Immunological Significance of Meconium-Stained Amniotic Fluid with Complement Factors C3 and CD4 Levels for Pregnancy Health

Hanan Fawzi Salman¹, Imtithal I. J.¹, Nagam Khudhair ¹*

Abstract
Background: Meconium stained liquor (MSL) occurs when the fetal anal sphincter muscles relax in utero, tinting the amniotic fluid green, yellow, or brown. It is less common in premature fetuses. Method: Complement factors (C3 and CD4) were assessed in maternal serum and amniotic fluid from 30 women with MSL undergoing diagnostic fetoscopy at gestational week. Serum levels were consistently higher than control levels, which were higher than levels in amniotic fluid. Results: In the MSL group versus the control group, mean C3 levels in serum and amniotic fluid were (200.3913±37.6689 vs. 155.1321.8257) and (100.912±22.1869 vs. 98.0027±30.0989 pg/ml), respectively, showing statistically significant differences (p<0.01). Significant differences (p<0.01) were also observed between amniotic fluid and serum samples (155.1321 vs. 200.3913 pg/ml) in the MSL group, but not in the control group (100.912 vs. 98.0027 pg/ml). Mean CD4 levels in serum and amniotic fluid in the MSL group versus the control group were (13.1072 ± 4.2871 vs. 7.8651±2.5954) and (4.0372 ± 1.766 vs. 4.2328 ± 1.3943 pg/ml), respectively, with statistically significant differences (p<0.01). Significant differences (p<0.01) were noted between amniotic fluid and serum samples (7.8651 vs. 13.1072 pg/ml) in the MSL group, but not in the control group (4.2328 vs. 4.0372 pg/ml). Conclusion: Elevated levels of C3 and CD4 in serum and meconium-stained amniotic fluid indicate potential issues in pregnancy, suggesting their utility for early detection of this pathological condition to safeguard fetal health.

Keywords: Meconium-Stained Liquor (MSL), Amniotic Fluid (AF), Cluster differentiation CD4, Complement factor (C3)

Significance
This research demonstrated the immune factors C3 and CD4 activity in amniotic fluid on prenatal health, which can diagnose the meconium-stained liquor complications early.

*Correspondence. Nagam Khudhair, Biology Department, Education College for Women, University Of Anbar, Ramadi 31001, Iraq. E-mail: nagam.mahdi@yahoo.com. Phone No. 07724568544

Introduction
The amniotic fluid, a complex biological fluid surrounding the developing fetus, serves as a critical interface between the maternal and fetal compartments, playing pivotal roles in fetal development, protection, and immune modulation. Comprising both soluble and cellular components, amniotic fluid contributes to the maintenance of a suitable intrauterine environment essential for the growth and well-being of the fetus.

The amniotic fluid contains soluble and cellular components (Underwood, 2005). Soluble components encompass carbohydrates, proteins, peptides, lipids, lactate, pyruvate, electrolytes, enzymes, and hormones, among others (Maddipati et al., 2016), many of which serve as the first line of defense against invading pathogens in the amniotic cavity (Pierce et al., 2016). Cellular components consist of various cell types derived from exfoliating surfaces of the developing fetus, including the skin, respiratory system, urinary tract, gastrointestinal tract, and stem...
Meconium stained liquor (MSL) occurs when fetal anal sphincter muscles relax within the womb, resulting in the amniotic fluid appearing green, yellowish, or brownish. This condition is rare in premature fetuses (less than 37 weeks). MSL is considered uncommon before 37 weeks of pregnancy, with 7 to 22% of term pregnancies (37 to 42 weeks gestation) being complicated by MSL globally, leading to respiratory distress in some infants who may require oxygen supplementation (Mundhara and Agarwal, 2013; Madhuri, 2013).

Meconium is a germ-free, thick, black-green, odorless material first recognized in the fetal intestine around 12 weeks of gestation and stored in the fetal colon throughout gestation (Jain et al., 2017; Kashikar et al., 2021). Passage of meconium into the amniotic fluid does not occur before 37 weeks and is attributed to intestinal peristalsis and sphincter relaxation (Dobhit et al., 2018). Meconium-stained liquor (MSL) may be thick or thin compared to clear fluid, with thicker meconium associated with higher morbidity and mortality (Biradar et al., 2018; Shrestha et al., 2018).

The amniotic fluid (AF) is primarily composed of water (99% - 99%) along with electrolytes, carbohydrates, proteins, lipids, peptides, and hormones (Machtejevien, 2013).

Meconium is a complex mixture of water, epithelial cells, bile, mucus, and amniotic fluid, containing numerous substances and danger motifs that may induce inflammation (de Beaufort et al., 2003). Newborns may experience dyspnea when amniotic fluid (AF) is aspirated into the fetal lungs, regardless of the presence of meconium pollution. Studies have reported a significant amount of amniotic fluid aspiration in postmortem brain tissue (Lavezzi et al., 2019).

C3 plays a pivotal role in activating the complement system, necessary for both classical and alternative pathways. Individuals deficient in C3 are more susceptible to bacterial infections (Matsuyma et al., 2001).

The immune system, particularly the complement system and CD4 T helper cells, emerges as central players in the intricate interplay between amniotic fluid composition and pregnancy outcomes. Complement factor C3, pivotal in activating the complement cascade, exhibits significant alterations in both serum and amniotic fluid of MSL cases, reflecting potential immune dysregulation associated with this condition. Moreover, CD4 T helper cells, essential components of the adaptive immune response, demonstrate elevated levels in MSL cases, underscoring their role in orchestrating immune responses within the amniotic cavity. During pregnancy, the mother’s immune and cardiovascular systems undergo remarkable changes to ensure a healthy delivery. The complement system is no exception, displaying significant alterations to protect the fetus from complement system attack. Recent research suggests that the complement system is crucial for normal development and infection protection (Mamidi et al., 2014). Comprising over 50 different proteins, the complement system maintains a modest degree of steady-state activity. Activation through one or more of the three pathways—classical, lectin, or alternative—can amplify the complement cascade. C3 activation leads to C3 deposition, involving the covalent attachment of the C3b fragment to invaders or self. The fluid-phase activation products C3a and C5a engage with G protein-linked receptors on various cell types, triggering inflammation and activating immune cells. CD4 plays a fundamental role in the adaptive immune response, leading to the activation of naive B cells and plasma cells, which subsequently secrete neutralizing and opsonizing antibodies (Zhu and Paul, 2008). CD4 T cells induce macrophage activation, recruit phagocytes—primarily neutrophils and macrophages—to the site of infection, and stimulate the activation of cytotoxic T cells (CTLs) (Nicholson, 2016). CD4 T helper cells are essential components of the immune system, working in conjunction with other immune and non-immune cells to protect the body from illnesses throughout life. Arising in the thymus after derivation from common lymphoid progenitors in the bone marrow, CD4 T cells primarily function in peripheral tissues and various lymphoid organs (Kumar et al., 2018).

Effector CD4 T cells aid in host defense against infections by producing effector cytokines, which stimulate other immune cells to destroy infected cells—such as macrophages and CD8 T cells—and assist B cells in antibody production to mediate humoral immune responses (Ruterbusch et al., 2020). CD4+ T cells can develop into various effector subsets, including Th1, Th2, Th9, and Th17 cells, depending on the surrounding microenvironment (Fang et al., 2018).

Initially observed around 20 years ago, T helper cells gained recognition as a unique lineage of CD4 T cells crucial for germinal center (GC) development upon the identification of their master transcription factor Bcl6 (Yu et al., 2009).

Interleukin-16 (IL-16), a soluble ligand for CD4, is known for its ability to chemoattract various CD4+ cells, including lymphocytes, eosinophils, monocytes/macrophages, and dendritic cells. IL-16 can activate CD4+ T cells in the presence of IL-2 (Kaser et al., 1999). High concentrations of CD4+ lymphocytes are present in the human fetal intestine from approximately week 11 of gestation (Spencer et al., 1986).

The present study aimed to elucidate the immune-mediated mechanisms underlying MSL by investigating the levels of complement factor C3 and CD4 in serum and amniotic fluid samples collected from pregnant women with MSL compared to controls. Through a comprehensive analysis of immune markers and their implications for pregnancy outcomes, this research endeavors to advance our understanding of MSL pathophysiology.
and contribute to the development of diagnostic and therapeutic strategies for mitigating adverse pregnancy outcomes associated with this condition. By unraveling the intricate relationship between immune factors and amniotic fluid composition, this study seeks to pave the way for improved management and care of pregnant individuals at risk of MSL and its associated complications.

Materials and Methods

Study Design

The study comprised 30 women with Meconium Stain Liquor (MSL) as the studied group, alongside 30 cases with clear liquor serving as the control group. This study adheres to the principles outlined in the Declaration of Helsinki and its subsequent amendments. The research protocol was approved by the institutional review boards of AL-Liqa Hospital for Maternity and Al-Yarmouk Teaching Hospital.

Participant Criteria

Participants were women aged 16 to 39 years with a gestational age between 37 to 41 weeks. Those who smoked were excluded from the study.

Sample Collection

Samples were obtained from AL-Liqa Hospital for Maternity and Al-Yarmouk Teaching Hospital between February and July 2022.

Blood Sample Collection:

Five milliliters of blood samples were collected from both the MSL and clear liquor groups. Samples were transferred to gel tubes and centrifuged at 1600 RPM for 10 minutes at room temperature. The separated serum was then stored at -20°C until testing.

Amniotic Fluid Collection:

Amniotic fluid was collected through childbirth by inserting a syringe through the amniotic sac membranes to obtain 10 ml of fluid via the vagina from both the MSL and control groups. The collected fluid was stored in plain tubes, centrifuged at 1600 RPM for 10 minutes, and then stored at -20°C until testing.

Laboratory Testing

The quantitative sandwich enzyme immunoassay technique was employed, wherein an antibody specific for the parameters C3 and CD4 was immobilized on a solid surface ELISA multi-well plate (96-well). The protocol for each immunological test was followed.

Measurement of C3 and CD4:

C3 and CD4 levels were measured using commercially available ELISA kits from Cusa Bio, USA. Tests were conducted according to the manufacturer’s instructions to determine the concentration of C3 and CD4.

Statistical analysis

Statistical calculations were carried out using MINI tab 13. ANOVA one-way test (for independent measures) was used at the level of probability (p ≤ 0.01) and results were verified as the arithmetic mean ± Standard Deviation.

Result

The mean C3 levels in serum and amniotic fluid specimens in the MSL group and control group were (200.3913±37.6689 vs. 155.1321±8.2527 and 100.912±22.1869 vs. 98.0027±30.0989 pg/ml, respectively), with a statistically significant difference (p<0.01) between the control group and MSL group, indicating higher C3 levels in the serum and amniotic fluid of the MSL group compared to the control group (Table 1-1).

Significant differences (p<0.01) were observed between amniotic fluid and serum samples (155.1321 vs. 200.3913 pg/ml, respectively) for the MSL group, while no significant differences (p<0.01) were found between the studied samples (100.912 vs. 98.0027 pg/ml, respectively) of the control group.

Similarly, the mean CD4 levels in serum and amniotic fluid specimens in the MSL group and control group were (13.1072 ± 4.2871 vs. 7.8651±2.5954 and 4.0372 1.766± vs. 4.2328±1.3943 pg/ml, respectively), with a statistically significant difference (p<0.01) between the control group and MSL group, indicating higher CD4 levels in the serum and amniotic fluid of the MSL group compared to the control group (Table 1-2).

Significant differences (p<0.01) were observed between amniotic fluid and serum samples (7.8651 vs. 13.1072 pg/ml, respectively) for the MSL group, while no significant differences (p<0.01) were found between the studied samples (4.2328 vs. 4.0372 pg/ml, respectively) of the control group.

Discussion

Complement factor C3

Amniotic fluid harbors a diverse immune cell composition during the second and third trimesters of pregnancy. Between 15 and 20 weeks, innate lymphoid cells (ILCs) are most abundant in the amniotic fluid. T cells and ILCs, followed by NK cells, are more abundant between 15 and 30 weeks gestation than at birth. B cells are rare between 15 and 20 weeks but become a stable population of immune cells until full term. Neutrophils increase as pregnancy progresses, while monocytes/macrophages appear after 20 weeks and remain constant until delivery. In cases of intra-amniotic infection/inflammation, all amniotic fluid immune cells, except ILCs, are increased (Gomez-Lopez et al., 2018).

Previous research indicates that mothers at term exhibit higher serum levels of C3 compared to non-pregnant women (Millar and Mills, 1972). Additionally, studies have shown elevated levels of C3 in amniotic fluid compared to maternal serum (Stabile et al., 1988). C3 supplementation in amniotic fluid is valuable for diagnosing intra-amniotic infection in preterm labor (Elimian et al., 1998). In pathological conditions such as a short cervix, higher C3 levels were found compared to C5 levels in the amniotic fluid (Kim et al., 2018).
Table 1. The level of complement C3 in the MSL group and control group.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Groups</th>
<th>Mean± SD</th>
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<tbody>
<tr>
<td>Amniotic fluid</td>
<td>MSL</td>
<td>155.13±31.8257</td>
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<tr>
<td></td>
<td>Control</td>
<td>98.00±30.0989</td>
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<tr>
<td>P value</td>
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<td>0.00001</td>
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<tr>
<td>Serum</td>
<td>MSL</td>
<td>200.39±37.6689</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>100.91±22.1869</td>
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<td>P value</td>
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P value: 0.00001

Table 2. The level of complement CD4 in the MSL group and control group.

<table>
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<td>Amniotic fluid</td>
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<tr>
<td></td>
<td>Control</td>
<td>4.233±1.3943</td>
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<tr>
<td>P value</td>
<td></td>
<td>0.00001</td>
</tr>
<tr>
<td>Serum</td>
<td>MSL</td>
<td>13.107±4.2871</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>4.037±1.766</td>
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<td>P value</td>
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<tr>
<td>P value</td>
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P value: 0.00001

MSL: Meconium Stained Liquor, SD: Standard Deviation, p<0.05
Earlier research revealed that meconium is a potent activator of complement, a key mediator of inflammation. Meconium activates the alternative complement pathway, as evidenced by a significant increase in the C3bBbP-transforming alternative pathway in human umbilical cord serum (Castellheim et al., 2004). A recent study by Hong et al. (2020) found elevated cervicovaginal fluid (CVF) levels of C5a and IGFBP-1 to be significantly associated with intra-amniotic infection (IAI) and spontaneous preterm delivery (SPTD) at <34 weeks, while C3a levels were associated with IAI but not SPTD. Syukri et al. (2015) discovered that serum C3 levels were significantly different between patients with recurrent urinary tract infections (UTI) and a group of healthy young women (mean 42.08 μg/ml ± 1.20 vs. 42.75 μg/ml ± 0.71, p = 0.008), although this difference was not clinically relevant. In a study by Soto et al. (2005) focusing on various pathological conditions, the mean plasma concentration of C5a in pregnant patients with acute pyelonephritis was significantly higher than in normal pregnant women (p<0.001). However, there was no statistical difference in the average plasma concentration of C3a and C4a between the two groups. A recent study by Salman et al. (2022) identified significant differences in the levels of IL-1β, IL-6, and IL-17 between mothers with meconium-stained fluid and mothers with clear amniotic fluid. Salvesen et al. (2009) observed that meconium activates the lectin-complement pathway in addition to the alternative pathway. They found that C1 inhibitor (C1-INH) efficiently reduced a wide range of inflammatory mediators even at the lowest concentration, suggesting that C1-INH administration may reduce the inflammatory response in meconium aspiration syndrome (MAS).

**Complement factor CD4**

A previous study by Salvesen et al. (2008) found that combined inhibition of complement and CD14 almost completely abolished meconium-induced cytokine and chemokine formation and markedly reduced the formation of growth factors. In late pregnancy, amniotic fluid contains CD4+ T-cell chemotactic activity, which is reduced by 58% in the presence of anti-IL-16 antibodies (Thornton et al., 2009). According to a recent study by Galaz et al. (2020), women with preterm labor rupture of membranes (pPROM) had a higher concentration of CD4+ and CD8+ T lymphocytes than B cells in their amniotic fluid. Additionally, increased levels of interleukins and CD4CD8 were found in the amniotic fluid of women who experienced premature birth (Gomez-Lopez et al., 2019). To prevent disease throughout life, the body requires CD4 T helper cells, which work in concert with other immune and non-immune cells. Originating from common lymphoid progenitors in the bone marrow, CD4 T lymphocytes develop in the thymus and primarily function in peripheral tissues and other lymphoid organs (Kumar et al., 2018).

**Conclusions**

The study elucidated the intricate interplay between the immune system and amniotic fluid composition, shedding light on potential implications for pregnancy complications such as meconium-stained amniotic fluid (MSL). Amniotic fluid, rich in diverse immune cell populations, serves as a crucial milieu for maintaining fetal health, while alterations in its composition may signify underlying pathological conditions. Elevated levels of complement factor C3 and CD4 observed in both serum and amniotic fluid of MSL cases underscore potential immune dysregulation associated with this condition. The findings corroborate previous research highlighting the role of complement activation in response to meconium exposure and emphasize the diagnostic value of immune markers in identifying intra-amniotic infections and preterm labor risks. Collectively, these insights advance our understanding of immune-mediated mechanisms underlying MSL and underscore the importance of vigilant monitoring and intervention strategies to mitigate adverse pregnancy outcomes.

**Author contributions**

H.F.S. contributed to the development of the concept and the design of the study. Laboratory studies were conducted and clinical data were collected by I.I.J., N.K. conducted the study design, analyzed the data, and wrote the draft of the manuscript.

**Acknowledgment**

Authors were grateful to their department.

**Competing financial interests**

The authors have no conflict of interest.

**References**


infection. Obstetrics and Gynecology, 92(1), 72–76.
Spencer, J. O., MacDonald, T. T., Finn, T., & Isaeson, P. G. (1986). The development of gut associated lymphoid tissue in the terminal ileum of fetal human intestine. Clinical and Experimental Immunology, 64(3), 536


