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Bacteria and endotoxin in meconium-stained amniotic fluid at term: could intra-amniotic infection cause meconium passage?

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Abstract

Background—Meconium-stained amniotic fluid (MSAF) is a common occurrence among women in spontaneous labor at term, and has been associated with adverse outcomes in both mother and neonate. MSAF is a risk factor for microbial invasion of the amniotic cavity (MIAC) and preterm birth among women with preterm labor and intact membranes. We now report the frequency of MIAC and the presence of bacterial endotoxin in the amniotic fluid of patients with MSAF at term.

Materials and methods—We conducted a cross-sectional study including women in presumed preterm labor because of uncertain dates who underwent amniocentesis, and were later determined to be at term (n=108). Patients were allocated into two groups: (1) MSAF (n=66) and (2) clear amniotic fluid (n=42). The presence of bacteria was determined by microbiologic techniques, and

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endotoxin was detected using the Limulus amebocyte lysate (LAL) gel clot assay. Statistical analyses were performed to test for normality and bivariate comparisons.

Results—Bacteria were more frequently present in patients with MSAF compared to those with clear amniotic fluid [19.6% (13/66) versus 4.7% (2/42); p<0.05]. The microorganisms were Gram-negative rods (n=7), *Ureaplasma urealyticum* (n=4), Gram-positive rods (n=2) and *Mycoplasma hominis* (n=1). The LAL gel clot assay was positive in 46.9% (31/66) of patients with MSAF, and in 4.7% (2/42) of those with clear amniotic fluid (p <0.001). After heat treatment, the frequency of a positive LAL gel clot assay remained higher in the MSAF group [18.1% (12/66) versus 2.3% (1/42), p<0.05]. Median amniotic fluid IL-6 concentration (ng/mL) was higher [1.3 (0.7–1.9) versus 0.6 (0.3–1.2), p=0.04], and median amniotic fluid glucose concentration (mg/dL) was lower [6 (0–8.9) versus 9 (7.4–12.6), p<0.001] in the MSAF group, than in those with clear amniotic fluid.

Conclusion—MSAF at term was associated with an increased incidence of MIAC. The index of suspicion for an infection-related process in postpartum women and their neonates should be increased in the presence of MSAF.

Keywords

amniotic fluid glucose; fetal bowel function; fetal defecation; fetal diarrhea; interleukin-6; intrauterine inflammation/infection; Limulus amebocyte lysate (LAL); white blood cell

Introduction

Meconium-stained amniotic fluid (MSAF) occurs when there is passage of the fetal colonic contents into the amniotic cavity [1–6]. The frequency of this condition increases as a function of gestational age [7–10]. The frequency of MSAF ranges from 5% to 20% (400,000–600,000 deliveries per year in the U.S. alone) [4,11–14].

The presence of meconium predisposes to meconium aspiration syndrome (MAS) [4,10,11,15–30], which only occurs in 5% of all neonates born to mothers with MSAF [8,10,13,14,31]. MSAF is a risk factor for clinical chorioamnionitis [15,32–38], neonatal hypoxic-ischemic encephalopathy [4,39–41], neonatal sepsis [4,18,42–45], seizures [4,18,42,46,47] and cerebral palsy [48–51]. Therefore, the presence of MSAF is considered a warning sign by obstetricians [2,16,52–63], even though most neonates do not have evidence of hypoxia or metabolic acidemia [21,64–70].

We have previously reported that MSAF is associated with microbial invasion of the amniotic cavity (MIAC) in patients presenting with preterm labor and intact membranes [32]. We now report a study of the frequency of MIAC and the presence of bacterial endotoxin in the amniotic fluid of patients with MSAF at or near term.

Material and methods

Study design and population

This was a cross-sectional study which included patients at term with MSAF and clear amniotic fluid. The study was conducted by searching the clinical database and bank of

biologic samples of the Sótero del Río Hospital in Chile, which had been subsequently shared with the faculty of Yale University, Wayne State University, the Detroit Medical Center and the Perinatology Research Branch of NICHD/NIH/DHHS.

Patients presenting with an episode of preterm labor with intact membranes (rupture of membranes was ruled out by a sterile speculum examination, nitrazine, fering and pooling) were offered an amniocentesis to evaluate fetal lung maturity to determine whether tocolysis and steroids should have been administered, and the microbial status of the amniotic cavity. The lung maturity tests included a “shake” test (or Clemens test), or counting the number of orange cells. The amniotic fluid was also processed for Gram stain, aerobic and anaerobic cultures and genital *Mycoplasmas.*

The patients included in this study were women with singleton gestations who presented with an episode of preterm labor who had uncertain dates. Sonographic fetal biometry had not been performed, as it was largely unavailable at the time as part of routine prenatal care either in the U.S. or in other countries. Retrospectively, these patients were considered to be at term due to the following characteristics: (1) spontaneous labor; (2) delivery within 48 hours of amniocentesis; (3) analysis of amniotic fluid consistent with fetal lung maturity; (4) birthweight >2500 g; (5) absence of respiratory distress syndrome or other complications of prematurity; and (6) physical examination by a pediatrician which was consistent with that of a term neonate.

The following groups were included: (1) patients with MSAF (n=66) and (2) patients with clear amniotic fluid selected as controls (n=42). All women provided written informed consent before collection of the amniotic fluid samples. The collection and utilization of the samples was approved by the Human Investigation Committee of the participating institutions and the IRB of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD/NIH/DHHS).

Clinical definitions

Clinical chorioamnionitis was diagnosed by the presence of a temperature elevation to 37.8°C or higher, and two or more of the following criteria: uterine tenderness, malodorous vaginal discharge, fetal tachycardia (heart rate >160 beats/min), maternal tachycardia (heart rate >100 beats/min) and maternal leukocytosis (leukocyte count >15 000 cells/mm^3) [71,72]. Intra-amniotic infection was defined as a positive microbiological culture in amniotic fluid or the presence of a positive Gram stain [73]. Endometritis was defined as postpartum temperature elevation to 38°C or higher on two occasions, 4 hours apart, excluding the day of delivery, with uterine tenderness, foul-smelling lochia and no other apparent source of fever. Neonatal sepsis was diagnosed in the presence of a positive culture of blood, urine, or cerebrospinal fluid similar to previously published criteria [74,75].

Sample collection and microbiological studies

Amniotic fluid samples were obtained by transabdominal amniocentesis. Samples of amniotic fluid were transported to the laboratory in a sterile capped syringe immediately after collection. Gram stain examination was performed in all samples using commercial reagents (crystal violet, safranin and Gram’s iodine; Difco Laboratories, Detroit, MI) under

standard conditions [32]. Stained slides were examined by trained technologists and the presence or absence of microorganisms was noted.

Detection of endotoxin

Bacterial endotoxin was detected using the Limulus amebocyte lysate (LAL) gel clot assay as previously described [76–79]. Briefly, the LAL gel clot assay was performed by adding 200 μL of amniotic fluid to 100 μL of LAL (Associates of Cape Cod, Inc., Woodshole, MA) and incubating the mixture in a motionless water bath at 37 °C for one hour. A positive result was scored when a solid adherent gel was present on inversion of the tube. A negative control (pyrogen-free water) was run with each assay. The sensitivity of the test was 50 pg/mL. Positive samples were reassayed after heat treatment (100 °C for 5 minutes).

Statistical analysis

The Kolmogorov–Smirnov and Shapiro–Wilk tests were used to determine if data were normally distributed. The Mann–Whitney *U* test was used to compare continuous nonparametric variables between groups. Comparisons between proportions were performed using Chi-square or Fisher's exact tests. A p value <0.05 was considered statistically significant.

Results

Demographic and clinical characteristics of the study population

Table 1 displays the demographic and clinical characteristics of patients. Among patients with a suspicion of preterm labor, 66.1% (66/108) had MSAF and 33.9% (42/108) had clear amniotic fluid. The frequency of cesarean delivery was significantly higher in the MSAF group than in the group with clear amniotic fluid (28.7% versus 9.5%; $p<0.05$). Otherwise, there were no significant differences in characteristics between the study groups ($p>0.05$).

Bacteria are more frequently present in MSAF than in clear amniotic fluid

Microorganisms were identified in 19.6% (13/66) of patients with MSAF and in 4.7% (2/42) of those with clear amniotic fluid ($p<0.05$). Table 2 displays the microorganisms found in patients with MSAF. The most common microorganisms were Gram-negative rods (n = 7), followed by *Ureaplasma urealyticum* (n = 4), Gram-positive rods (n = 2) and *Mycoplasma hominis* (n = 1). The amniotic fluid of one patient had a Gram-positive rod and *Mycoplasma hominis*. The clinical laboratory did not pursue organism characterization at the time. Two patients with clear amniotic fluid had positive cultures for bacteria (*Ureaplasma urealyticum*). One patient with MSAF who delivered by cesarean section developed postpartum endometritis; however, there were no cases with clinical chorioamnionitis or neonatal sepsis. The median amniotic fluid IL-6 concentration (ng/mL) was significantly higher [1.3 (0.7–1.9) versus 0.6 (0.3–1.2), $p=0.04$] and the median glucose concentration (mg/dL) was significantly lower [6 (0–8.9) versus 9 (7.4–12.6), $p<0.001$] in the MSAF group than in those with clear amniotic fluid (Table 1 and Figure 1).

Endotoxin was more frequently found in MSAF than in clear amniotic fluid

The LAL gel clot assay was positive in 46.9% (31/66) of patients with MSAF, but in only 4.7% (2/42) of those with clear amniotic fluid (p <0.001) (Table 3). After heat treatment, the LAL assay remained positive in 38.7% (12/31) of samples in the MSAF group and 50% (1/2) in the clear amniotic fluid group. The frequency of a positive LAL assay was still significantly higher in the MSAF group compared to those with clear amniotic fluid, even after heat treatment [18.1% (12/66) versus 2.3% (1/42); p <0.05] (Table 3).

Discussion

Principal findings of the study

(1) Patients with MSAF in spontaneous labor at term have a higher frequency of microbial invasion of the amniotic cavity (defined as a positive amniotic fluid culture for microorganisms) than those with clear amniotic fluid; (2) MSAF at term was associated with a lower median amniotic fluid glucose, and a higher median amniotic fluid IL-6 concentration than women with clear amniotic fluid in spontaneous labor at term. Therefore, patients with MSAF had findings consistent with intra-amniotic inflammation; (3) Gram-negative bacteria were the most common isolates in the amniotic fluid of patients with MSAF; and (4) bacterial endotoxin assayed by the LAL assay was present in the amniotic fluid of 46.9% of patients with MSAF and only 4.7% of patients with clear amniotic fluid in spontaneous labor at term. We propose that fetal ingestion of amniotic fluid containing microorganisms, microbial products and/or inflammatory mediators may cause enteritis, accelerate colonic motility and result in the intrauterine passage of meconium.

Meconium – what is it and when is it formed?

“Meconium” is derived from the Greek word *meconiumarion*, which means “poppy juice” or “opium-like” [80]. Aristotle is often credited with the observation that MSAF was associated with neonatal depression [80], and obstetricians have generally considered MSAF as a sign of fetal distress [2,16,52–63]. Meconium is frequently present in the amniotic fluid in cases of fetal death [81,82]. Hence, the detection of meconium, either through amnioscopy or amniocentesis, was a method used to identify the fetus at impending risk of fetal death [83–86]. The most common indications for such an approach were post-term gestations [87,88], intrahepatic cholestasis of pregnancy [89–91] and other complications of pregnancy associated with an increased risk of fetal death [83,89]. Further, the presence of echogenic or particulate matter in the amniotic fluid, thought to be consistent with intrauterine passage of meconium, has been documented by ultrasound [92,93].

Meconium represents the colonic content, and consists of water, swallowed amniotic fluid and cellular components exfoliated from the gastrointestinal tract [1,94,95]. The conventional view is that meconium is sterile (does not contain bacteria), and is first detected between 70 and 85 days of gestation [80,96,97]. The typical green coloration of meconium is attributed to bile pigments, which are products of heme catabolism. They are first detected in the bile of the fetus at 14 weeks of gestation, and their concentrations increase with advancing gestational age [98,99]. Many drugs are metabolized in the liver, excreted into the bile and subsequently enter into the small bowel, and therefore can be detected in meconium

obtained from the neonate at the time of birth [100–105]. This provides information about *in utero* exposure to a wide range of agents, such as cocaine and cannabinoids [100–105]. The contents of the large bowel are colorless, or very light yellow in young fetuses, and colorless with a few specks of brown-green material between 12 and 23 weeks [98]. The mechanism of meconium passage *in utero* has been previously reviewed [4,5].

Meconium passage in fetal life: is fetal defecation physiologic?

The traditional view has been that fetal defecation normally does not occur until the second trimester of pregnancy (until approximately 24 weeks of gestation) because at this time, the anus is patent and the sphincter is non-functional. After approximately 24 weeks of gestation, the anal sphincter is thought to be innervated and closed [106,107]. Clinicians have traditionally considered the passage of meconium after this time to reflect a pathologic state, often attributed to fetal hypoxia/anoxia or other stimuli thought to induce peristalsis and relaxation of the anal sphincter. Contrary to this view, there is now a considerable body of experimental and clinical data indicating that fetal defecation is a physiologic phenomenon, and much of these data derives from ultrasound examination of the fetal perineum (see below) [108–111].

Kizilcan et al. reported a series of experiments in which a nonhydrosoluble contrast media (preferred over hydrosoluble, which could be absorbed through the gastrointestinal tract) was administered to fetal goats via a nasogastric tube [112]. All fetuses ($n = 8$) began to pass contrast media into the amniotic cavity within 16–22 hours. This was documented by radiopacity of the amniotic cavity. The contrast media was detected in the stomach, small bowel and rectum. Importantly, there was no evidence of fetal acidemia, hypoxemia, or hypercapnea assessed by fetal blood analysis (blood obtained by catheterization). Consequently, the authors concluded that fetal defecation occurs physiologically in the absence of distress detected by changes in the pH and blood gas analyses. It is not possible to exclude, however, that defecation could have been due to other forms of stress. In another set of experiments, Ciftci et al. injected radioactive technetium (^{99m}TC-HIDA) intramuscularly into fetal rabbits [113]. Technetium was excreted through the fetal liver into the gastrointestinal tract, and subsequently detected in the amniotic fluid [113]. However, the placement of a purse-string suture to close the anus of the animals prevented the detection of technetium in the amniotic fluid [114]. Collectively, these findings suggest that fetal defecation occurs under normal circumstances. Some authors have argued that the concept of physiologic fetal defecation is not a surprise, given that fetuses normally urinate and swallow intermittently (and there is no reason to believe that the fetus should be constipated) [115].

Experimental observations could always be attributed to the effect of the procedures (e.g. anesthesia, trauma, open surgery and intramuscular administration of technetium). Therefore, the question of whether fetal defecation is a physiologic phenomenon in the human fetus could only be addressed with a non-invasive imaging modality. Lopez and Ocampo reported that defecation could be detected in all fetuses ($n = 240$) using high resolution ultrasound between 15 and 41 weeks of gestation [109]. The highest frequency of defecation was observed between 28 and 34 weeks of gestation. The authors concluded that

the activity of the external anal sphincter was consistent with primitive neuroendocrine control of the sphincter in which relaxation of the anus was fairly maintained. However, after 22–24weeks of gestation, the external sphincter of the anus was closed. An interesting observation from this sonographic study was that opening or closure of the fetal anus could be observed, and this was not necessarily associated with defecation.

If defecation occurs in all fetuses, and the intestinal content is thought to be colored by bile pigments, why is clear amniotic fluid the norm? In a different study, Lopez and Ocampo reported performing amniocentesis after defecation had been documented by examining the anus for 10–15 minutes in 70 fetuses between 14 and 22 weeks of gestation [110]. All samples of amniotic fluid were clear, but contained a whitish material, which was interpreted as representing fetal stool [110]. The authors reported that the amniotic fluid was clear because fetal intestinal content is not green in color at this gestational age window [110]. An unsettled issue is whether amniotic fluid (and the pellet) obtained shortly after defecation in the third trimester would also be clear in the absence of pathology (i.e. stress, hypoxia or infection).

If the fetus defecates regularly, why is the amniotic fluid clear in the third trimester? Ciftci et al. proposed that MSAF may be due to an inadequate clearance of meconium [114]. This concept is based on studies in rabbits, in which technetium was administered intramuscularly to fetal rabbits, whose mothers were allocated to two groups: (1) a control group which was sham-operated and (2) an experimental group in which the maternal aorta was constricted below the level of the renal arteries [114]. Fetal defecation occurred with the same frequency in both groups. However, the concentration of radioactive technetium was higher in the amniotic fluid of the animals exposed to hypoxemia than in the control group. In contrast, the concentration of technetium was lower in the maternal blood of rabbits exposed to hypoxemia than in the control group [114]. The authors proposed that MSAF is not primarily due to a change in the frequency of defecation with hypoxia, but rather in the clearance of amniotic fluid [114]. The mechanisms responsible for such clearance remain unknown at this time. The extent to which these concepts apply to the human fetus and, in particular, to the intrauterine passage of meconium at term, is unclear.

Meconium-stained amniotic fluid and intra-amniotic infection in preterm gestations

Nearly two decades ago, we reported that MSAF from patients in preterm labor with intact membranes was associated with culture-proven MIAC [32]. Among 707 patients who underwent an amniocentesis presenting with preterm labor and intact membranes, the frequency of a positive amniotic fluid culture was significantly higher in women with MSAF than in those with clear fluid [33% (10/ 30) versus 11% (75/677) p =0.001] [32]. At that time, we proposed that fetal meconium passage in cases of MIAC may occur after the swallowed bacteria stimulates peristalsis in the bowel. An alternative explanation was that MSAF predisposes to microbial invasion [32,33,116–119], given experimental evidence that meconium impairs the antimicrobial activity of amniotic fluid [18,120–123]. Subsequently, Mazor et al. [33] reported similar findings in a nested case-control study which included 45 women with preterm labor and MSAF, and 135 patients with preterm labor and clear amniotic fluid. The frequency of MIAC and clinical chorioamnionitis was higher in patients

with MSAF than in those with clear amniotic fluid [MIAC (38% versus 11%; p <0.001) and clinical chorioamnionitis (22% versus 6%; p =0.003)] [33]. Collectively, these observations, and those reported by others [32,33,116–119], provide evidence that in the context of preterm gestations, MSAF is associated with MIAC and clinical infection-related complications, such as chorioamnionitis. Yet, most cases of MSAF occur at term. Does the association reported in preterm gestations also occur in term gestations?

Microbial invasion of the amniotic cavity in frequently present in patients with meconium-stained amniotic fluid at term

We report, for the first time, that patients with MSAF at term can have microorganisms in amniotic fluid using cultivation techniques. The most frequently isolated microorganisms were Gram-negative rods. Patients with MSAF also had a lower median amniotic fluid glucose concentration and a higher median amniotic fluid IL-6 concentration than those with clear fluid. Hsieh et al. also reported a higher mean amniotic fluid IL-6 concentration in patients with MSAF at term [124]. Collectively, these data suggest that MIAC is present in nearly 20% of patients with MSAF at term, and that there is evidence of an intra-amniotic inflammatory response.

The observation that Gram-negative rods were the most frequently found microorganisms in MSAF is in contrast to previous studies (both term and preterm gestations) reported by our group [73,125–152] and others [117,153–167], in which the most frequent microorganisms isolated from the amniotic fluid were genital Mycoplasmas (in particular, *Ureaplasma* species). One possible explanation for this finding is that Gram-negative bacteria may be a more potent inducer of bowel peristalsis and meconium passage into the amniotic fluid than other microorganisms. Indeed, in a previous study, most patients with MIAC with genital Mycoplasmas had clear amniotic fluid [32]. Further work is required to describe the microbial profile of MSAF in patients at term. The application of sequence-based techniques should provide a more comprehensive description of microbial diversity and burden in amniotic fluid, including the identification of non-culturable microorganisms. One of the authors has found enterovirus in the amniotic fluid of a patient with MSAF at term undergoing amniocentesis for fetal lung maturity, suggesting that an enterovirus from the mother can cross the placenta, cause fetal infection and induce the equivalent of fetal diarrhea. Therefore, future studies should also include a search for viruses (Romero R. – personal communication).

An important implication of this study is that meconium with bacteria and inflammatory mediators may be aspirated *in utero* in cases in which there is a stressful event with or without acidemia.

Bacterial endotoxin in meconium stained of amniotic fluid

The LAL gel clot assay, the standard method to detect endotoxin (or lipopolysaccharide), has been previously used to detect endotoxin in amniotic fluid [76–79]. The gel clot assay was positive in 46.9% (31/66) of cases with MSAF. Given the high prevalence of a positive LAL test in MSAF, we suspected that meconium may contain a factor with trypsin-like activity [168], which would cross-react with bacterial endotoxin in the LAL test [169]. A

simple approach to address this question was to repeat the LAL test after heat treatment of all LAL-positive samples. Trypsin, and other mammalian proteases which could yield a positive LAL test, are heat-labile, while bacterial endotoxin is heat-stable. After heat treatment of the amniotic fluid samples, 18.1% (12/66) were still positive for endotoxin. The precise nature of the substances, which may induce clotting of the LAL assay, remain to be determined. However, trypsin has been demonstrated in the meconium and feces of healthy newborns [168], and therefore, must be considered a potential candidate for interference with the LAL assay. Cross-reactivity in the bioassay has been documented with microbial products other than endotoxin, such as products of Gram-positive bacteria and fungi [170–172]. The presence of endotoxin in amniotic fluid, as well as other microbial products which have not been characterized to date, along with inflammatory mediators, may predispose to short- and long-term disorders of bowel function and MAS.

The effect of endotoxin on the fetal gastrointestinal tract

There is an extensive body of literature about the effect of bacteria and several microbial products (exotoxins and endotoxins) on the intestine [173–185]. This line of investigation has been pursued largely because infection-related diarrhea is a major illness responsible for substantial morbidity and mortality, particularly in children [186,187]. Recently, Wolfs et al., working in the laboratory of Boris Kramer, reported the effects of experimental intra-amniotic injection of endotoxin on the bowel of fetal sheep [188]. Endotoxin was administered at different gestational ages, and the bowel was studied after 2, 14 or 30 days of endotoxin administration. The investigators reported that: (1) endotoxin could be detected in the fetal stomach in 2 days, demonstrating that this microbial product was swallowed; (2) the expression of tight junction protein ZO-1 (zonula occludens protein-1) in the fetal intestine increases as a function of gestational age [188]. However, exposure to endotoxin impaired maturation of tight junctions, an effect that lasted as long as 30 days. This has potential implications because inadequate tight junction distribution can result in easy access of microbial toxins to the bowel mucosa; (3) interestingly, LPS did not induce an early (2 days) inflammatory response in the gut of preterm animals. However, exposure to endotoxin for 14 days was associated with an increased number of T-lymphocytes, myeloperoxidase positive cells and gammadelta T-cells (an inflammatory response); and (4) there was a gestational-age dependent increase in the intestinal expression of TLR4 and MD-2 mRNA in the control group; yet, endotoxin exposure reduced the expression of these two molecules implicated in Gram-negative microbial recognition [188]. Altogether, the findings of this study suggest that exposure to endotoxin in the amniotic fluid during fetal life disturbs fetal intestinal development, which may predispose to necrotizing enterocolitis and other disorders. The response of the intestine to endotoxin or bacterial exposure appears to be quite different from that of the fetal lung [188–201].

Wolfs et al. subsequently demonstrated that exposure to IL-1α in the amniotic cavity of sheep at 1, 3 or 7 days before cesarean delivery (performed at 125 days of gestation) led to an intestinal inflammatory process, characterized by overexpression of mRNA levels for interferon-γ, TNF-α, IL-4 and IL-10, as well as an increase in CD3+ and CD4+ lymphocytes and myeloperoxidase positive cells [198]. This was coupled with a decreased number of cells expressing FoxP3+, which are generally considered a marker for T

regulatory cell identification. This decline in T regulatory cells may be permissive of the inflammatory phenomenon reported in the ileum. The latter observation is relevant to the human fetus, because we [77,79] and others [202–205] have previously demonstrated not only the presence of endotoxin in patients with preterm labor, but also increased concentrations of IL-1β [128,131] and IL-1α [128,131] in the amniotic fluid of patients in preterm labor (and in some cases, term labor).

Meconium-stained amniotic fluid at term as a risk factor for maternal and neonatal infection

Clinical observations demonstrate an association between MSAF at term and the presence of clinical or subclinical infection in the mother [11,15,35,36,38,206,207] and neonate [11,26,42,208,209]. In the largest retrospective cohort study reported to date, in which 43,200 women delivered at term, Tran et al. reported that patients with MSAF had higher rates of clinical chorioamnionitis and puerperal endomyometritis than those with clear amniotic fluid [clinical chorioamnionitis (18.9% versus 2.3%, p <0.001) and puerperal endomyometritis (1.7% versus 1%; p <0.0001)] [38]. Moreover, patients with thick meconium in the amniotic fluid also had a higher frequency of these two infection-related complications [38]. Indeed, a multivariable model showed that patients with moderate to thick MSAF had an increased risk for chorioamnionitis (OR 1.39; 95% CI 1.20–1.61) and endomyometritis (OR 1.51; 95% CI 1.19–1.93) after adjusting for maternal age, parity, education, ethnicity, length of labor, birthweight and mode of delivery [38]. These observations are consistent with those of other investigators who have provided evidence that MSAF is a risk factor for maternal infection-related complications [34,35,38,206].

The frequency of suspected or proven neonatal sepsis in patients with MSAF has varied among reports. Based on a large database of 18,299 neonates ≥2000 g, Escobar et al. reported the neonatal outcomes following a workup for sepsis from mothers who had and had not received intrapartum antibiotics [43]. Sepsis was defined based on clinical and microbiologic criteria. Among neonates of mothers who had not received intrapartum antibiotics (n = 1568), MSAF was significantly associated with neonatal sepsis [OR 2.23, 95% CI (1.18–4.21)]. Even when mothers had received intrapartum antibiotics (n =1217), the association between MSAF and neonatal sepsis was observed [OR 2.73, 95% CI (1.08–6.94)] [43].

Subsequently, Kayange et al. reported a prospective cross-sectional study of 300 neonates born >28 weeks of gestation with clinical and culture-proven sepsis conducted in Tanzania [44]. The frequencies of neonatal positive blood culture in the first 72 hours of life (early-onset sepsis) and after 72 hours of life (late-onset sepsis) were higher in pregnancies with MSAF than in those with clear amniotic fluid [early-onset: 81% (34/42) versus 29.1% (23/79); p =0.0001 and late-onset:85.7% (42/49) versus 38.5% (50/130); p =0.0001)] [44]. Similar observations were reported from a secondary analysis of a randomized controlled trial conducted in South Africa for the prevention of perinatal sepsis (PoPS Trial) [45]. MSAF was associated with both early-onset (aRR = 2.8; 95% CI 2.2–3.7; p <0.05) and late-onset sepsis (aRR = 2.4, 95% CI 1.1–5.0; p <0.05) in both preterm and term neonates while adjusting for mode of delivery, duration of labor, primiparity, birthweight and preterm birth

(<37 weeks of gestation) [45]. In this study, early-onset sepsis was defined as sepsis occurring within the first two days of life, while late-onset sepsis was defined as that occurring on days 3–28 of life [45]. Therefore, MSAF has been associated with neonatal sepsis in both preterm and term gestations.

Could intra-amniotic infection explain why only some neonates with meconium stained amniotic fluid develop meconium aspiration syndrome?

One of every seven pregnancies has MSAF [210], and this is particularly the case in term and post-term gestations. Yet, only 5% develop MAS [8,10,13,14,31] – why?

The pathophysiology of MAS has been attributed to one of the following factors: (1) the mechanical effect of meconium, which can obstruct (partially or completely) segments of the distal airways [14,211–213]; (2) the chemical effect of the material contained in meconium (i.e. free fatty acids on the surface of the airway) which may inactivate surfactant [14,214,215]; (3) inflammatory mediators which are contained in meconium, including a broad range of chemokines and cytokines [17,213,216,217], which can recruit inflammatory cells, such as neutrophils, into the lung and mediate a local inflammatory response; (4) complement activation [218–221]; (5) phospholipase A2, which has been detected in human meconium and meconium-contaminated lungs [14,222], and implicated in the induction of lung inflammation; and (6) apoptosis or programmed cell death [17,213,223,224]. Vidyasagar and Zagariya proposed that chemokines and cytokines in MAS can lead to angiotensin II-induced apoptosis of lung cells [19,224,225]. While the picture is complex, severe inflammation seems to be a convergent point in the pathophysiology of MAS.

We have previously demonstrated that MIAC or intraamniotic inflammation are associated with high concentrations of cytokines (such as IL-1α and β, TNFα, IL-6, IL-18, IL-16, leukemia-inhibiting factor, IL-10) [74,131,226–233], chemokines (such as IL-8, monocyte chemoattractant protein-1 [MCP-1], CXCL-10 [IP-10], macrophage inflammatory protein-1α [MIP-1α], growth regulated oncogene-α [GRO-α]) [234–239] complement-split products [240,241], phospholipase A2 (Romero R – unpublished observations) and matrix-degrading enzymes (MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9) [242–248], as well as other components which participate in the regulation of programmed cell death [249–251]. Therefore, in the context of MIAC and intra-amniotic infection, amniotic fluid contains a high concentration of mediators that, when aspirated *in utero*, could induce lung inflammation.

But can meconium be aspirated *in utero*? Studies in which indwelling catheters have been placed in the respiratory tract of fetal sheep demonstrate that the egress of lung fluid is toward the amniotic cavity [252,253]. Does the fetus inhale amniotic fluid? Using ultrasound and color Doppler, the evidence is now clear that there is an influx and efflux of fluid not only in the nasopharynx and nose [254–259], but also in the trachea [259–261]. Fetal gasping was reported in humans by Boddy and Dawes, who describe gasping 24–72 hours before death in the absence of labor [262]. Subsequently, Patrick et al. reported a study of 16 lambs that died from infection, hypoxia and other causes prior to the onset of labor [263]. Gasping could be seen for up to 16.5 hours before death, and the investigators documented negative tracheal pressure by monitoring continuous pressure with indwelling catheters

[263]. Manning et al. reported gasping in primate fetuses (*Macaca mulatta*) before death, while investigating breathing movements with continuous tracheal pressure recordings [264]. Altogether, this evidence suggests that amniotic fluid can be inhaled *in utero*, particularly when the fetus is in a pre-agonal state [264–267].

Is there documentation that meconium can be found in the fetal lung before birth? There is now conclusive evidence that meconium can be found in the fetal lung in cases of stillbirth [266,268]. Mortensen and Kearney recently reported autopsy findings that the frequency of intrauterine meconium aspiration in the midtrimester was 9% in 21 cases [268]. Importantly, in a study that included stillbirths (with gestational ages ranging from 31 to 39 weeks), 80% of fetuses had meconium aspiration [266]. Perhaps the finding that meconium aspiration can occur before birth explains the limited success of intratracheal removal of meconium at birth to prevent MAS. If a fetus with MSAF gasped before birth, the particulate material may have already reached the distal airways, and the beneficial effect of removing the meconium from the airways may be limited.

Based on the available evidence, we propose that *in utero* aspiration of MSAF containing bacteria, endotoxin and high concentrations of inflammatory mediators (such as chemokines, cytokines, phospholipase A2 and complement) can create conditions predisposing to MAS. Other factors, such as the density of the particulate matter (thick meconium), duration of meconium exposure during the intrauterine stay, and existence of other morbidities that may induce gasping, would favor the occurrence of MAS. For example, fetuses with oligohydramnios and umbilical cord blood occlusions who have developed MIAC may be at particular risk for MAS. If the duration of exposure to infected meconium (which contains inflammatory mediators) is sufficient, this may also elicit a systemic fetal inflammatory response which would further predispose to MAS – perhaps this is the explanation of why MAS is rarely observed in patients who initially had clear amniotic fluid during labor, and only developed MSAF just prior to delivery.

Strengths and limitations

We report, for the first time, that MIAC is present in nearly 20% of all patients with MSAF in spontaneous labor at term, and that there is evidence of an intra-amniotic inflammatory response. Limitations of this study include that microbiologic workup was performed in a low-resource setting, and therefore, genus and species characterization of bacteria was not available. Further studies are required using sequence-based techniques to identify non-culturable microorganisms.

Clinical implications

MSAF is a frequent occurrence in labor and delivery units. The observations reported herein indicate that meconium is associated with the presence of bacteria in the amniotic fluid; therefore, the neonate is at risk for congenital infection. A randomized clinical trial in which patients with MSAF were allocated to ampicillin/sulbactam versus placebo showed that antibiotics reduced the incidence of clinical chorioamnionitis (6.3% vs 23.3%; RR 0.48, 95% CI 0.22–0.92; p =0.02) and postpartum endometritis (16.7% versus 8.3%; RR 0.64, 95% CI 0.30–1.33; p = 0.16) [269]. This provides therapeutic evidence that treatment of

patients with MSAF may reduce the frequency of infection-related complications in the mother. Whether antibiotic administration when meconium is identified during labor can reduce the frequency of suspected or proven neonatal sepsis remains to be determined – such hypothesis would be aided by the availability of a means to identify intra-amniotic inflammation in cases of meconium in a non-invasive way. Other implications of our findings include that MSAF, in cases of MIAC or intra-amniotic inflammation, may be a risk factor for bowel disorders (e.g. irritable bowel syndrome) and MAS. Neonatologists have searched intensively for an explanation of why only a fraction of neonates exposed to meconium develop MAS. Perhaps meconium containing bacteria, microbial products (such as endotoxin) and high concentrations of inflammatory mediators may play a role in amplifying the lung injury caused by exposure to the particulate matter contained in meconium.

Conclusions

Meconium-stained amniotic fluid at term is associated with MIAC and higher concentrations of amniotic fluid IL-6. Therefore, the presence of this clinical sign should raise the index of suspicion for maternal and/or neonatal infection related complications. Further studies are required to determine if meconium containing bacteria, microbial products and inflammatory mediators may predispose to MAS, and whether interventions aimed at treating intra-amniotic infection/inflammation, neonatal sepsis or even aspiration of infected meconium, can reduce neonatal morbidity (e.g. pneumonia, sepsis, MAS).

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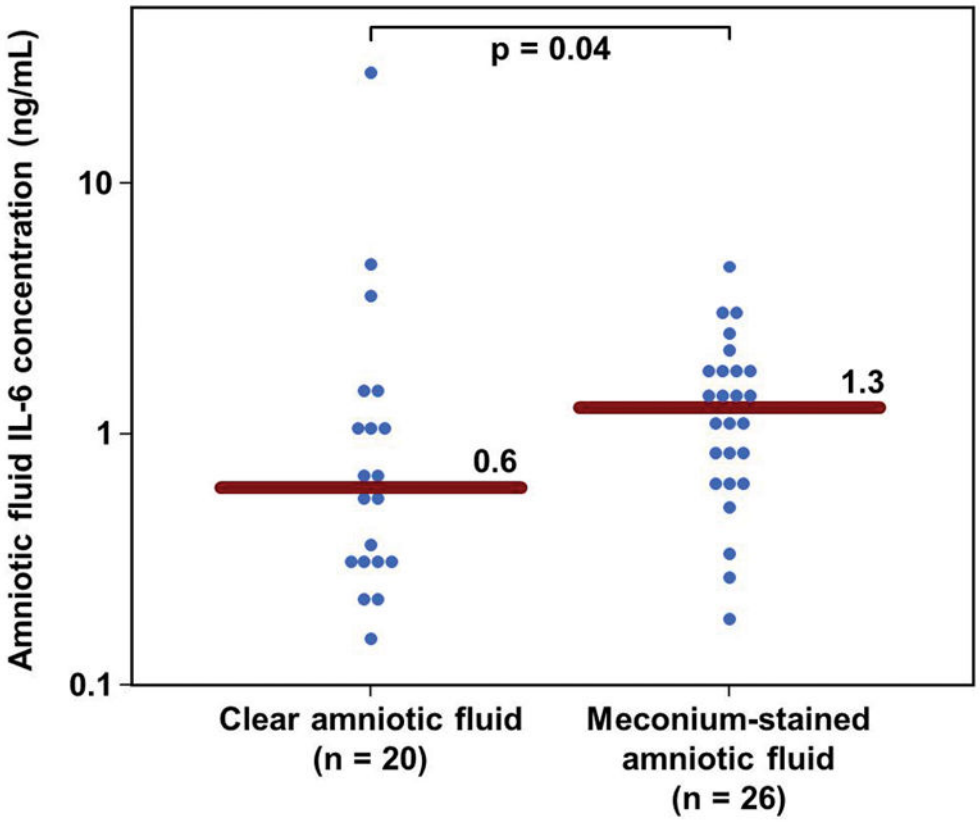

Figure 1. Amniotic fluid interleukin-6 (IL-6) concentration in women at term with clear and meconium-stained amniotic fluid

Patients with meconium-stained amniotic fluid had a significantly higher median amniotic fluid IL-6 concentration (ng/mL) than in those with clear amniotic fluid [1.3 (0.7–1.9) versus 0.6 (0.3–1.2); $p = 0.04$].

Table 1

Clinical characteristics of the study population

	Clear amniotic fluid (n=42)	Meconium-stained amniotic fluid (n=66)	P value
Maternal age (years)	21.5 (18–25.5)	24.5 (21–28)	0.02
Gestational age (weeks)	39.3 (38.1–40)	39 (38.4–40.3)	0.9
Nulliparity	25 (59.5%)	34 (57.6%)	0.4
Birth weight (grams)	3145 (3010–3612)	3290 (3030–3497.5)	0.6
No. of cesarean sections	5 (11.9%)	18 (32.1%)	0.02
Cervical dilatation (cms)	3 (2–5)	3 (2–4.25)	0.4
Apgar score at 5 minutes < 7	0	1	0.8
Amniotic fluid glucose (mg/dL)	9 (7.4–12.6) *	6 (0–8.9) **	<0.001
Amniotic fluid interleukin-6 (ng/mL)	0.6 (0.3–1.2)#	1.3 (0.7–1.9)##	0.04

Data are presented as mean (interquartile) or n (%).

* n=40;

** n=61.

n=20;

n=26.

Table 2

Microbiologic findings and the results of Limulus amebocyte lysate assay (LAL) from patients with meconium-stained amniotic fluid

Microorganism	Incidence (n)	Positive LAL (n)	Positive LAL after heat treatment (n)
Gram-negative rod	7	4	2
Ureaplasma urealyticum	4	2	1
Gram-positive rod	2	2	2
Mycoplasma hominis	1*	1	1

LAL: Limulus amebocyte lysate assay.

*Combined infection with Gram-positive rod.

Table 3

Limulus amebocyte lysate assay (LAL) results before and after heat treatment

	Clear amniotic fluid (n=42)	Meconium-stained amniotic fluid (n=66)	P value
Positive LAL	2 (4.7%)	31 (46.9%)	<0.001
Positive LAL after heating	1 (2.3%)	12 (18.1%)	<0.05

Data are presented as n (%).

LAL: Limulus amebocyte lysate assay