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Preterm Prelabor Rupture of the Membranes: A Disease of the Fetal Membranes

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Abstract

Preterm prelabor rupture of the membranes (pPROM) remains a significant obstetric problem that affects 3-4% of all pregnancies and precedes 40% to 50% of all preterm births. pPROM arises from complex, multifaceted pathways. In this review, we summarize some old concepts and introduce some novel theories related to pPROM pathophysiology. Specifically, we introduce the concept that pPROM is a disease of the fetal membranes where inflammation-oxidative stress axis plays a major role in producing pathways that can lead to membrane weakening through a variety of processes. In addition, we report microfractures in fetal membranes that are likely sites of tissue remodeling during gestation; however, increase in number and morphometry (width and depth) of these microfractures in pPROM membranes suggests reduced remodeling capacity of membranes. Microfractures can act as channels for amniotic fluid leak, and inflammatory cell and microbial migration. Further studies on senescence activation and microfracture formation and their role in maintaining membrane homeostasis are needed to fill the knowledge gaps in our understanding of pPROM as well as provide better screening (biomarker and imaging based) tools for predicting women at high risk for pPROM and subsequent preterm birth.

Keywords

Fetal signals; parturition; exosomes; biomarker; aging; fetal membranes; amniochorion

Introduction

Preterm prelabor rupture of the membranes (pPROM) is defined as rupture of the fetal membranes prior to 37 weeks of completed gestation. This significant obstetric problem occurs in about 3-4% of all pregnancies and is directly antecedent to 40% to 50% of all preterm births^{1,2}. Since about 560,000 babies are born prematurely each year in the U.S. (~12.0% of all births), this correlates to about 150,000 spontaneous preterm births (sPTBs) that are complicated by pPROM³. The number of pPROM cases exceeds that of

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preeclampsia and gestational diabetes and other iatrogenic preterm births. In addition, neonatal mortality and morbidities are higher in pPROM group than any other subclasses of preterm births. Yet, pPROM is an often-ignored and understudied adverse outcome of pregnancy. Despite remarkable improvements in prenatal care over the past three decades, rates of pPROM and subsequent preterm delivery have worsened⁴.

While several tests are available to confirm a diagnosis of pPROM post facto (e.g. pooling, fern tests, nitrazine, and Amnisure®), no method to reliably predict pPROM is available. This dilemma is mostly attributable to the fact that precise causes or risk factors are unknown and pathways resulting in pPROM have only recently been delineated⁴. Empirical treatment approaches that ignore the complexity and heterogeneity of pPROM pathophysiology have to date been futile⁵. Proper diagnosis and management of pPROM is likely to require thorough investigation of specific exposure-induced pathophysiologic pathways and the development of biomolecular markers that can predict pPROM.

pPROM arises from complex, multifaceted pathways. Several epidemiological and clinical factors are considered precursors to pPROM. These include maternal reproductive tract infections (e.g., bacterial vaginosis [BV], trichomoniasis, gonorrhea, Chlamydia and occult chorioamnionitis), behavioral factors (e.g., cigarette smoking, substance abuse, poor nutritional status, and coitus during pregnancy), obstetric complications (e.g., multiple gestation, polyhydramnios, incompetent cervix, gestational bleeding, prior cervical surgery, and antenatal trauma)⁶⁻⁸. Environmental factors (e.g., stress, toxin exposure) and genetic predisposition also have been proposed. In addition, biochemical signals from the fetus, including endocrine signals that promote fetal membrane apoptosis, have also been implicated in the initiation of pPROM⁵⁻⁹. In this review, we summarize some old concepts and introduce some novel theories related to pPROM pathophysiology.

Fetal membranes: Structure and function

Human fetal membranes, also referred as placental membranes or amniochorionic membranes, is the inner lining of the pregnant intrauterine cavity. These fetal tissues are distinct from placenta and serves as a barrier between the fetoplacental and the maternal compartments. Fetal membranes are comprised of the amnion (innermost layer of the intraamniotic cavity) and the chorion (fetal tissue connected to maternal decidua), and are connected by collagen-rich extra cellular matrix (ECM)⁹. ECM, which is made up of fibrous proteins along with various types of collagen, provides the architectural and structural framework of the fetal membranes (Figure 1).^{9,10} The amnion is constantly bathed in amniotic fluid, signifying its importance as a primary responder to changes in the amniotic cavity. The chorion is in close proximity to maternal decidua and maintains the immune tolerance at the maternal-fetal interface.¹¹⁻¹⁴

The amnion and chorion are fetal tissues in origin and play major roles in maintaining pregnancy by providing multi-level protection to the growing fetus. Fetal membranes accommodate constant challenges (immune, structural, mechanical and endocrine) during pregnancy; continue to grow and mechanistically, as well as biochemically, maintains elasticity to the stretch forces experienced during fetal growth. Despite the fact that membranes overlaying the placenta and cervix face distinctly different environments and

insults during pregnancy, the membranes still maintain the homeostatic balance necessary to sustain fetal growth without interruption. This companionship between the fetus and the membranes continues until term when the fetus reaches maturity and the membranes reach longevity.

Development of fetal membranes

The development of amnion and chorion begins with embryogenesis, although they do not participate directly in the formation of the embryo or fetus.¹⁵ Like the fetus, early growth of the amnion and chorion layers is rapid and independent of each other. The formation of amniochorion as a unit structure is complete by the 12th week of gestation.¹⁵ The composition of the membrane and its ability to produce a broad spectrum of biomarkers at different stages of gestation illustrate the possible role of the fetal membrane's influence on the growing fetus, as well as in adverse pregnancy outcomes.¹⁰ In addition, fetal membrane cells continue to divide throughout pregnancy and its growth plateaus at term, retaining the capacity for DNA replication.¹¹ Lastly, the presence of stem cells in fetal membranes further depicts the likely important role of fetal membranes during in utero fetal life.^{9;12-14}

pPROM: A disease of the fetal membranes

Unlike the placenta, fetal membranes are not involved in transport of nutrients or other materials. One of the major functions of fetal membranes is to protect the fetus during its growth and development in utero. Specifically, the fetal membrane functions to provide mechanical^{16,17} and immune protection and acts as a barrier for microbial access¹⁸⁻²⁰. This protective role is supported by the biomarkers that are produced by fetal membranes during gestation and parturition²¹. Compromise in the immune and mechanical properties of the fetal membranes allows for microbial invasion from genital tract²², activation of host inflammatory response leading to collagenolysis mediated mechanical disruption^{16,23-25}, and membrane weakening predisposing the membranes to pPROM. Abruption associated thrombin, matrix metalloproteinase (MMP) activation and collagenolytic processes have also been reported in fetal membrane weakening and pPROM²⁶. Clearly, the dysfunctional status of fetal membranes is more evident in pPROM than sPTB with no ROM. Thus, pPROM is considered as a disease of the fetal membranes and likely a separate entity from sPTB with no ROM. Current research on this disease of the fetal membranes is focused on addressing three major questions: 1) what are the initiators of proteolytic activity in the fetal membranes resulting in pPROM, 2) what are the effectors (biochemicals) of proteolysis, membrane weakening/rupture and can these biochemicals serve as markers to predict risk of pPROM and 3) how can we reduce the risk of pPROM prior to its occurrence and minimize the impact on maternal-fetal wellbeing²⁷.

Risk factors for pPROM

Approximately 70% of pPROM cases are associated with intraamniotic infection (IAI), as documented by positive amniotic fluid (AF) cultures or by clinical evidence of infection^{4,28}. However, it has been debated whether infection is a cause or consequence of pPROM. Histological and microbiological findings indicate that focal infection and inflammation may play a primary or secondary role in the pathogenesis of pPROM²⁹. Evidence of inflammatory changes is reported to be adjacent to the putative site of membrane rupture,

suggesting that bacterial infection may be an initiator of pPROM¹. However, bacterial toxins and bacteria-derived matrix degrading enzymes have been discounted as a primary mediators of pPROM for the following reasons: 1) the concentration of bacterial toxins required to produce deleterious effects on isolated membranes are not achieved in AF; 2) pPROM is seen in women with IAI due to organisms that are not known to produce proteases; 3) most bacterial matrix degrading enzymes do not have the catalytic specificity to cleave human ECM collagens; and 4) pPROM has been documented in many cases after effective antibacterial therapy for periodontal disease, BV and IAI³⁰⁻³². Recent reports have indicated that pPROM may be associated with sterile inflammation in the fetal membranes (an inflammatory condition mimicking infection³³ with no evidence of microbial presence either through culture or molecular methods). We hypothesize that certain risk factors of pPROM (ie smoking, bleeding)³⁴, can produce sterile inflammation in the fetal membranes leading to an immunocompromised state that allows for microbial invasion. Infection in pPROM is likely a secondary phenomenon rather than a causal factor. In the remainder of this review, we will provide new perspectives in our understanding of fetal membrane dysfunction and pPROM (that involves factors other than infection-associated inflammation) and provide novel concepts to be tested in future research.

Oxidative stress (OS) and pPROM

One of the pathophysiologic factors that has been overlooked in pPROM is oxidative stress (OS) and generation of reactive oxygen species (ROS) as mediators of OS. Healthy pregnancy, like other physiological states, is characterized by a stable redox balance between reactive oxygen species (ROS) and antioxidants³⁵⁻³⁹. The two most common sources of ROS are leakage from the electron transport system in the inner mitochondrial membrane during cellular respiration and release by immune cells during phagocytosis. OS increases placental mitochondrial activity and production of ROS when the energy demands of the fetoplacental unit are high, such as high altitude pregnancy, antioxidant nutrient deficiency, or intrinsic microvascular disease. This results in an imbalanced redox state⁴⁰. Behavioral risk factors associated with pPROM, particularly cigarette smoking, generate superoxide, hydrogen peroxide, hydroxyl ions and nitric oxide that could damage the collagen matrix and consume antioxidant defenses⁴¹⁻⁴⁸. Collagen has been shown to be a primary target for ROS. Superoxide, stimulates the in vitro cleavage of fibrillar collagen to 4-hydroxyproline⁴⁹. Chorioamniotic collagenolytic enzymes are vulnerable to ROS activation⁵⁰, with MMP9 being suppressed by superoxide dismutase (SOD) or the glutathione precursor N-acetylcysteine. Lappas and colleagues have reported inhibition of MMP9 and other inflammatory markers using antioxidant N-Acetyl-cysteine (NAC)⁵¹. NAC inhibits an NF- κ B-activated pathway and subsequent phospholipid metabolism, proinflammatory cytokine release, and protease activity in human fetal membranes⁵¹. The evidence above indicates that OS and inflammation are major phenomena associated with pPROM. These events are initiated in response to exposures with dramatic biochemical consequences that can lower the threshold for membrane rupture.

Collagenolysis, extracellular matrix (ECM) degradation and rupture

Degradation of the collagen-rich ECM, connecting the amnion and chorion layers of the fetal membranes, is one of the key events leading to ROM⁵²⁻⁵⁷. This process is mediated by

several MMPs, each of which catalyze collagen type-specific substrate turnover. MMP activity is tightly regulated in the fetal membranes by transcription, translation and post-translational modification of MMPs and their endogenous inhibitors, the tissue-specific inhibitors of metalloproteinases (TIMPs)^{58,59}. A balanced MMP/TIMP system in human fetal membranes plays a role both during normal labor and in pPROM. Although studies have yet to clearly define how this balance is disrupted in pPROM, it is clear that proteases associated with infection or inflammation can activate endogenous MMPs in fetal membranes, leading to collagen turnover and membrane weakening^{23,60-65}.

Apoptosis of fetal membranes

Over a decade ago, fetal membrane apoptosis has been reported as a pathological mechanism associated with pPROM⁶⁶⁻⁷¹. This was further supported by the fact that elements of apoptosis are predominantly seen in membranes from women with pPROM than sPTB⁶⁷. Infection and other endotoxins are capable of inducing many of these proapoptotic factors during pPROM that are generally absent in membranes collected after sPTB with no ROM^{69,72,73}. Classic DNA fragmentation pattern (used in diagnosing apoptosis) has been reported in fetal membranes obtained from women with pPROM. However, apoptosis is not often associated with the massive inflammatory response that is seen in pPROM. In addition, DNA fragmentation (as shown by TUNEL assay or LmPCR) that is used as evidence of apoptosis can be seen with other forms of tissue/cell death such as necroptosis^{74,75}. OS also causes DNA fragmentation from fetal membrane cells⁷⁶. These factors raise ambiguity in the role of apoptosis as a pathologic mechanism in predisposing membranes to pPROM. It is likely that pPROM may have elements of all forms of cell deaths as inflammatory mediators can induce various forms of cell deaths depending on their kinetics and dose.

In summary, the roles of infection, inflammation, collagenolytic enzymes and apoptosis in pPROM have been independently characterized with the expectation that pathways responding to specific risk factors should indicate which initiators and effectors of pPROM are operative in any given case. However, to date, findings from such studies have not produced useful biomarkers or improvements in the rates of pPROM and sPTB. We will focus the rest of this review on some novel concepts that can mechanistically lead to pPROM.

Novel Concepts in pPROM

Phenotypic heterogeneity in studies that have evaluated pPROM is one of the major reasons why identifying a biomarker or clinical indicator that predicts pPROM has been elusive. For instance, sPTB and pPROM overlap in several ways; 1) etiologic similarities, 2) association between amniotic fluid and cervico-vaginal fluid genital mycoplasma colonization and infections and sPTB and pPROM, 3) infection-associated inflammation and biochemical markers determining underlying pathophysiology, 4) clinical and histologic evidence of chorioamionitis in cases of sPTB and pPROM. However, approximately 40% of women rupture their membrane prior to labor initiation whereas majority will not experience ROM. This suggests that although similarities may exist in etiologies and clinical presentations, the underlying mechanistic pathways leading to pPROM and sPTB with intact membranes are

distinct and therefore they should be considered as independent phenotypes. The following sections will review new evidences from our own laboratories that showed differences in biomarkers between these phenotypes and provide evidence for a novel concept on pPROM pathology. Based on basic and clinical evidences, pPROM is considered a disease of the fetal membranes²⁷. Therefore, we investigated changes to the fetal membranes in pPROM, sPTB with intact membranes and normal term pregnancies (regardless of labor status).

Oxidative stress in pPROM, sPTB and normal term birth

Using fetal membranes as a model, we examine inflammation and OS as underlying pathophysiologies for both pPROM and sPTB^{77,78}. Although inflammation and OS are inseparable components of innate immune mechanism, OS markers (antioxidants, OS induced damages and products of OS induced damages) are not readily investigated compared to pro-inflammatory biomarkers. OS biomarkers are hardly reported in the literature in association with both sPTB and pPROM⁷⁹. Using clinical specimens (amniotic fluid and fetal membranes) from these two conditions and in vitro model of fetal membrane organ explant system⁸⁰, we conducted studies to examine OS in these conditions and investigated the source and mechanisms of inflammation. We used normal term membranes as our comparison group and some of the findings from our report are described below.

Generation of ROS as a part of the aerobic energy building process is inevitable and is well balanced (redox status) by an array of enzymatic and non-enzymatic antioxidant systems^{40,77,78,81-84}. Redox balance is maintained through the production and subsequent elimination of ROS. Cells are able to protect themselves against OS by the finely tuned regulation of redox status through endogenous enzymes, antioxidants, and other cellular mechanisms. At low levels, ROS, often generated in biological systems, is essential for cell division and survival, cell signaling, inflammation and immune functions, autophagy, and stress response^{85,86}. However, an overwhelming redox imbalance compromises a biological system's ability to detoxify these highly reactive molecules or to repair any damage caused by them⁸⁵. Therefore, the former is termed "OS" and the latter "oxidative damage."³⁸ Oxidative damage due to ROS generation has been linked to the development of adult diseases, including cardiovascular disease, cancer, chronic inflammation, and neurologic disorders.

F2-Isop is a lipid peroxidation product and a biomarker of OS associated damages⁸⁷⁻⁸⁹. To determine OS damage in membranes and release of damaged cellular elements to the tissue environment or biological fluid, we measured F2-isoprostanes (F2-IsoP) in the amniotic fluid of women from pPROM, sPTB and normal term birth⁹⁰. We found that F2-Isop levels were significantly higher in women with pPROM and normal term birth compared to those who had sPTB with intact membranes⁹⁰. Similar increases in F2-IsoP were also reported by Longini et al⁸⁴. We also noted that women who smoked (OS inducer) during pregnancy (regardless of pregnancy outcome) also had elevated F2-IsoP. Cases with infection also had higher F2-IsoP; however, the levels were not so high as seen in women who smoked⁹⁰. This suggests that OS damage is a factor associated with pPROM and normal term birth.

Reduction of telomeres in pPROM and normal term birth

Telomeres are cap structures that protect chromosomal ends and their length is gradually reduced as cells divide and age⁹¹. Hence, telomere length is considered a measure of biological aging^{91,92}. Telomere length is reduced in response to OS as well⁹³. After documenting OS damages associated with pPROM and normal term birth, we tested the impact of stress on fetal tissue telomere length. Telomere length is not only an indicator of aging it is also a biomarker for OS damage as the guanine repeat sequences (TTAGGG repeats that dominates telomere cap structure) in telomeres are highly vulnerable OS damages. Telomere length analysis in fetal leukocyte DNA (cord blood) and fetal membranes obtained from women with pPROM showed significantly lower telomere length compared to gestational age matched samples obtained from women with sPTB with intact membranes, especially in samples < 34 weeks⁹⁴. Fetal membrane telomere lengths were also reduced in these conditions and correlated with leukocyte DNA telomere lengths⁹⁴. These data showing identical telomere lengths in pPROM and normal term births suggest that OS and OS associated telomere length reduction occurs in both conditions. As mentioned above, telomere lengths are indicators of biologic aging and this data led us to hypothesize that pPROM and normal term births are linked to OS and OS damages and these factors can accelerate fetal membrane aging. Aging of fetal membranes is likely a natural and physiologic event in normal term pregnancies; however, this process is likely accelerated in response to various OS inducing risk factors of pPROM.

Oxidative stress at term

The pregnant uterine environment, especially fetal tissues (ie placenta), progress through various states of oxidative status as the fetus matures⁹⁵⁻⁹⁸. Fetal and placental growth are linked to changing status of oxygen tension and OS in response to various oxidative conditions. ROS generated in response to cellular respiration are involved in cell signaling and growth. Therefore, OS status in the pregnant uterine environment plays a major role in fetoplacental growth and development^{97,99,100}. Once the fetal development is complete, OS and inflammation build up at term is a factor responsible for triggering parturition. Oxidative stress and presence of ROS rises at term due to following factors: 1) increased metabolic demands from the fetus who is ready for its independent existence outside the uterus⁷⁸, 2) depleted fetal antioxidant reserve in the intrauterine compartments¹⁰¹, 3) no change in supply of substrate to meet increased metabolic demands by the fetus^{102,103} and 4) uterine over-distension and fetal stretching¹⁰⁴. In preterm pregnancies, especially those complicated by pPROM, OS buildup can occur in response to risk factors such as infection, nutrient insufficiency (specifically antioxidants), behavioral factors (cigarette smoking, drug and alcohol abuse), and low or high BMI.

OS induces fetal membrane aging at term and in pPROM

Oxidative damage, as described above, affects the functioning of lipids, proteins and nucleic acids along with other cellular structures. In human fetal membranes, OS causes activation of p38 mitogen activated protein kinase (MAPK). p38MAPKs are a family of evolutionarily conserved serine/threonine kinases (mitogen activated protein kinases) that contains four stress response signaling isoforms (p38 α , β , γ , δ) whose functions differ

significantly^{105,106}. ROS-mediated phosphorylation and activation p38MAPK activates its downstream effectors, p16Ink4 and p19arf, resulting in cell cycle arrest and senescence¹⁰⁷⁻¹⁰⁹. Senescence is a mechanism that can predispose to cellular aging. Senescent cells are inflammatory and can persist in cellular environment to cause further damage to the tissues^{110,111}. Examination of fetal membranes obtained from women who underwent labor at term compared to women who did not labor at term showed increased OS³⁹ and induced p38MAPK mediated senescence¹¹².

Sterile inflammation (also known as senescence associated secretory phenotype – SASP) is a feature of senescence associated aging and this is characterized mostly by the presence inflammatory biomarkers, growth factors and matrix degrading enzymes¹¹⁰. SASP markers are known mediators of uterotonic activity at term and have been widely reported in the OB literature¹¹². In vitro, using amnion cells, we have shown that OS can transition a normal term not in labor membrane into a senescence stage and produce SASP. This transition, histologic and biochemical, is similar to that seen in term labor. Progressive activation of telomere-associated, p38MAPK-mediated development of senescence was also seen in murine pregnancy. Telomere length reduction is maximum in fetal membranes compared to all other tissues (placenta, and uterine decidua) that were tested suggesting that the fetal membranes play a major role in the readiness of parturition. In addition to SASP, senescent cells also release highly inflammatory damage associated molecular pattern markers (DAMPs). These are released in response to senescent associated tissue damage. High mobility group box (HMGB) 1, cell free fetal cell DNA and telomere fragments, uric acid are some of the DAMPs that have been linked to parturition¹¹³⁻¹¹⁵. We postulate that at term, senescence causes increased inflammation due to tissue injury and this enhances the overall inflammatory load in feto-maternal tissues to initiate labor-associated changes.

Although not proven mechanistically or in animal model studies, similar events are expected to happen in membranes prior to rupture. Experimental data has shown that membranes from women with pPROM exhibit the following when compared to membranes obtained from women with sPTB with intact membranes: 1) increased DNA damage and reduced telomere length^{94,116}, 2) reduced antioxidant capacity and increased oxidative stress^{84,90,117-120} and 3) increased p38MAPK activation and senescence.¹¹⁷ In addition, pPROM is associated with increased matrix metalloproteinase activity, a constituent of SASP that is required to degrade membrane II^{23,62,63,65,121}

In summary, several molecular and histologic factors are similar at term and pPROM supporting our hypothesis that term labor is associated with a natural and physiologic aging of the fetal membranes rendering them dysfunctional. Inflammation arising from an aging membrane can increase the overall inflammatory load of the uterine cavity. Premature aging of fetal membranes can also cause membrane dysfunction and preterm rupture. Table 1 summarizes the key factors that are common and different in fetal membranes between pPROM, sPTB and term birth. Figure 2 provides pathways in which fetal membranes play a role in normal term birth and pPROM. However, given the data to date, one question still remains, which is if sPTB with intact membranes does devoid of OS? The likely answer is that the extent of OS and OS damage are minimal in sPTB when membranes are intact. This may depend on the type and dose of exposure capable of inducing uterine OS. Lastly,

vulnerability of subject to specific risk factors and their innate response to control OS may determine their phenotypic outcome.

Microfractures of fetal membranes – sites of membrane architectural damage?

Recently, we examined fetal membrane cellular and collagen structures using nonlinear microscopy from term and preterm labor revealed development of microfractures in fetal membranes¹²³. In this study, the combined methods of multi-photon autofluorescence (MPAM) and second harmonic generation (SHG) were applied to investigate the multilayered 3-dimensional micro-organization of human fetal membranes without the use of exogenous contrast agents. Microfractures are areas of structural changes and are likely initiated by disturbances in the cellular layers. In our study, the following four criteria were used to identify and characterize microfractures: 1) contain altered amnion epithelial layer or site of epithelial shedding, 2) deterioration of basement membrane layer, 3) presence of migrating cells in the ECM portions of the membrane and 4) basement membrane damage and migrating cells develop tunnels in the ECM that extends from basement membrane through the spongy layer. In summary, microfractures are channels or tunnels that appear as extensions of areas often vacated by amnion cells due to shedding or topographically altered due to senescence. Microfractures extend through the spongy layer of the ECM thus connecting its contents with the sparse collagen of the reticular layer. This connection could potentially be considered as channels of cross talk between amnion and chorion layers throughout gestation and likely get resealed to facilitate tissue remodeling. The number of microfractures and their dimensions (wider and deeper) are significantly higher in fetal membranes from pPROM than gestational age matched sPTB tissues. We postulate that these microfractures are areas where tissue remodeling was insufficient or ineffective due to underlying pathological reasons or premature senescence. These regions are also associated with the large amounts of collagen degradation. Persistent microfractures can act as channels for amniotic fluid and inflammatory cell infiltration as seen in pPROM. Figure 3 shows an example of microfracture in pPROM membranes that have a 2.5 fold higher prevalence than membranes obtained from women with sPTB or preeclampsia.

Summary

pPROM is a disease of the fetal membranes where the inflammation-oxidative stress axis plays a major role in producing pathways that can lead to membrane weakening through a variety of processes. Recent data provide molecular evidences for aging of fetal membranes in response to OS (physiologically at term and pathologically at preterm) can cause a telomere dependent, p38MAPK signaler driven senescence activation of the fetal membranes. Senescence, a mechanism contributing to aging of fetal membranes, produce sterile inflammation that can cause further damage to fetal membranes leading to weakening and or rupture. In addition, we report microfractures in fetal membranes that are likely sites of tissue remodeling during gestation; however, increase in number and morphometry (width and depth) of these microfractures in pPROM membranes suggests reduced remodeling capacity of membranes. In addition, these fractures can act as channels for amniotic fluid leak, and inflammatory cell and microbial migration. Further studies on senescence activation and microfracture formation and their role in maintaining membrane homeostasis

are expected to fill more knowledge gaps in our understanding of pPROM pathology as well as provide better screening (biomarker and imaging based) tools for predicting women at high risk for pPROM.

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Figure 1. Characterization of Fetal Membranes with Non-Linear Optical Microscopy

A schematic of the fetal membrane layers (A) compared to the layers imaged by a two-photon laser using multiphoton autofluorescence (MPAM) (red) and second harmonic generation microscopy (SHG) (green) (B).

A: Schematic of the fetal membrane layers: The amnion epithelial layer (blue cells), constantly bathed in amniotic fluid, is a single layer of cuboidal cells held together by neighboring cells gap junctions. Amnion epithelial cells secrete collagen Types III and IV, and glycoproteins to form the basement membrane (orange) on their basal side. This forms a junction and allows communication between the two layers. Stromal cells (purple cells) secreting Type 1 and 3 collagens create the compact layer (orange stripes) and fibroblast layer forming a fibrous skeleton helpful in maintaining amnion integrity. The fibroblast layer is connected to the spongy layer made up of proteoglycans, glycoproteins and Type 3 collagen producing a spongy meshwork (black). The reticular layer, made up of fibrillar bundles of diffused collagen, is attached to the spongy layer on its apical side and the chorion (tan cells) on its basal side. The extra cellular matrix layers, filled with mesenchymal stromal cells, can interact with each other, degrade collagen layers, and communicate with cellular layers to help with the fetal membrane remodeling. The chorion (tan cells) is a typical epithelium layer, with its cell polarity facing the maternal side connecting to the decidua. The decidua (green cells), a maternally derived tissue, contains enlarged mesenchymal stromal cells and maternal immune cells that play a critical role in regulating inflammation at term. The maternal myometrium, is connected to the basal side of the decidua, and is made up of smooth muscle cells that form contractions around the fetus at the end of gestation.

B: MPAM/SHG: Multiphoton images of the fetal membranes show cellular content in red and collagen in green. Amnion cells are seen in a monolayer with their nuclei appearing darker than the cytoplasm. The basement membrane in green, mimics the outline of the epithelial cells and is connected to the organized/wavy collagen in the fibroblast layer. Elongated stromal cells in red are seen interspersed through the collagen. The spongy layer is made up of tightly packed fibular collagen and is attached to the reticular layer comprised of loose collagen and stromal cells (red). The extra cellular matrix averages around 80 μm in depth. The trophoblast cells are tightly packed close to the basement membrane of the extra cellular matrix and spread out as they approach the decidua. The decidua is made up of enlarged, elongated, mesenchymal cells with intermittent red blood cells.

Figure 2.

Similarities exist between normal term birth and pPROM. OS increase (factors are depicted in figure) seen towards term transition a non-labor phenotype into a laboring phenotype. Similarly, increase in intrauterine OS during gestation in response to various risk factors (see figure, *list is not complete*) can cause similar transition at preterm. Both these processes, culminate in similar pathways leading to telomere attrition, peroxidation of cellular elements and organelles resulting in senescence and senescence associated inflammation. The increased load of inflammation is linked to human parturition¹²². In pPROM, similar activation of these pathways occur and premature senescence leads to sterile inflammation, membrane dysfunction, loss or reduction in microbial defense capacity. This can lead to microbial invasion and infectious inflammation. Therefore, term parturition is likely linked to sterile inflammation but pPROM may have both sterile and infectious inflammation. This is hard to distinguish as biomarkers of both forms of inflammation are similar.

Figure 3. Characteristics of microfractures in term and preterm birth

A. Cartoon representation of a microfracture with a cell migrating through a collagen tunnel and a multiphoton image of a microfracture. Cells (red) collagen (green). 1) area of altered amnion morphology. 2) Multiphoton image of a degraded basement membrane (grey arrow) around an area of altered amnion morphology (white astric). 3) Multiphoton image of a cell in a collagen tunnel (grey arrow). Other stromal cells represented in this image are not in collagen tunnels. 4) IMARIS reconstruction of a collagen tunnel. B) Multiphoton image of TL tissue. 1) Amnion morphology above the microfracture. 2) Degraded basement membrane around the migrating cell of interest highlighted with yellow cross hairs. These cross hairs are constant throughout figure 3B. 3) Degraded collagen tunnel with cell migrating through the spongy layer. 4) Orthogonal view of a microfracture with migrating cell in degraded collagen tunnel. Non-Linear Optical Microscopy analysis of collagen degradation by second harmonic generation microscopy (SHG). C) PPROM clinical samples imaged with SHG visualizing areas of collagen degradation (white circles). These areas of collagen degradation, or microfractures, were reconstructed in blue as seen in 1,2, and 4 highlighting the depth, width, and location of microfractures. PPROM samples presented with wider and larger quantify of microfractures compared to SPTB and pre-eclamptic samples. D) SPTB samples only contained one true microfracture that was reconstructed and sliced as seen in images 1,2, and 4. The other blue area in image 4 may show the beginning of microfracture formation due to the indentation but not tunnel formation seen at this location. E) Preeclampsia samples also showed low number and smaller microfractures compared to PPROM samples. Regions of $360\mu\text{m} \times 260\mu\text{m}$ ($9.4 \times 10^4 \mu\text{m}^3$) were analyzed for each sample.

Table 1
Oxidative stress, senescence and inflammatory markers in pPROM, PTB and normal term births

Comparison between pPROM, spontaneous PTB with intact membranes and normal term birth				
Measure	Sample	Indication	pPROM vs PTB	pPROM vs Normal term birth
F2-IsoP	Amniotic fluid	Oxidative stress	Higher in pPROM	Same as pPROM
3-NT staining	Fetal membranes	Oxidative stress	Higher in pPROM	Higher in pPROM
Salivary proteases	Maternal saliva	Proteolysis	Higher in pPROM	Same as pPROM
MMPs/TIMPs	Amniotic fluid	Proteolysis	Higher in pPROM	Higher in pPROM
Cytokines (IL-1, TNF, IL-6, IL-8)	Amniotic fluid	Inflammation	No Difference	Lower in normal birth
Telomere length	Fetal DNA	Senescence	Higher in PTB	Same as pPROM
p38MAPK	Fetal membranes	Senescence	Higher in pPROM	Same as pPROM
p53	Fetal membranes	Senescence	Higher in pPROM	Same as pPROM
Ras-GTPase	Fetal membranes	Signaling	Higher in PTB	Same as pPROM