fetal deaths occurred in small-for-gestational-age cases. The median interval from diagnosis to delivery was 0.1 weeks [range 0–0.5 weeks], one patient delivered spontaneously, 37 had induction of labor, and two were delivered by cesarean section. A Kleihauer-Betke test was performed in 33 patients and only one had a positive result.

Placental lesions

The most frequent lesions were those consistent with maternal vascular underperfusion (63.2%; 24/38), followed by chronic inflammatory lesions (57.9%; 22/38), acute inflammatory lesions consistent with AFI (28.2%; 11/39), and fetal vascular thrombo-occlusive disease (23.1%; 9/39) (Figure 2). More than one type of lesion was diagnosed in 61.5% (24/39) of the placentas. The most frequent combination of lesions (14/22) included those consistent with maternal vascular underperfusion and chronic placental inflammation (Figure 3). Chronic placental inflammatory lesions consisted of 18 cases with chronic chorioamnionitis (17/18 cases were preterm gestation), six cases with VUE (5/6 cases were preterm gestation) and seven cases with chronic deciduitis. In regard to the staging and grading of chronic chorioamnionitis, 61.1% (11/18) were stage 2 (involvement of the chorionic connective tissue) (Figure 1), 38.9% (7/18) were grade 2 (a diffuse, rather than localized, inflammatory process), and 33.3% (6/18) also involved the chorionic plate. In four of the cases, chronic deciduitis was also present.

All six cases of VUE had basal, low-grade lesions (five cases were preterm gestation). In all six cases, the AF was negative for bacteria and viruses. In one-half of the VUE cases (3/6), there was evidence of chronic chorioamnionitis, and equally in 3/6 cases, there was chronic deciduitis.

Amniotic fluid concentrations of CXCL10 and interleukin-6 in fetal death

The median AF concentration of CXCL10 (a chemokine elevated in cases of allograft rejection) was 2.9 ng/mL [IQR 1.7–7.6] – this is higher than the 95th centile of CXCL10 measured in the mid-trimester AF (2.2 ng/mL) of patients with a normal pregnancy outcome (see Supplemental Material Figure 1). An elevated AF CXCL10 concentration was present in 60% (24/40) of patients with a fetal death.

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The AF CXCL10 concentration was elevated for 17 of 22 (77.2%) patients with chronic placental inflammatory lesions, including two cases with both chronic chorioamnionitis and VUE – this included 13 cases with chronic chorioamnionitis and five with VUE. There was a positive correlation between the concentration of CXCL10 and the occurrence of chronic placental inflammatory lesions ($r = 0.37$, $P = 0.02$). We found no correlation between gestational age and CXCL10 for the entire study population ($r = -0.23$; $P = 0.16$).

The median AF concentration of CXCL10 in patients with and without chronic placental inflammatory lesions was [5.2 (2.2–9.2) vs. 2.1 (1.6–3.3), $P = 0.01$], respectively. In the current study, a receiver operating characteristics (ROC) curve was plotted using sensitivity and 1-specificity; an AF CXCL10 concentration of 2.9 ng/mL had a sensitivity of 73%, a specificity of 75%, a positive likelihood ratio of 2.9, and a negative likelihood ratio of 0.4 with an area under the curve of 0.72 ($P = 0.02$) for the presence of chronic placental inflammatory lesions. Of note, using the ROC curve, the previously reported cut-off for the 95th percentile of CXCL10 (2.2 ng/mL) [146] would have had a sensitivity of 77%, a specificity of 56%, a positive likelihood ratio of 1.8, and a negative likelihood ratio of 0.4 for the presence of placental lesions consistent with chronic inflammation.

Forty-five percent (18/40) of patients had intra-amniotic inflammation (defined as an AF IL-6 concentration ≥2.6 ng/ml) [157, 168, 169]. The median concentration of AF IL-6 was 2.2 [0.5–5.8] ng/ml; neither acute inflammatory lesions of the placenta nor microbial invasion of the amniotic cavity was associated with AF IL-6 ($P = 0.14$, $P = 0.26$, respectively).

In 13 of 40 (32.5%) patients, both AF markers of acute (IL-6) and chronic (CXCL10) inflammation were greater than the 95th percentile. The odds of co-occurrence of elevated AF CXCL10 and IL-6 were nine times higher among patients who had mixed placental histologic lesions compared to those with an isolated lesion or no lesion (OR 9; 95% CI 1.2–62); adjustment for gestational age at diagnosis slightly increased the odds ratio (OR 11; 1.4–83).

Microbial invasion of the amniotic cavity

A positive AF result for microorganisms was present in 12.5% (5/40) of the study population (Table 1). In most cases, the organisms were viruses (i.e. parvovirus); in one case, it was *Lactobacillus sakei* (Table 2). The organisms, clinical characteristics, results of AF analysis, IL-6, and CXCL10, as well as the results of placental pathologic findings, are described in Table 2.

Standard cultivation techniques identified one bacteria and one virus. *Cytomegalovirus* was identified by culture and confirmed with molecular microbiology. One culture of AF was positive for coagulase negative staphylococci – it is noteworthy that this patient had only four AF white blood cells per cubic milliliter, a glucose of 10 mg/dL, and a normal IL-6/CXCL10, and no evidence of acute inflammatory lesions of the placenta (acute chorioamnionitis, funisitis, or chorionic villitis). Therefore, it is possible that this represents contamination of the specimen, given that this organism is frequently present on the skin.

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Molecular microbiologic techniques identified two cases of parvovirus B-19, one of *Lactobacillus sakei* and one of human enterovirus.

None of the cases with microbial invasion of the amniotic cavity had acute inflammatory lesions of the placenta. Indeed, if pathological lesions were present, these cases had either chronic inflammatory lesions or maternal underperfusion or sometimes both. Three cases with chronic inflammatory lesions had microbial invasion: two with parvovirus B-19 and one case with *Lactobacillus sakei*.

Discussion

Principal findings of the study

(1) Chronic placental inflammatory lesions were present in 57.9% (22/38) of cases; (2) chronic chorioamnionitis was the most frequent chronic inflammatory lesion of the placenta, followed by chronic deciduitis with plasma cells and VUE; (3) one-half of the VUE cases (3/6) also demonstrated chronic chorioamnionitis; (4) most cases of chronic chorioamnionitis had no evidence of microbial invasion of the amniotic cavity, except for three (two with parvovirus B-19, one with *Lactobacillus sakei*). However, these cases did not have acute inflammatory lesions, indicating the absence of either a maternal inflammatory response (acute chorioamnionitis) or a fetal inflammatory response (acute chorionic vasculitis or funisitis); (5) an elevated AF concentration of CXCL10 (above the 95th centile) was present in 60% of cases with fetal death; (6) an elevated AF CXCL10 concentration of 2.9 ng/mL had a sensitivity of 73% and a specificity of 75% in the identification of chronic placental inflammatory lesions; (7) microbial invasion of the amniotic cavity, detected by a combination of cultivation and molecular microbiologic techniques, was present in only 12.5% of fetal death cases. Importantly, most of the organisms were viruses (parvovirus B-19, *Cytomegalovirus* and enterovirus); (8) acute placental inflammatory lesions were infrequent in cases of fetal death; and (9) the placentas of pregnancies complicated with fetal death often had more than one lesion – the most frequent combination included maternal vascular lesions of underperfusion and chronic inflammatory lesions; 10) the odds of both an elevation in acute (IL-6) and chronic (CXCL10) inflammation markers were nine times greater when more than one class of placental lesion was diagnosed compared to a single class or when no lesion was found. Collectively, this evidence suggests that maternal anti-fetal rejection plays a role in unexplained fetal death. This is supported in patients with a fetal death by a high frequency of chronic placental inflammatory lesions, a high concentration of CXCL10, and a low frequency of microbial invasion of the amniotic cavity.

Fetal death: a manifestation of allograft failure

Fetal death is syndromic in nature and can be caused by maternal insults (i.e. maternal trauma leading to placental abruption [182, 183], a metabolic disorder such as diabetic ketoacidosis [184–186], maternal sepsis [187, 188], etc.), placental disease (i.e. observed in patients who have absent or reversed end-diastolic velocities of the umbilical artery [189, 190], massive perivillous fibrin deposition [191–193], etc.), or fetal disorders (such as cardiac arrhythmias [31, 32], hydrops fetalis [194, 195], critical congenital anomalies [33, 34] and some chromosomal abnormalities [196, 197]).

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Although the fetus and placenta are known to be semi-allografts [87] and immune disorders can cause fetal death (i.e. Rh alloimmunization [198, 199]), the role of maternal anti-fetal rejection as a mechanism of disease responsible for fetal death has been considered only recently [121, 126].

Allograft rejection is a dynamic process that occurs frequently in transplanted organs [200, 201]. Infiltration of lymphocytes from the recipient into the donor organ is not uncommon [113–115], as is the deposition of complement by antibody-mediated rejection [202, 203]. However, histopathologic evidence of an immune response in the graft is not sufficient to define rejection – the latter requires organ dysfunction, which is clinically manifested by a functional disorder of the transplanted organ, such as renal failure, congestive heart failure, pulmonary insufficiency, etc. [204–207].

The placenta is a multi-functional organ that has respiratory, nutritive, excretory, endocrine and immune functions, among others [208–210]. Therefore, severe placental dysfunction could manifest in a number of ways, such as inadequate transport of oxygen, leading to fetal myocardial failure and subsequent death [5]. Nutritive failure of the placenta could lead to fetal growth restriction, a risk factor of fetal death; however, this in and of itself would not be sufficient to cause death unless placental respiratory failure also occurs. Other forms of placental dysfunction may be more subtle, e.g. placental sulfatase deficiency can lead to an enzymatic inability to hydrolyze the sulfate group from DHA sulfate (DHA-S) and 16a-hydroxy-DHA sulfate. This condition is associated with low levels of estrogens and is clinically manifested with post-term pregnancy [211].

The immune functions of the placenta are complex and involve host defense against microorganisms that may gain access to the uterus via an ascending pathway or through a hematogenous route [73, 212–220]. Some infections are known to be a cause of fetal death (i.e. syphilis [15–19]). Another major immunological function is the successful establishment and maintenance of maternal-fetal tolerance [72, 75, 83, 84, 92, 94, 99, 104, 111], the breakdown of which may lead to maternal antifetal rejection. The clinical spectrum of maternal antifetal rejection may include subclinical semi-allograft rejection detected only at the time of examination of the placenta (by identification of chronic chorioamnionitis [118–122, 124, 126, 128, 129], VUE [122, 123, 126, 130, 131], or chronic deciduitis [122, 126, 129]), preterm labor with intact membranes [118–120, 122, 124–126], PPROM [118], maternal perivillous fibrin deposition [127], and other obstetrical syndromes [121, 122, 126]. However, the extreme form of rejection would include a functional failure of the placenta of such magnitude that it would lead to fetal death [121, 126].

The precise mechanisms whereby rejection may lead to fetal death are not yet known; however, we have described a new entity, the fetal inflammatory response syndrome type 2 (FIRS type 2), which occurs in fetuses whose placentas have lesions consistent with maternal anti-fetal rejection [122, 126]. FIRS type 2 is characterized by a unique change in the profile of cytokines in the peripheral blood, with an elevation of CXCL10, and by a stereotypic transcriptome [122]. Gene ontology analysis of differentially expressed genes in fetal white blood cells with FIRS type 2 indicates enrichment for genes that participate in immune-related processes [122]. In contrast, in FIRS type 1, the systemic inflammatory

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response associated with microorganisms or “danger signals” can lead to a situation that resembles septic shock in the fetus and newborn, and some reports of fetal death [221–225]. However, the natural history of FIRS type 2 has not been elucidated; thus, it is unclear how maternal antifetal rejection and FIRS type 2 can progress toward fetal death. However, the data presented herein show a clear association between fetal death, placental lesions of maternal anti-fetal rejection, and an elevation of CXCL10 in the AF, as well as the absence of microbial invasion of the amniotic cavity in most cases. Therefore, the immune disorder detected in the placenta and AF cannot be attributed to a viral or bacterial infection.

Placental pathology in fetal death

The most common lesion associated with fetal death was maternal vascular lesions of underperfusion [6, 7, 61, 144, 226–233]. These lesions fall into two categories: villous changes and vascular lesions. At the most recent meeting of the Amsterdam Placental Workshop, experts recommended to re-label this category of lesions into “maternal vascular malperfusion” [234]. It is believed that an inadequate supply of nutrients and oxygen would occur in patients who have lesions such as massive perivillous fibrin deposition and massive placental infarction [235].

An important finding of this study is that chronic placental inflammatory lesions were observed in 57.9% (22/38) of cases. Chronic inflammatory lesions included chronic chorioamnionitis, VUE, and chronic deciduitis with plasma cells. Chronic placental inflammatory lesions were associated in 63.6% (14/22) of cases with lesions of maternal vascular underperfusion. The precise mechanism responsible for the association between chronic placental inflammatory lesions and maternal vascular lesions of underperfusion is unknown. It is noteworthy that in one condition, massive perivillous fibrin deposition, there is evidence of antibody-mediated maternal anti-fetal rejection (documented by a mismatch of HLA antigens between the mother and the fetus, maternal sensitization demonstrated by the presence of anti-HLA antibodies, and complement fixation of the umbilical vein shown as C4a deposit) [127]. This condition has also been found to be associated with an abnormal, severe angiogenic/antiangiogenic profile which begins in early pregnancy [236] and has been successfully treated with pravastatin [237]. Further studies are required to determine the extent of the association between chronic placental inflammation and maternal vascular lesions of underperfusion in other obstetrical syndromes.

Acute placental inflammatory lesions were present in 28.2% of cases. Although placental pathologists have considered that acute chorioamnionitis, funisitis, and chorionic vasculitis are manifestations of an amniotic fluid infection syndrome, it is now known that these lesions can occur in the absence of bacteria or viruses in the amniotic cavity and that these processes have been attributed to danger signals that can initiate an inflammatory response in the absence of microbial products [139, 173–176]. These danger signals are considered to be alarmins [238] (e.g. high-mobility group box-1 (HMGB1) [169, 239] and IL-1α [240]), which are present in the AF. It is noteworthy that only 12.5% of patients in our study had bacteria or viruses in the AF. Therefore, an infection-related etiology appears to be rare in high-income countries. In low-income countries, infection appears to play a more important role [21]. The frequency of the acute placental inflammatory lesions (28.2%) is at odds with

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the frequency of microbial invasion of the amniotic cavity, which was 12.5%. A possible explanation is that some patients may have developed acute inflammatory lesions and, specifically, acute chorioamnionitis during the induction of labor because of fetal death. The induction process may result in the administration of agents such as prostaglandins [241–252], insertion of a Foley catheter [253], or any other method of induction of labor that may predispose to inflammation. Additionally, labor per se is an inflammatory state associated with the infiltration of inflammatory cells into the cervix [254–264], myometrium [256, 265–271] and chorioamniotic membranes [272–277]. It should also be considered that amniocenteses were performed at the time of diagnosis and placentas were examined after delivery. While there is a temporal dissociation between the assessment of the microbial state of the amniotic cavity and placental examination, the overwhelming majority (92.5%) of patients in our study were induced and the median interval from diagnosis to delivery was short [0.1 (0–0.5) weeks]. Therefore, a smaller fraction of those with acute inflammation may not be fully explained by the process of labor induction.

The least common of the four major categories of lesions recognized by the Society of Pediatric Pathology was fetal vascular thrombo-occlusive disease – this was present in 23.1% of cases and associated with maternal vascular lesions of underperfusion in two-thirds of cases. Whether this represents simultaneous activation of the coagulation system in the mother and fetus, due to acquired or congenital thrombophilic states, remains to be determined. Some investigators have suggested that thrombophilia is a risk factor for fetal death; however, a systematic review and meta-analysis have not confirmed the initial reports [278, 279]. This may represent a lack of association or an incomplete knowledge of thrombophilic mutations at the present time.

In only 10.3% of cases did placental pathology not reveal a lesion belonging to the four major categories recognized by the Society of Pediatric Pathology. The circumstances of these fetal deaths were examined and there were no explanations for these occurrences (i.e. cord accident, maternal fetal hemorrhage, etc.).

Amniotic fluid cytokines in fetal death

The current study focuses on the examination of the concentration of two cytokines: IL-6, a marker of acute inflammatory lesions of the placenta [150], and CXCL10, a marker of chronic placental inflammatory lesions [126]. The widely used cut-off for intra-amniotic inflammation is an AF IL-6 concentration of 2.6 ng/mL [157, 168, 169]. Only one patient with microbial invasion of the amniotic cavity had an elevated AF IL-6 concentration – parvovirus B19 was detected and the IL-6 concentration was 13.7 ng/mL. This patient did not have evidence of acute chorioamnionitis but rather of chronic placental inflammatory lesions and, specifically, chronic chorioamnionitis, as well as maternal vascular lesions of underperfusion. Parvovirus B19 is typically transmitted transplacentally [280] and this may be the reason for the absence of acute chorioamnionitis.

Mixed histologic lesions were the most frequently observed placental finding, occurring in 61.5% (24/39) of placentas. The odds of a woman having a combined elevation of AF IL-6 and CXCL10 were nine times greater when mixed placental lesions were present than when no lesion or only a solitary lesion class was diagnosed. The most common classes of

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placental lesions found when mixed lesions were present were those of maternal vascular underperfusion and chronic inflammation. Microbial invasion of the amniotic cavity does not fully explain the elevation of IL-6 seen in unexplained fetal death. This finding raises the question of whether dysregulation of inflammatory pathways (both acute and chronic) is underway as mixed lesions are developing in the placenta. More investigation into the overlap of acute and chronic inflammation may help to answer this question.

The association between chronic placental inflammatory lesions, especially chronic chorioamnionitis, and fetal death has been previously reported by our group [121, 126]. The novel aspect of this report was the use of PCR techniques to evaluate for the presence of microorganisms in the AF. Using these advanced microbiological techniques, we determined that chronic inflammatory lesions, particularly VUE, could not be attributed to intraamniotic infection by bacteria or viruses.

The diagnostic performance of CXCL10 in the identification of patients with chronic inflammatory lesions indicted a sensitivity of 73%, a specificity of 75%, a positive likelihood ratio of 2.9, and a negative likelihood ratio of 0.4. Further studies are required to determine whether examination of the behavior of the inflammatory/cytokine network can enhance the diagnostic performance of CXCL10. CXCL10, as well as CXCL9 and CXCL11, are T-cell chemokines, which may play a role in the rejection of the semi-allograft fetus [118, 121, 124]. Similarly, the receptor for these T-cell chemokines is CXCR3 and its soluble form is increased in the AF of women who underwent spontaneous preterm labor and whose placentas presented lesions of maternal anti-fetal rejection [281]. Thus, it is possible that a combination of chemokines and their receptors may be of help in identifying the patients with maternal antifetal rejection. The same can be said for the determination of these factors in maternal and fetal blood.

Strengths and limitations

A strength of this study is that we investigated placental pathology, AF cytokines, and microbial invasion of the amniotic cavity in a cohort of patients with fetal death. One of the limitations of the study is the time interval between amniocentesis and delivery, even though the majority of patients had labor induced following the diagnosis of fetal death at admission. Another limitation is the uncertainty about the relationship between the frequency of acute inflammatory lesions of the placenta, microbial invasion of the amniotic cavity, and the interval to delivery. This relationship questions whether acute inflammatory lesions may be due to the process of induction of labor and labor per se rather than a pre-existing pathologic process related to fetal death.

Conclusions

This cohort study of patients with unexplained fetal death suggests that placental lesions associated with chronic placental inflammatory lesions are second only to the prevalence of lesions consistent with maternal underperfusion; and there is a strong correlation with the AF concentration of CXCL10, a chemokine involved in allograft rejection and fetal death. The mechanisms whereby maternal anti-fetal rejection may lead to fetal death require further investigation and may be related to the deployment of FIRS type 2.

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Supplementary Material

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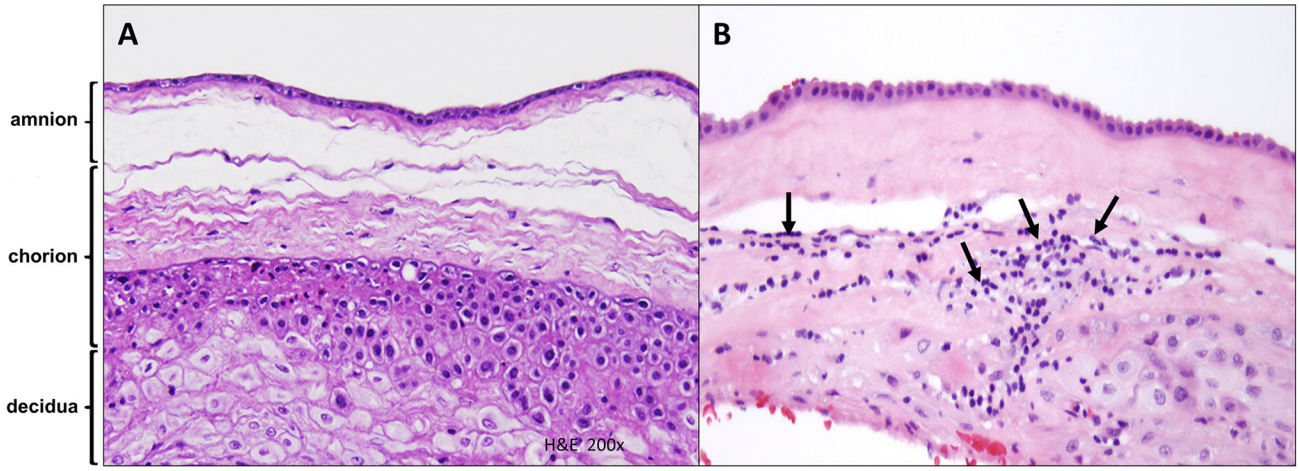

Figure 1. Histology of the extraplacental chorioamniotic membranes

(A) Normal chorioamniotic membranes with no evidence of chronic inflammatory cell infiltration in the amnion and choriodecidua.

(B) Chronic chorioamnionitis, stage 2: chorioamniotic membranes from FD, gestational age 31.4 weeks. Chronic inflammatory cells (arrows) infiltrate into the chorioamniotic connective tissues beyond the chorionic trophoblast layer. FD: fetal death.

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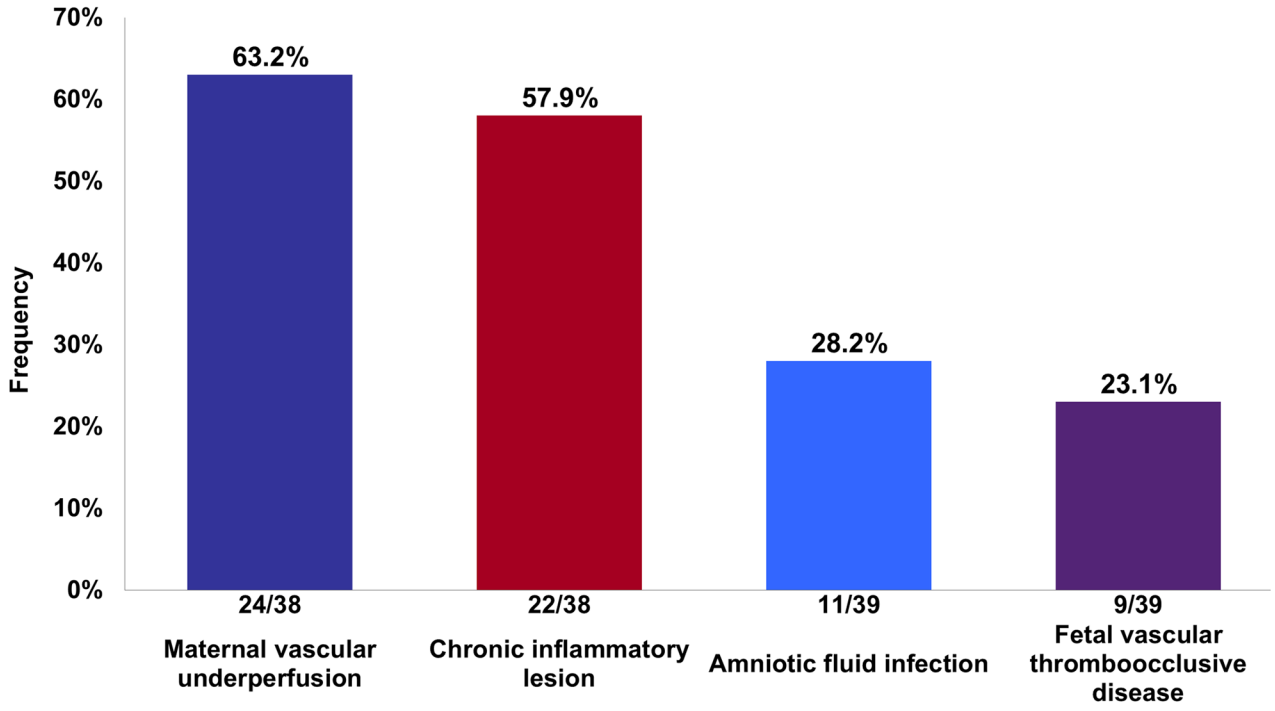

Figure 2. Frequency of placental lesions in fetal death

The classification of placental lesions was based on those categorized by the Society for Pediatric Pathology. Chronic inflammatory lesions encompassed chronic chorioamnionitis, villitis of unknown etiology and chronic deciduitis. Chronic inflammatory lesions were the second most predominant histopathological lesion observed in unexplained fetal death.

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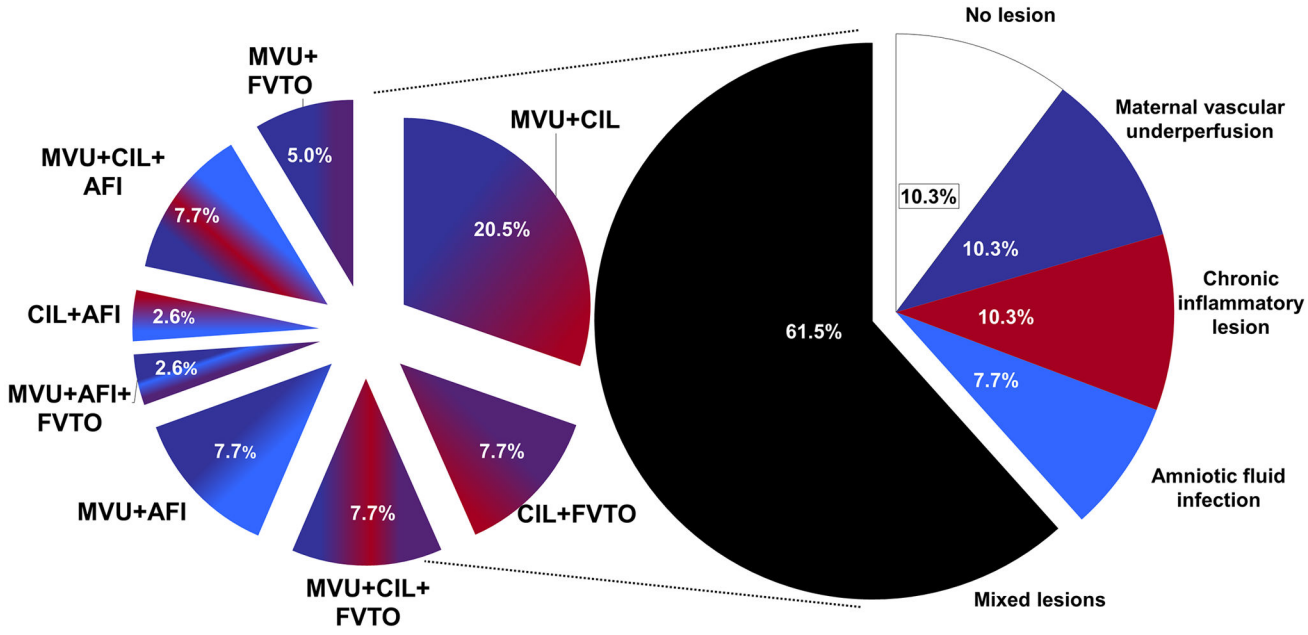

Figure 3. Frequency of isolated and mixed placental lesions in unexplained fetal death

The placental examination demonstrated lesions from multiple histological classes (mixed) more frequently than those confined to one class (isolated). The most abundant findings were lesions of maternal vascular underperfusion and chronic inflammation. Some placentas even had lesions from 3 different classes. The frequency of isolated lesions is represented in solid colors and the frequency of mixed lesions in colors derived from the blend of the classes involved. AFI: amniotic fluid infection; CIL: chronic inflammatory lesion; FVTO: fetal vascular thrombo-occlusive disease; MVU: maternal vascular underperfusion.

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Table 1

Clinical, Obstetrical and laboratory results of patients with fetal death

	Stillbirths (n=40)
Maternal age (years)	25.0 (±4.6)
Race/Ethnicity	
African American	87.5% (35/40)
Caucasian	2.5% (1/40)
Hispanic	5% (2/40)
Other	5% (2/40)
Body mass index (kg/(m)^2)	30.2 (±8.7)
Mean arterial pressure (mm Hg)	95 (±13)
Nulliparity	37.5% (15/40)
Prior preterm birth	17.5% (7/40)
Maternal medical comorbidities[ǂ]	37.5% (15/40)
Active tobacco smoker	20% (8/40)
Drug use during index pregnancy (positive urine drug screen)	28.2% (11/39)
Gestational age at diagnosis & amniocentesis (weeks)	30.9 [25.2–35.7]
Interval from diagnosis to delivery (weeks)	0.1 [0–0.5]
Induction of labor	92.5% (37/40)
Stillborn birthweight (grams)	1403 [625–2283]
Birthweight <10th percentile	27.5% (11/40)
Male fetus	57.5% (23/40)
Kleihauer-Betke positive	3% (1/33)
Amniotic fluid	
White blood cell count (cells/ml ^{3})	1 [0–13]
Glucose (mg/dL)	18 [10–22]
CXCL-10 (ng/ml)	2.9 [1.7–7. 6)]
IL-6 (ng/ml)	2.2 [0.5–5.8]
Positive bacterial culture	2.5% (1/40)
Positive viral culture	2.9% (1/34)
Positive bacterial molecular study	2.5% (1/40)
Positive viral molecular study	10% (4/40)

Data presented as median [IQR] or mean (± SD) and % (n/N).

[ǂ]Medical comorbidities included (n): asthma (7), cerebral palsy (1), chronic hypertension (3), Crohn's disease (1), drug abuse (2), gastroesophageal reflux disease (1), liver disease (1), systemic lupus erythematosus (1), psychiatric mood disorder (1), Wiskott-Aldridge Carrier (1).

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Table 2

Microbial invasion of the amniotic cavity in fetal death: microorganisms, amniotic fluid analysis and placental findings.

Organism	Culture	PCR	Genome/ well	GA diagnosis/ amniocentesis (wks)	AF WBC (cells/ml^3	AF glucose (mg/dl)	AF IL-6 (ng/ml)	AF CXCL-10 (ng/ml)	Placental histological classes			
									AFI	CIL	FVTO	MVU
Parvovirus B-19	NS	+	921	26	---	11	13.8	1.7	--	+≠	--	+
Parvovirus B-19	NS	+	931	39	260	22	0.3	1.2	--	+≠	--	--
Cytomegalovirus	+	+	685	27	0	18	0.4	2.0	--	--	--	--
Human enterovirus§	--	+	23	33	4	10	1.2	1.9	--	--	--	+
Coagulase negative staphylococci§	+	--	NA									
Lactobacillus sakei	--	+	15	29	10	9	0.5	7. 7	--	+≠	--	+

NS, no specimen. NA, not applicable. PCR, polymerase chain reaction. GA, gestational age. AF, amniotic fluid. WBC, white blood cell. AFI, amniotic fluid infection. CIL, chronic inflammatory lesion. FVTO, fetal vascular thromboocclusive disease. MVU, maternal vascular underperfusion.

§ These microorganisms were recovered from the same AF specimen.

≠ Chronic chorioamnionitis was the only chronic inflammatory lesion present—there were no chronic villitis cases.