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MULTI-APPROACH ANALYSIS OF MMP-9 IN PROM AND PPROM: HISTOPATHOLOGICAL AND NETWORK-BASED PERSPECTIVES

PROM VE PPROM'DA MMP-9'UN ÇOK YÖNLÜ ANALİZİ: HİSTOPATOLOJİK VE AĞ TABANLI YAKLAŞIMLAR

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ABSTRACT

Objective: This study investigates the role of matrix metalloproteinase-9 (MMP-9) in prelabor rupture of membranes (PROM) and preterm PROM (PPROM) by integrating histological analysis with bioinformatics-based molecular network assessment.

Methods: Placental tissue samples were collected from 25 control, 25 PROM, and 25 PPROM cases. MMP-9 expression was analyzed via immunohistochemistry, and histological changes were examined. Modular network analysis of MMP-9 was performed using Cytoscape and STRING databases, with clustering conducted using the MCODE plugin and functional enrichment assessed via Enrichr platform. P-values less than 0.05 were considered statistically significant.

Results: Immunohistochemical analysis revealed a progressive increase in MMP-9 expression from control to PROM and PPROM placentas, with the highest expression observed in PPROM cases. Structural disruptions, including fibrin deposition and connective tissue irregularities, were more pronounced in PPROM ($p<0.05$). Network analysis identified four MMP-9-associated molecular clusters, of which two were significantly enriched in key biological pathways ($p<0.05$). Module 1 was associated with interleukin signaling (IL-4, IL-10, and IL-13) and extracellular matrix (ECM) degradation, while Module 2 was linked to neutrophil degranulation and collagen breakdown.

Conclusion: These findings indicate that MMP-9 plays a crucial role in ECM remodeling and inflammatory activation, contributing to membrane weakening and rupture in PROM and PPROM. The identified molecular clusters and signaling pathways provide insights into the underlying mechanisms and highlight MMP-9 as a therapeutic target for preventing premature rupture of membranes.

Keywords: MMP-9, prelabor rupture of membranes, placenta, immunohistochemistry, bioinformatics

ÖZ

Amaç: Bu çalışma, matriks metalloproteinaz-9 (MMP-9) ekspresyonunun preterm doğum öncesi membran rüptürü (PROM) ve preterm PROM (PPROM) patogenezindeki rolünü histolojik ve biyoinformatik yaklaşımlarla araştırmayı amaçlamaktadır.

Yöntem: Çalışmada 25 kontrol, 25 PROM ve 25 PPROM vakasından plasental doku örnekleri toplandı. MMP-9 ekspresyonu immünohistokimya yöntemiyle analiz edildi ve histolojik değişiklikler incelendi. MMP-9 modüler ağ analizi Cytoscape ve STRING veri tabanları kullanılarak gerçekleştirildi; kümelenme MCODE eklentisiyle tespit edildi ve fonksiyonel zenginleştirme analizi Enrichr platformu kullanılarak yapıldı. $P<0,05$ olan değerler istatistiksel olarak anlamlı kabul edildi.

Bulgular: İmmünohistokimyasal analiz sonuçları, kontrol grubundan PROM ve PPROM'a doğru MMP-9 ekspresyonunun kademeli olarak arttığını ve en yüksek seviyeye PPROM vakalarında ulaştığını ortaya koydu ($p<0,05$). Fibrin birikimi ve bağ dokusundaki düzensizlikler, özellikle PPROM grubunda daha belirgindi. Yolak analizinde, MMP-9 ile ilişkili dört moleküler küme tespit edildi; bunlardan ikisi biyolojik açıdan önemli yolaklarla anlamlı düzeyde zenginleşme gösterdi ($p<0,05$). Modül 1, interlökin sinyalleşmesi (IL-4, IL-10 ve IL-13) ve ekstrasellülar matriks (ECM) yıkımı ile ilişkilendirilirken, Modül 2 nötrofil degranülasyonu ve kollajen yıkımı ile bağlantılı olarak bulundu.

Sonuç: Bu bulgular, MMP-9'un ECM yeniden şekillenmesi ve inflamatuar aktivasyonda kritik bir rol oynadığını ve bunun membran zayıflaması ve rüptürüne katkıda bulunduğunu göstermektedir. Belirlenen moleküler kümeler ve sinyal yolları, PROM ve PPROM'un altında yatan mekanizmalara ışık tutmakta ve MMP-9'u potansiyel terapötik hedef olarak öne çıkarmaktadır.

Anahtar Kelimeler: MMP-9, doğum öncesi membran rüptürü, plasenta, immünohistokimya, biyoinformatik

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Introduction

The membranes typically rupture during labor. Prelabor rupture of membranes (PROM) occurs before the membranes begin labor after the 37th week.[1] Preterm PROM (PPROM) is defined as the rupture of membranes before 37 weeks of gestation. It contributes to 40% to 50% of all preterm births. Preterm PROM contributes more to neonatal mortality and morbidity than any other disease group.[2-4] The interaction of physiological weakening of the membranes with the pressure caused by uterine contractions and intraamniotic infection are the major risk factors for PROM. Risk factors for PPROM include a history of PROM, short cervix length, second or third trimester vaginal bleeding, excessive uterine distension, low body mass index (BMI), and smoking. Timely and accurate diagnosis of PROM is essential for the continuation of pregnancy and prevention of neonatal mortality and morbidity.[5,6]

PPROM can be triggered by bacterial infection or inflammation of the membranes, known as chorioamnionitis. Amniochorion membranes derive their tensile strength from a collagen-rich extracellular matrix (ECM); therefore, it is of great importance to understand the enzymes and processes that can degrade the membrane ECM. Matrix metalloproteinases (MMPs) are a class of enzymes that can degrade collagen and other components of the ECM and can be induced by inflammation.[7] Matrix metalloproteinase-9 (MMP-9) is the primary MMP involved in normal labor and also plays an important role in pathological labor. MMP-9 is the most active MMP in the amnion and has been found to be significantly increased in amniotic membranes after the onset of contractions. Matrix metalloproteinase, also known as matrixin, plays an important role in the breakdown and remodeling of the ECM, ultimately causing both preterm labor and PPROM. PPROM increases MMP-9 concentrations in amniotic fluid, causing amniotic fluid infection, threatened preterm labor, and poor birth outcome. Manipulation of MMP may play a role in preventing spontaneous preterm labor.[8]

Given its critical role in ECM remodeling and inflammation-induced membrane rupture, this study aims to elucidate the contribution of MMP-9 to PROM and PPROM pathophysiology through histopathological and in silico approaches by analyzing its interactions with key protein networks involved in these processes.

Methods

Placenta Collection

This study was conducted with the approval of the Non-Interventional Clinical Ethics Committee, Dicle University Faculty of Medicine (date 20.12.2023 and number 2023/6). In the study, pregnant women aged between 18-49 years and without any systemic disease or secondary disease were examined at the Gynecology and Obstetrics Clinic of Dicle University Faculty of Medicine. The patient groups consisted of 25 term pregnant women who gave birth in hospital with the diagnosis of PROM and 25 PROM pregnant women. The control group consisted of 25 pregnant women who gave birth in hospital with the diagnosis of healthy pregnancy. Placental tissue samples were taken from all three groups. The patients whose placental tissues would be collected after delivery were informed about the study and their informed consent was obtained.

Placenta Tissue Preparation

Placenta tissues taken from the maternity clinic after delivery were reduced in size in a manner suitable for histological follow-up. Placenta tissues were first kept in formalin solution for one day. Then, they were kept in running water overnight. Placenta tissues were passed through an ascending ethanol series (50%, 70%, 80%, 90%, 96% and absolute ethyl alcohol) to remove water from the tissues. Tissues were kept in xylene solution 3 times for 30 minutes to remove alcohol. Then, tissues were taken in molten paraffin liquid at 58°C. In the final stage, tissues were embedded in paraffin blocks and 4-6 µm thick sections were taken with a microtome (catalog no: Leica RM2265, Wetzlar, Germany).[9]

Hematoxylin-Eosin Staining

Sections obtained from paraffin blocks of placental tissues were placed in a bain-marie set at 50°C. Sections were left in a 60°C oven overnight to allow the tissues on the sections to stick to the slide and to melt excess paraffin. Sections were removed from the oven, left at room temperature and allowed to cool. To remove paraffin residues from the sections, sections were kept in renewed xylene solutions for 15 minutes three times. After excess paraffin melted, sections were kept in a decreasing ethanol series (100%, 96%, 90%, 70%, 50% ethyl alcohol) for 10 minutes and excess alcohol was cleaned in distilled water. Harris hematoxylin stain was first applied to the sections for 8 minutes. Sections were kept under tap water for 5 minutes to clean excess staining. Then, the cleaned sections were kept in alcoholic (5%) eosin for 6 minutes. After the staining stage was completed, the sections were quickly dipped in a rapidly rising ethanol series. In order for the tissue in the sections to appear clear and clean, the sections were kept in xylene solutions for 3x15 minutes. Covering medium was added to the sections, they were covered with a slide and stored for examination.[10]

Immunohistochemical Staining

Sections were washed in phosphate buffer solution (PBS) for 3x5 minutes. After epitope retrieval in ethyl diamine tetra-acetic acid (EDTA) solution (pH: 8.0, catalogue no: ab93680, Abcam, Cambridge, USA), sections were treated with hydrogen peroxide solution (catalogue no: TA-015-HP, ThermoFischer, Fremont, CA, USA) for 20 minutes. Nonspecific staining was blocked with blocking solution (catalog no: TA-015-UB, ThermoFischer, Fremont, CA, USA) Primary antibody MMP-9 (catalog no: sc-393859, Santa Cruz Biotechnology, Heidelberg,

Germany, dilution ratio: 1/100) was dipped onto the tissues and left overnight at +4°C. following biotinylated secondary antibody (catalog no: TP-015-BN, ThermoFischer, Fremont, CA, USA), biotin-streptavidin complex was formed (catalog no: TS-015-HR, ThermoFischer, Fremont, CA, USA). Diaminobenzidine (DAB) (catalog no: TA-001-HCX, ThermoFischer, Fremont, CA, USA) was used as a chromogen. Gill III hematoxylin staining was used as a counter stain. Sections were quickly passed through an increasing ethanol series, cleared in xylene and mounted analyze and visualize with a Zeiss Imager A2 light microscope.[11]

Semi-quantitative Histological Scoring

For the semiquantitative assessment of MMP-9 expression, staining intensity was measured using ImageJ software (version 1.53, http://imagej.nih.gov/ij). Ten microscopic fields per sample were analyzed in each group, and the quantification results were documented. Brown coloration indicated positive antibody staining, whereas blue represented negative staining. Signal intensity (expression) within each area was obtained by dividing the stained antibody area by the total examined area. For each sample, the ratio of positively stained area relative to the total area was calculated across ten fields, and subsequently, a mean value for each group was determined. These mean values were then used for semiquantitative immunohistochemical scoring.

Statistical Analysis

The statistical analysis of our study was performed using IBM SPSS 29.0 software (IBM, Armonk, New York, USA). Shapiro-Wilk's test was used to assess the normality assumption. Continuous variables were presented as mean ± standard deviation (SD). Comparisons of more than two groups were carried out with the one-way analysis of variance (ANOVA), and pairwise group comparisons were made with the Tukey and Dunnett tests. Significance was considered for p values <0.05.

Modular Network and Enrichment Analysis of MMP-9 Protein Targets

To investigate the potential mechanisms associated with MMP-9 in PROM and PPROM, a modular network and pathway enrichment analysis was conducted. Initially, a protein-protein interaction (PPI) network was generated in Cytoscape (v.10.3.3) using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, incorporating an additional 100 interacting proteins. The confidence score threshold was set at 0.400. Subsequently, module identification was performed using the MCODE plugin in Cytoscape to detect functionally relevant clusters within the PPI network. The MCODE parameters were defined as follows: maximum depth=100, node score cutoff=0.2, degree cutoff=2, and K-core=2. The identified modules were further analyzed for functional and pathway enrichment using the Reactome database via the Enrichr platform. Pathways with a p-value less than 0.05 were considered statistically significant, and the top 10 enriched pathways were ranked based on ascending p-values for further interpretation.

Results

Demographic properties of patients

The clinical and demographic characteristics of the study groups are summarized in Table 1. Maternal age and BMI did not differ significantly among the groups (p> 0.05). However, gravida and parity were significantly higher in PROM and PPROM cases (p<0.05). Gestational age at delivery and birth weight were significantly lower in these groups, with the most pronounced decrease in PPROM (p<0.001).

Table 1. Clinical and demographic of patients

Parameters (n=25)	Control (mean ± SD)	PROM (mean ± SD)	PPROM (mean ± SD)	Significance
Maternal age, year	28.35 ± 3.57	32.34 ± 2.68	34.72 ± 2.28	p>0.05
Gravida, n	1.73 ± 0.58	3.47 ± 1.28	4.72 ± 2.28	p<0.05
Parity, n	1.10 ± 0.45	2.25 ± 1.12	2.87 ± 1.66	p<0.05
Gestational age at delivery, week	38.61 ± 1.15	35.24 ± 2.26	32.15 ± 2.43	p<0.001
Birth weight, gr	3815.27 ± 357.49	2504.29 ± 415.82	2147.72 ± 358.46	p<0.001
BMI, kg/m²	28.25 ± 4.58	30.57 ± 4.75	31.24 ± 3.86	p>0.05

SD: Standard deviation

PPROM and PPROM Showed Significant Increase in MMP-9 Expression

Images labeled A, B, and C represent placentas from control, PROM, and PPROM groups, respectively, immunostained for MMP-9 (Figure 1). In the control group (Figure 1A), there was mild expression of MMP-9 in chorionic villi, which exhibited preserved structural integrity, regular connective tissue distribution, and low-level staining in the trophoblast layer. The PROM group (Figure 1B) showed increased MMP-9 expression compared to controls, with noticeable fibrin deposition, mild villous irregularities, and increased staining intensity in the trophoblast layer. Furthermore, moderate congestion was observed in the intervillous spaces. The PPROM group (Figure 1C) demonstrated significantly elevated and widespread MMP-9 expression along with pronounced fibrin accumulation, notable disruption of villous structural integrity, marked irregularities in

connective tissue, and intense staining within the trophoblast layers. Semi-quantitative measurement of MMP-9 expression in groups were presented in Figure 1D. Statistically, MMP-9 expression was increased in PROM and PPROM groups compared to control group, however, no statistical difference was observed between PROM and PPROM groups. These findings suggest that MMP-9 expression is progressively enhanced from control to PROM and most markedly in PPROM, correlating with structural disruptions and increased fibrin deposition.

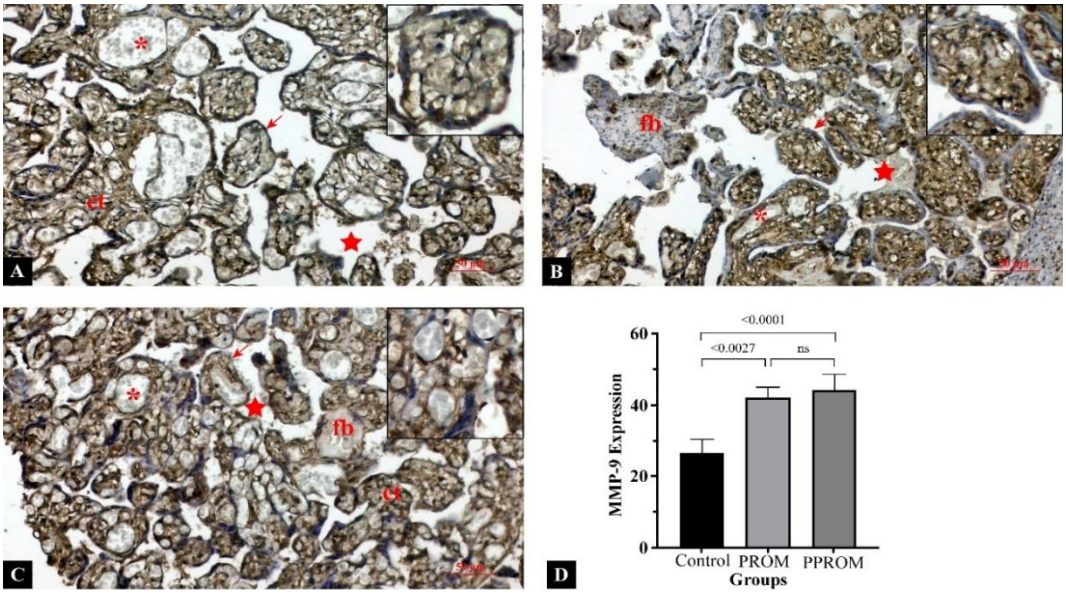

Figure 1. Cross sections of placental tissue in A: Control, B: PROM, C: PPROM groups.: A close-up of MMP-9 expression in placental tissues. D: Semi-quantitative MMP-9 expression in groups. inset Arrow: chorionic villus, ct: connective tissue, fb: fibrin deposition, *: capillary, star: intervillous area. MMP-9 immune staining, inset: Scale Bar: 20 µm (magnification: 40X).

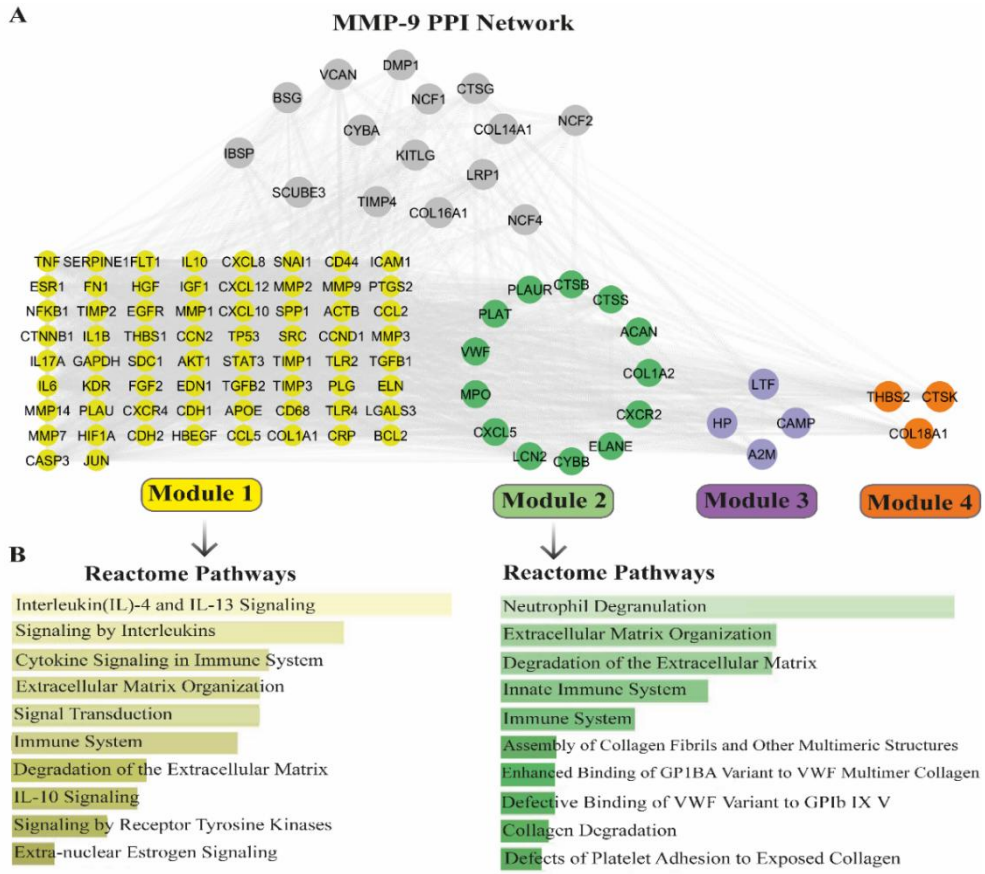

Figure 2. Modular network analysis of the MMP-9 PPI network. A. The MMP-9-centered PPI network was generated using STRING and clustered in Cytoscape with MCODE, identifying four modules: Module 1 (66 proteins), Module 2 (13 proteins), Module 3 (4 proteins), and Module 4 (3 proteins). B. Bar charts represent the significant functional annotation of Module 1 and Module 2, showing enriched pathways ranked in ascending order of p-values.

Modular Network Identification and Functional Enrichment Analysis of MMP-9 PPI Network

Modular network analysis of the MMP-9 PPI network identified four distinct clusters (Figure 2A). However, Module 3 (4 proteins) and Module 4 (3 proteins) were excluded from further analysis due to their small size and lack of biologically meaningful interaction networks. Functional annotation was performed on Module 1 (66 proteins) and Module 2 (13 proteins), which exhibited significant enrichment in pathways related to immune signaling, ECM remodeling, and inflammatory processes. Module 1 was primarily associated with Interleukin signaling (IL-4, IL-10, and IL-13), cytokine regulation, and ECM degradation, while Module 2 showed enrichment in neutrophil degranulation, innate immune response, and collagen degradation (Figure 2B).

Discussion

PROM refers to the spontaneous rupture of fetal membranes before the onset of labor at term (≥37 weeks of gestation). PPROM occurs before 37 weeks of gestation and is associated with significant maternal and neonatal complications, including preterm birth, neonatal sepsis, and increased perinatal morbidity and mortality.[2-4] MMP-9 is a member of the MMP family, which plays a critical role in ECM remodeling.[12] MMP-9 is primarily involved in degrading type IV collagen, a major component of the fetal membranes. Increased MMP-9 activity has been implicated in the weakening of the amniotic sac, contributing to membrane rupture. Its dysregulation has been associated with pathological conditions such as premature rupture of membranes, chorioamnionitis, and preterm labor.[13]

The pathophysiology of PROM and PPROM involves complex biochemical and mechanical processes. Inflammatory cytokines such as IL-6, IL-1β, and TNF-α upregulate MMP-9 expression, leading to ECM degradation and membrane weakening.[14] Increased reactive oxygen species (ROS) contribute to ECM degradation by stimulating MMP-9 expression. Microbial invasion of the amniotic cavity triggers an immune response, enhancing MMP-9 production and leading to premature membrane rupture.[15] Excessive mechanical forces on the fetal membranes may activate MMP-9, promoting ECM breakdown and membrane rupture.[16]

Our study demonstrated that MMP-9 expression was significantly elevated in PROM and PPROM placental tissues compared to the control group, with the highest expression observed in PPROM cases. These findings align with previous studies suggesting that excessive MMP-9 activity contributes to preterm membrane rupture and premature delivery. Studies have reported increased MMP-9 expression in preterm labor, particularly in cases associated with infection and inflammation. MMP-9 levels in amniotic fluid were significantly higher in patients with PPROM, correlating with microbial invasion and inflammatory responses. Furthermore, higher MMP-9 concentrations have been observed in fetal membranes of PPROM cases compared to term pregnancies, supporting the role of ECM degradation in preterm rupture.[17,18] In addition, our findings are consistent with the hypothesis that MMP-9-mediated degradation of type IV collagen weakens the fetal membranes, making them susceptible to rupture. This mechanism is exacerbated in PPROM due to persistent inflammatory stimulation and infection-associated upregulation of MMP-9.

The clinical significance of our findings highlights the potential role of MMP-9 as a biomarker for predicting membrane integrity and the risk of preterm birth. Elevated MMP-9 levels in placental tissues and amniotic fluid may serve as an early indicator of membrane weakening, guiding clinical decisions regarding patient management. Targeted Therapies: Inhibitors of MMP-9 (e.g., tissue inhibitors of metalloproteinases) may be explored as therapeutic agents to prevent premature membrane rupture in high-risk pregnancies. MMP-9 expression profiling in amniotic fluid or maternal blood could help identify women at risk for PROM and PPROM, allowing for early intervention strategies. Patients with elevated MMP-9 levels could benefit from close monitoring, prophylactic antibiotics (in cases of suspected infection), and corticosteroid administration to improve neonatal outcomes.[19,20]

Our bioinformatics analysis identified two key MMP-9-associated molecular clusters in PROM and PPROM, revealing distinct yet interconnected mechanisms. Module 1 was enriched in immune signaling pathways, notably interleukins (ILs) IL-4, IL-10, and IL-13, while Module 2 was primarily associated with neutrophil degranulation, innate immune activation, and collagen degradation. These findings align with our histological results, which showed progressively increased MMP-9 expression from control to PROM and PPROM, suggesting its role in extracellular matrix (ECM) remodeling and immune-mediated membrane weakening. The enrichment of cytokine signaling pathways in Module 1 implies that dysregulated immune responses may contribute to PROM and PPROM progression by promoting ECM degradation. Similarly, the pathways identified in Module 2 highlight the role of innate immune activation and proteolytic enzyme release in weakening fetal membranes. These observations are consistent with previous studies demonstrating MMP-9 upregulation in preterm labor, infection-induced inflammation, and preterm membrane rupture.[21,22] For instance, elevated MMP-9 levels have been detected in fetal plasma of cases with preterm PROM, implicating its role in membrane rupture mechanisms. Additionally, activated neutrophils have been shown to upregulate MMP-9 and prostaglandin E2 release in fetal membranes, contributing to their weakening.[18,22] Moreover, studies have shown that increased MMP-9 expression is associated with the degradation of type IV collagen in fetal membranes, leading to their weakening and rupture.[23] This suggests that MMP-9-mediated ECM remodeling plays a crucial role in the pathogenesis of PROM and PPROM. On the

other hand, polymorphisms in the MMP-9 promoter region have been linked to altered enzyme expression, potentially influencing individual susceptibility to PROM and PPROM.[24] Together, the integration of our modular network analysis with histological and literature-based evidence suggests that MMP-9 contributes to membrane weakening via IL-driven immune responses and neutrophil-mediated proteolytic activity. Moreover, its association with type IV collagen degradation and genetic polymorphisms further supports its involvement in membrane instability. Collectively, these insights highlight MMP-9 as a potential biomarker and therapeutic target, highlighting the importance of continued investigation into its regulatory pathways and potential therapeutic approaches.

This study provides valuable insights into the role of MMP-9 in PROM and PPROM through an integrated histopathological and bioinformatics approach. However, several limitations should be acknowledged. First, the sample size, while balanced across groups, remains relatively small, potentially limiting the statistical power and generalizability of the findings. Second, while the study utilized immunohistochemical methods to assess MMP-9 expression, quantitative assays such as ELISA or Western blotting could provide a more precise evaluation of protein levels. Third, the bioinformatics network analysis was based on publicly available databases and predicted interactions, which may not fully capture tissue-specific or gestational age-specific variations in protein-protein interactions. Additionally, functional validation of the identified molecular clusters and pathways was not performed, leaving open questions regarding the causal role of these pathways in membrane rupture. Future studies should aim to validate these findings in larger, multicentric cohorts and include in vitro or ex vivo experimental models to elucidate the functional consequences of MMP-9 modulation. Investigating upstream regulatory mechanisms and potential MMP-9 inhibitors may offer novel therapeutic avenues for preventing PROM and PPROM, especially in high-risk pregnancies. Furthermore, longitudinal studies examining the temporal dynamics of MMP-9 expression could improve understanding of its role in the progression from membrane weakening to rupture.

In conclusion, this study highlights MMP-9's pivotal role in PROM and PPROM pathogenesis, demonstrating its increased expression in placental tissues, particularly in PPROM cases, and its contribution to ECM remodeling and inflammatory activation leading to membrane rupture. A key novelty of this study is the integration of bioinformatics-driven modular network analysis, identifying the specific protein clusters through which MMP-9 exerts its effects. These clusters are primarily involved in immune signaling, neutrophil degranulation, and ECM degradation, shedding light on the molecular interactions underlying membrane weakening and preterm birth risk. Additionally, the findings support the hypothesis that excessive MMP-9 activity disrupts type IV collagen, making fetal membranes more vulnerable, especially in the presence of chronic inflammation and infection. By combining histological analysis with network-based profiling, this study offers a systems biology perspective, providing potential biomarkers and therapeutic targets for preventing preterm birth in high-risk pregnancies. These insights highlight MMP-9 as a crucial mediator in PROM and PPROM, emphasizing the need for further research into its regulatory mechanisms and therapeutic targeting to improve maternal-fetal health outcomes.

Ethical Approval

This study was conducted with the approval of the Non-Interventional Clinical Ethics Committee, Dicle University Faculty of Medicine (date 20.12.2023 and number 2023/6).

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

TK: Study design, hypothesis, in silico analysis, manuscript preparation; GA, MGBA, MK: Evaluation of molecular aspects, critical review of manuscript; HA, FA: Material preparation, experimental data analysis; EA and AA: Evaluation of clinical aspects, review.

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