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Fetal membrane architecture, aging and inflammation in pregnancy and parturition

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ABSTRACT

Preterm birth is the single major cause of infant mortality. Short and long term outcomes for infants are often worse in cases of preterm premature rupture of the fetal membranes (pPROM). Thus, increased knowledge of the structure characteristics of fetal membranes as well as the mechanisms of membrane rupture are essential if we are to develop effective treatment strategies to prevent pPROM. In this review, we focus on the role of inflammation and senescence in fetal membrane biology.

1. Introduction

Human fetal membranes (placental membranes or amniochorionic membranes) is the innermost tissue layer that forms the intrauterine cavity [1,2]. Fetal membranes are comprised of amnion (innermost layer of the intraamniotic cavity) and chorion (fetal tissue connected to maternal decidua) connected by collagen-rich extracellular matrix (ECM) containing amnion mesenchymal cells [1,3,4]. ECM, made of fibrous proteins embedded in a polysaccharide gel along with various types of collagen, form the architectural framework and various collagen types provide structural framework of the fetal membranes [3–5]. The amnion component is continuously rinsed in amniotic fluid [6], signifying its importance as a primary responder to changes in the amniotic cavity. The chorion is in close proximity to maternal decidua [7] and maintains the immune tolerance at the feto-maternal interface (Fig. 1). As a single unit structure, amniochorion functions as one of the major fetal tissues that support and protect fetoplacental growth to maintain pregnancy [8]. In this review, we will summarise the growth and microfractures of the fetal membranes. Additionally, this review will highlight two major concepts related to the function of fetal membranes during pregnancy – senescence and inflammation - and their role in parturition.

2. Fetal membrane growth and microfractures

Amnion and chorion takes distinct growth trajectory upon embryogenesis [9]. The development of amnion and chorion begins with embryogenesis although they do not participate in the formation of the embryo or fetus [10–12]. Like the fetus, early growth of the amnion and chorion layers is a rapid process and independent of each other [10]. The formation of amniochorion as a unit structure gets completed between 14th – 17th week of gestation [13]. The fetal membranes are remodeled throughout gestation to accommodate the increasing volume of the intrauterine cavity. Fetal membrane remodeling occurs both at the cellular and matrix level. Matrix metalloproteinase (MMP) mediated ECM degradation is a well balanced inflammatory process where MMPs and their specific inhibitors in tandem achieve matrix turnover [14,15]. However, cellular level turnover is hardly studied as it is impractical to examine fetal membranes until after delivery. Recent findings from our laboratory has shown that fetal membranes are not always intact, specifically the highly elastic amnion membrane [16,17]. Amnion membrane is a single layer of cuboidal epithelial cells connected to the ECM via a Type IV collagen-rich basement membrane [3,4]. Due to the shedding of amnion epithelial cells, gap formation similar to that observed in the gut epithelial lining has been identified in the amnion epithelial layer [4]. These sites were associated with

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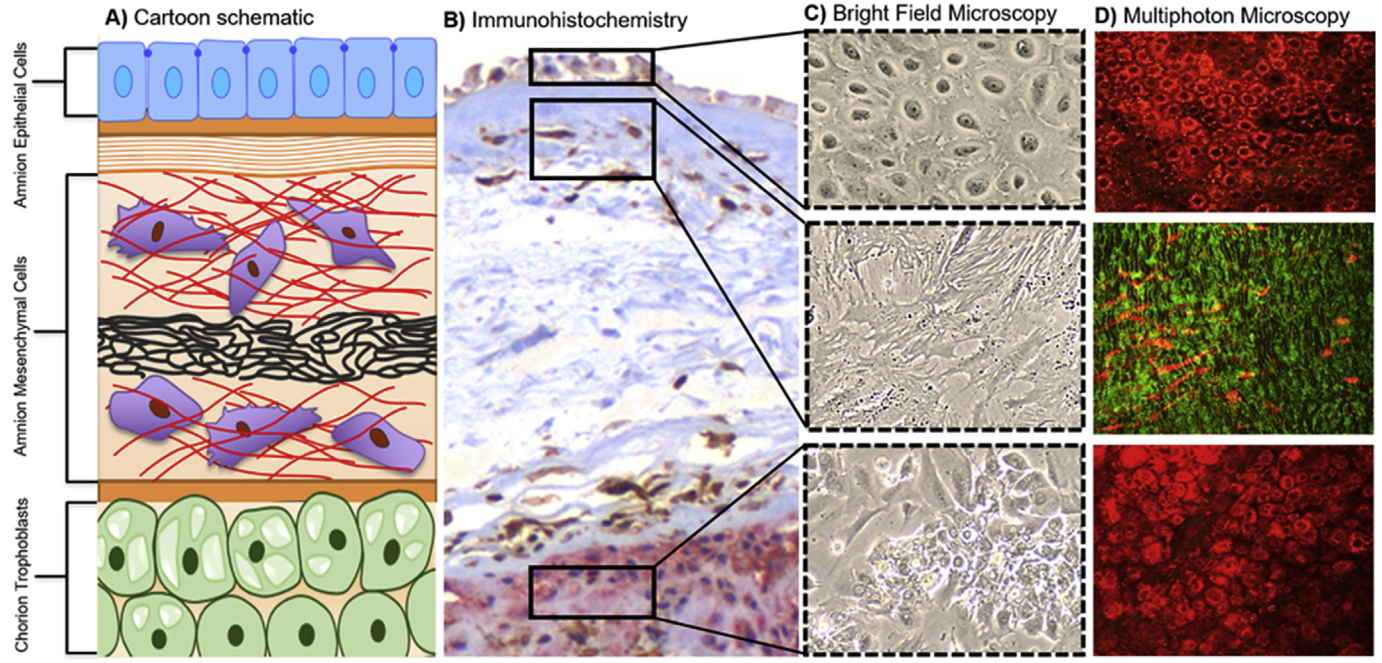

Fig. 1. Fetal Membrane structure and characteristics. **A) Proposed structures of fetal membranes**. The description starts from the innermost layer (amnion) and ends at the initial layer of chorion. Amnion epithelial cells (blue) are connected to the first layer of the extracellular matrix termed the basement membrane (orange)/ compact layer (orange strips). The fibroblast (top red), spongy (black), and reticular layers (bottom red) follow, containing amnion mesenchymal and stromal cells (purple). The chorion (green) is connected to the extracellular matrix through a pseudo-basement membrane (orange). The chorion is made up of two types of cells: (1) chorion leave cells that contain vacuoles (light green) and (2) the chorion trophoblast cells. The chorion interfaces with the maternal decidua, connecting the fetal to the maternal compartments of the uterus. **B)** Immunohistochemistry staining of fetal membranes for comparison of cellular and collagen layers. **C)** Bright flied microscopy of human fetal membrane primary cells reveling morphology in culture. Amnion and chorion epithelial cells express cuboidal epithelial morphology, while amnion mesenchymal cells in the extracellular matrix demonstrated elongated fibroblastoid shape with the presence of actin filaments. Images were captured at 10x and cropped. **D)** Multiphoton autoflorescent microscopy and second harmonic generation microscopy single-plain images confirm cellular and collagen morphology. Cellular components-red, Collagen-green. Images were captured at 25x and cropped.

areas of collagen degradation that extend through ECM potentially forming channels for cells and amniotic fluid. These biological structural aberrations in the membranes are termed as microfractures [16–18]. They are characterized by 1) altered amnion epithelial layer or site of epithelial shedding, 2) deterioration and damage of the basement membrane 3) presence of a migrating cells in the ECM 4) tunnels in the ECM that extends from basement membrane through the spongy layer [17]. Number of microfractures are similar between term not in labor and term labor fetal membranes. However, their morphometric measures (width and depth) were higher in term labor. In vitro experiments showed that morphometric changes seen at term labor membranes can be induced in not in labor fetal membranes by oxidative stress treatment [17]. Although microfractures are visible on fetal membranes delivered preterm (gestational ages between 28 and 34 weeks) due to maternal indications like preeclampsia (PE), their numbers and morphometries are smaller than those seen in membranes from gestational age-matched preterm premature rupture of the fetal membranes (pPROM) [17]. This is indicative of the existence of biologic fractures during gestational periods and not just at term. pPROM is termed as a disease of the fetal membranes whereas membrane pathology in PE is hardly reported. It is likely that an increased number of microfractures may be predisposing the membranes for rupture. Additionally, we have reported similarities between term labor and pPROM membranes and microfracture biology further supports that concept [17].

3. Understanding microfracture biology and its role at term and preterm parturition

Although the discovery of microfractures are rather new, based on their numbers and morphometry we have hypothesized microfractures are cellular and matrix-associated aberrations that contribute to membrane integrity and function. To test whether these microfractures are sites of tissue remodeling, in vitro scratch assays were performed using amnion epithelial cells (mimicking microfractures). Data demonstrated that pluripotent amnion epithelial cells are capable of healing these scratches [19] and we postulated that microfractures are likely sites of cellular turn over and transitions required for remodeling of the membrane cells. Gaps created by cellular exfoliation is likely healed by the transition of shed epithelial cells into mesenchymal cells (epithelial-to-mesenchymal-transition – EMT) that are more migratory and produce localized inflammation that eventually heals biological fractures. We further noted that oxidative stress prevents scratch healing and maintain amnion epithelial cells at a mesenchymal pro-inflammatory state [19]. This further supports our findings in term labor and pPROM membranes as both these conditions are associated with oxidative stress in the amniotic cavity and amniochorion membranes. A balanced redox system exists in the intrauterine cavity during gestation. As shown by Burton et al., hypoxic first and third trimester and hyperoxic second trimester produce distinct species of reactive oxygen species required for fetoplacental growth. Balanced redox activity maintains membrane integrity and likely remodel them through controlled cellular transition processes with localized inflammation. Overwhelming oxidative stress and an imbalance in reactive oxygen radicals can prevent cellular transitions, sustain membrane microfractures, and produce an inflammatory collagenolytic process capable of promoting parturition. On the contrary, in women with pPROM it is a pathologic event in response to various risk factors where persistence of microfractures without remodeling of the site can contribute to premature weakening.

In summary, remodeling of fetal membranes occurs throughout gestation and this tightly regulated process involves both cellular level transitions and migration to assist remodeling gaps in the membrane as

well as restructuring degraded collagen. Membranes contribute to parturition at term when oxidative stress diminishes cellular capabilities to remodel biologic microfractures. These sites are susceptible to inflammatory changes further weakening the membranes. This process emphasizes fetal membranes' contribution to uterine inflammatory pool required for promoting fetal delivery.

4. Senescence of fetal membranes

Discovery of microfractures were made while studying yet another biologic process associated with aging of the fetal membranes. Generation of balanced inflammation maintains membrane integrity through controlled remodeling whereas persistence of inflammation either due to physiologic (term) or pathologic (preterm) can be considered as fetal/host inflammatory response causing membrane weakening, rupture [16]. The inflammation observed in fetal membranes is 'sterile inflammation' [20] during normal pregnancy that are physiologic host response whereas the contributors of inflammation in preterm membranes are often 'infectious' [21] in response to microbial invasion of the amniotic cavity. Sterile inflammation is also reported in the absence of infection in pregnancy pathologies in response to various non-infectious risk factors [20]. Examination of sources of sterile inflammation in normal pregnancy membranes led us to identify senescence in fetal membranes [22]. Senescence is a mechanism associated with biologic aging due to active cell growth in fetal membranes [23,24]. Proliferation of cells during tissue remodeling contribute to telomere-dependent replicative senescence [25]. Telomeres are DNA cap structures that protect chromosomal ends and reduction of their length is considered as a marker of biologic aging [26]. Examination of fetal membranes at various stages of gestation, we have reported a progressive decrease in telomere length in both humans and animals [26–31]. Oxidative stress at term can accelerate this process and therefore the loss of telomere length is at its peek at term [8,25,31]. This reduction and aging of membrane corresponds with fetal growth and fetal membrane aging can be considered as a biologic mechanism indicative of fetal growth. Senescent cells are unique in generating specific inflammatory mediators (senescence-associated secretory phenotype – SASP [sterile inflammation]) [23,32] and these include cytokines, chemokines, growth factors, matrix-degrading enzymes and various other biochemicals. Senescent cells are not dead cells and therefore they can persists in their tissue environment and accelerate the senescent process in a feedforward fashion. Experimental evidence suggest that SASP as well as damaged cellular factors (damage-associated molecular pattern markers (DAMPs) released from senescent cells such as high mobility group box (HMGB)1 proteins, uric acid, and cell-free telomere fragments are capable of enhancing senescence [20,31]. Senescence and sterile inflammation mediated by SASPs and DAMPs are indicators of fetal membranes aging and enhance the overall inflammatory status of the uterine cavity. As mentioned above, fetal membranes aging corresponds with fetal growth and development. At term, inflammatory mediators released from fetal membranes are considered as yet another signaling mechanism the fetus utilizes to indicate its maturation and readiness for delivery. We have shown that senescent fetal membrane cells can propagate sterile inflammatory signals to maternal decidua, myometrium and cervix via paracrine signalers like extracellular vesicles, specifically exosomes, to increase inflammation in maternal tissues to cause parturition associated changes [33]. Thus, fetal membranes play a major role in contributing to parturition process. Examination of pPROM membranes indicated signs of premature senescence activation (or accelerated aging of membranes) prior to term to oxidative stress-inducing risk factors [34,35]. In vitro, we have shown that infection, inflammation, and environmental and behavioral risk factors can accelerate cell senescence in fetal membranes [20,21,24,36]. Thus, senescence-associated cellular level changes in fetal membranes contribute to an inflammatory process by not only weakening the membranes but disabling their functional capabilities. These changes in fetal membrane cells at term depicts their longivity and can be considered as mechanism to signal delivery of the fetus.

5. Inflammation: a heterogeneous condition in fetal membranes

As mentioned in the above paragraphs, inflammation is critical for membrane homeostasis during gestation as well as to signal membranes dysfunctional status to the mother to initiate birthing process. The latter could be normal (term) or abnormal (preterm). To note, the signature of inflammation, i.e. biochemical markers involved in inflammation is not homogeneous and in fact it is unique under different conditions. Overall an inflammatory imbalance is evident in membranes and even that imbalance during pregnancy (to remodel membranes) and at parturition are distinct. Inflammatory mediators triggering normal term parturition and preterm parturition are also different. The Menon laboratory has demonstrated that inflammatory profile is different based on type of risk. In summary, inflammation is a heterogeneous process during pregnancy and parturition. Understanding inflammatory markers is a critical tasks for several reasons: 1. Understanding the underlying physiology or pathophysiology associated with pregnancy 2. Development of membrane based biomarkers to predict fetal status during normal and abnormal pregnancy and 3. To develop intervention targets to minimize the risk generated by inflammatory imbalances. The next segment is dedicated to highlight several inflammatory markers and their potential functional contributions in normal and preterm pregnancies.

6. Inflammation of fetal membranes

In order to understand the physiology of membrane rupture, it is first necessary to identify the rupture site. Bourne [37] was the first to attempt to identify the rupture site. He transcervically marked the area of the fetal membranes over the cervix during the early stages of labour. After their spontaneous rupture, this area was always detected within the final rupture line. Seminal studies by Bell and colleagues [38] mapped the fetal membranes obtained post labour. These studies were able to identify an area of "high morphological change" in the region of fetal membranes along the line of rupture. Later, they were able to show that this area was also present in pre-labour fetal membranes. Subsequent studies by our group [39] and Moore and his team [40] have been able to identify this site in pre-labour fetal membranes using a similar technique to that described by Bourne in 1962 where a dye is transcervically applied to the chorion-facing fetal membranes before elective caesarean delivery at term. After delivery, samples of fetal membranes are obtained from the supracervical site, where the membrane was marked by the dye (approximately 8 cm diameter) and compared with samples from a distal site (2 cm from the placental edge). This site is referred to as fetal membranes overlying the cervix, zone of altered morphology, supracervical site or the para-cervical weak zone. Collectively, these study demonstrate that fetal membranes obtained from the area overlying the cervix have altered morphology as evidenced by increased connective tissue thickness (swelling), decreased thickness of the cellular layers, thinning or absence of decidua, and reduced tensile strength. Biochemical changes associated with this site include decreased membrane collagen content, and increased matrix degrading enzymes, apoptosis, inflammatory markers, and oxidative stress.

With respect to inflammatory markers, our studies have shown that pre-labour supracervical fetal membranes are characterised with increased expression of nuclear factor kappa B (NF-κB) and its transcriptional cofactor proteins CBP and p300 [41] and mitogen activated protein kinase (MAPK) proteins p-JNK and p-p38 MAPK [42] when compared to fetal membranes obtained close to the peri-placental edge. Furthermore, our studies have also shown that this site is associated with increased expression of pro-inflammatory cytokines and

chemokines and these changes are even more pronounced in post-labour versus pre-labour fetal membranes (Lappas, unpublished).

7. Targeting inflammation to prevent fetal membrane rupture

Research into membrane rupture is limited because animal models fail to mimic the human condition and because cell culture studies do not address adequately the major tissue changes that are associated weakening and rupture of the fetal membranes. However, in vitro fetal membranes explant models have been helpful in elucidating the role of inflammation in fetal membrane rupture. Pro-inflammatory cytokines such as TNF and IL1B can induce the biochemical and histologic tissue changes that mimic those seen in the physiologic weak zone in the fetal membranes region overlying the cervix [42,43]. They can also markedly weaken the fetal membranes [43]. Bacterial/viral products and pro-inflammatory cytokines, have all been shown to induce the expression of pro-inflammatory cytokines, prostaglandins and enzyme degrading enzymes in fetal membranes, which all contribute to the rupture of membranes. In all, these models can recapitulate aspects of inflammation and/or membranes weakening associated with the zone of altered morphology. Studies by the Lappas group have used these models to study if polyphenols – bioactive components of plants – can prevent fetal membrane weakening pathways and/or inflammation associate with membrane rupture. Our studies have shown that numerous polyphenols have been shown to prevent inflammation in fetal membranes. Resveratrol, a stilbene, found largely in the skins of red grapes, decreases LPS-induced pro-inflammatory cytokines and prostaglandin secretion in human fetal membranes [44]. Various flavonoids have also been shown to reduce LPS- and/or IL-1β-induced IL-6 and IL-8 mRNA expression and secretion, COX-2 mRNA expression, prostaglandins PGE_2 and $PGF_{2\alpha}$ secretion, and MMP-9 expression and activity in fetal membranes or primary amnion cells [45–49]. These polyphenols include, naringenin (flavanone from grapefruit), apigenin (flavone from parsley and chamomile), luteolin (flavone found in high amounts in parsley, thyme, peppermint, basil, herb, celery, and artichoke), kaempferol (flavonol found in apples, grapes, broccoli, cabbage, kale, beans, tomatoes and strawberries), nobiletin (flavone that abundantly exist in the bitter, white pith beneath peels of citrus genus and in smaller amounts in the juices of these fruits), silibinin (flavonolignan derived from milk thistle the main component of milk thistle) and honokiol (a lignan, derived from the bark, leaves and seed cones of the Magnolia officcinalis tree). Notably, nobiletin [47] and silibinin [48] were also able to reduce the expression and production of pro-inflammatory cytokines and MMP-9 in fetal membranes taken from women after spontaneous preterm birth.

Studies from our group have also identified a few proteins/pathways that play a role in regulating inflammation in fetal membranes. These include SIRT1 (Sirtuin 1), SLIT3, the inflammasome, endoplasmic stress proteins, cIAP1 and 2 (cellular inhibitors of apoptosis protein), MAPK proteins, GSK3 (glycogen synthase kinase), AMPK (AMP-activated protein kinase) and PIM1 kinase (proviral integration site for moloney murine leukemia virus). Our studies have shown that these signaling molecules have altered in expression in fetal membranes with labour, pPROM and with preterm chorioamnionitis. We have also shown that targeting these proteins using pharmacological agents can inhibit the pathways involved in rupture of membranes. While some of these pharmacological agents are approved for use in humans, there is a paucity of data on their safety in pregnancy.

The expression of the endoplasmic reticulum (ER) proteins GRP78, IRE1, and spliced XBP1 (XBP1s) are significantly increased in fetal membranes after term and preterm labor compared to non-laboring samples [50]. Furthermore, increased expression of ER stress proteins was also observed in fetal membranes treated with bacterial endotoxin LPS. The chemical chaperones 4-phenylbutyric acid (4-PBA) and tauroursodeoxycholic acid (TUDCA) ameliorated the increase in LPS-induced pro-inflammatory cytokine and chemokine expression and secretion from fetal membranes and IL-1β-induced MMP-9 expression and secretion of pro MMP-9 from primary amnion cells [50]. These findings suggest that the ER stress pathway may be a potential therapeutic target for the treatment of preterm birth. Although the U.S. Food and Drug Administration has approved the use of both 4-PBA and TUDCA as therapeutics in humans, there are no studies on the use of ER stress inhibitors in pregnant women.

Glycogen synthase kinase 3 (GSK3) activity is increased in fetal membranes after term and preterm labour [51]. In fetal membranes, the GSK3 inhibitor CHIR99021 significantly attenuated TNF-α, IL-1β, IL-6 and IL-8 mRNA expression and secretion induced by the bacterial products and TLR ligands LPS (TLR4 ligand), fsl-1 (TLR2/6 ligand) and flagellin (TLR5 ligand) [51]. The involvement of GSK3 as a regulator of inflammation makes inhibition of GSK3 using synthetic compounds an attractive avenue of research.

In fetal membranes, adenosine monophosphate (AMP)-activated kinase (AMPK) activity is decreased with term, but not preterm, labour [52]. Notably AMPK activity was decreased in preterm fetal membranes with PROM compared to intact membranes. In fetal membranes, the AMPK activators AICAR, phenformin and A769662 significantly decreased IL-6 and IL-8 mRNA expression and secretion stimulated by LPS, flagellin and the viral dsRNA analogue and TLR3 ligand poly(I:C) [52]. Additionally, in primary amnion cells, treatment with AMPK activators significantly decreased IL-1β-induced MMP-9 expression [52]. Interestingly, metformin which is a known AMPK activator, is safe to use in pregnancy and should also be investigated as a potential therapeutic.

Proviral integration site for Moloney murine leukemic virus 1 (PIM1) kinase expression is significantly increased in fetal membranes after spontaneous term labour and in amnion with preterm chorioamnionitis [53]. In human fetal membranes, the PIM1 inhibitors SMI-4a and AZD1208 significantly decreased the expression and secretion of the pro-inflammatory cytokine IL-6 and the chemokines IL-8 and MCP-1 mRNA and release, the secretion of PGF2α, and the release of the oxidative stress marker 8-isoprostane when stimulated with the bacterial products LPS or flagellin [53]. The crucial role of PIM1 kinase in regulating pro-inflammatory and pro-labour mediators makes it an attractive target. Noteworthy, numerous PIM1 inhibitors are currently in clinical trials of various cancers including hematologic malignancies [54].

8. Conclusions and introduction of the fetal membrane club

Fetal membranes are an integral component of the intrauterine cavity providing structural support for the growing fetus until their disruption at term. As described above, research into fetal membranes is often restricted to tissues obtained at term after vaginal deliveries or cesareans that has undergone several structural and mechanical disruptions. This has led to a stigma in the reproductive biology field that fetal membranes are a “dead tissue” or a “difficult to study tissue” to study. Fetal membranes are often dubbed as a part of placenta that has also mislead reproductive scientists from studies specifically utilizing fetal membranes. Fetal membranes are indeed called placental membranes but they are just “companions” of placenta and partly attached to placenta. Membrane cells may have origin linked to placental development but fetal membranes are structurally and functionally distinct from placenta. These perceptions have often led to difficulties for researchers in fetal membranes in getting funding or publishing innovative work in high impact journals, and most importantly projecting the contributions of an essential organ in pregnancy and parturition. This ignorance has been obscuring our understanding of membranes’ contributions in pregnancy and parturition. pPROM, a disease where membranes pathology is a major contributor, is hardly studied and it complicates 40% of all spontaneous preterm births. To note, the usefulness of postpartum fetal membranes that are stem cell rich have been well recognized by scientific community outside of reproductive and

perinatal biology. Membrane cells are effectively utilized by tissue engineers and regenerative medicine investigators for various purposes highlighting the importance of these cells [18,55–62].

To address these issues and to create an awareness on significance of membranes, an interest group promoting fetal membrane research called 'Fetal Membrane Club (FM Club)' has been created. The FM Club is a group of fetal membrane research enthusiasts interested in projecting and promoting fetal membranes' significance and importance to the Perinatal and Reproductive Biology world. Additionally, it is a forum to nurture friendship, insightful discussions, collaborations to support each other's work and most importantly have some fun while engaging in lifesaving research. The first meeting of the FM Club took place at the Society for Reproductive Investigation in 2018 where representative were elected to help facilitate meetings and a website was created to generate awareness of membranes' significance, promote for fetal membrane research and advocate for more funding from various global agencies. This inclusive club is open for all interested people in every areas of fetal membrane research.

This website (www.fetalmembraneclub.org) will continuously provide updates on fetal membranes' importance during pregnancy, labor, delivery and recent research development from World's experts. Additionally, this website is designed to facilitate research interactions and promote multinational collaboration of clinicians, research scientists, and students engaged in fetal membrane related research. Interactive forum section is open irrespective of topic of interest to post questions and blogs to report on current finding.

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