Investigation of correlation of biochemical marker expression and mechanical properties of preterm membranes

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Abstract:

The fetal membrane surrounds and supports the developing fetus during the pregnancy. The cellular structure of the fetal membrane and the proteins synthesized by the cells of amniotic and chorionic membranes, maintain the mechanical integrity of the tissue membrane. Rupture of the fetal membrane is a fundamental stage in normal term delivery. However, preterm premature rupture of membranes (PPROM) leads to preterm birth, which remains a major concern worldwide as the largest contributor of perinatal and neonatal mortality and morbidity. The underlying mechanism of PPROM is complicated and requires further investigation. The aim of this project is to newly reveal the underlying mechanisms of PPROM through the study of mechanical and biochemical properties of fetal membranes in both full term and preterm via new experimental protocols. With ethical approval, 42 fetal membranes were collected for this study from women who had full term normal vaginal delivery, preterm (less than 37 weeks) vaginal delivery (PPROM), full term and preterm delivery via caesarean sections. Mechanical characterisation was conducted for fetal membrane from full term and PPROM normal vaginal deliveries. Two new mechanical test techniques were established. Ball indentation test and Optical Coherence Elastography, were applied for the first time to study fetal membrane comprehensively in ruptured, non-ruptured areas and at different sublayers using optical coherence tomography for the thickness study. Biochemical assays; histological and immunological staining, plus Western Blotting were performed to study the microstructural and biochemical molecular (Collagen, sGAG, MMP 9 and 13, fibronectin, IL-1β & progesterone receptors) changes on the same categories of samples. Furthermore, this study investigated the changing biochemical molecule expression levels of the membranes in response to the applied external mechanical stimulation and maternal risk factors (smoking and maternal hypoglycaemia). In addition,

mechanotransduction signalling molecules (such as, nifedipine) and anti-inflammatory agent (dexamethasone) response was also studied in a new biomimetic experimental set up which was designed and implemented in full term and preterm fetal membranes from caesarean section deliveries. The mechanical test results revealed new evidence of noticeable difference between full term and preterm membranes. Preterm membranes showed great heterogeneity between ruptured and non-ruptured sites (creep property: 36%, modulus: 55%, thickness: 48%, collagen content: 59%, sGAG: 48%, and MMP 9: 57%) in comparison to their full term counterparts (creep property: 15%, modulus: 33%, thickness: 23%, collagen content: 24%, sGAG: 27% and MMP9: 23%). For the first time, a clear correlation between biomechanical and biochemical relations in full term and preterm was drawn. The *in vitro* loading model for the study of biochemical behaviours of fetal membrane in the presence of external force implied that cyclic force, either from fetal movement or fetal fluid can accelerate the synthesis of matrix degradation enzymes (MMPs) or trigger inflammation marker expression, leading to the weakening of the membranes. Preterm membrane was more sensitive to the force and biochemical environment than full term membrane, which might lead to premature rupture accidently. Downregulation of these biomarkers' expressions by addition of nifedipine during culture suggested that the calcium signalling pathway may play an important role in mechanotransduction in fetal membrane cells. In addition, maternal smoking correlated with membrane weakness; however, diabetes did not show any direct correlation with it under the influence of external force in the current experimental model. Finally, the addition of dexamethasone in the cyclic loading environment decreased expressions of membrane weakening biomarkers, which reinstated dexamethasone's beneficial effects on the prevention of fetal membrane rupture.

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ABBREVIATIONS:

BMI	Body mass index
CA	chorioamnion
CCD	Charge coupled device
COL	collagen
CS	Caesarean section
CX43	Connexion 43
DAPI	4',6-diamidino-2-phenylindole
DM	Diabetes Mellitus
ECM	Extra cellular matrix
Fn	Fibronectin
FM	Fetal membrane
GAGs	Glycosaminoglycan
IL	Interleukin
MMPs	Matrix metalloproteinase
OCE	Optical coherence elastography
OCT	Optical coherence tomography
OD	Optical densities
PPROM	Preterm Premature Rupture of Membrane
PR	Progesterone receptor

Publications:

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Sudeshna Bhunia, Shaughn O'Brien, Pensee Wu, Ying Yang. Induction of expression change of biochemical molecules in fetal membranes by external force and maternal risk factors. In preparation.

Conference Presentation (Oral)

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Sudeshna Bhunia, Shaughn O'Brien, Pensee Wu, Ying Yang. Investigation of the Mechanism in Preterm Premature Rupture of Membrane (PPROM). Obstetrics and Gynaecology meeting, University Hospital of North Staffordshire. UK, April 2016.

Sudeshna Bhunia, Shaughn O'Brien, Pensee Wu, Ying Yang, Correlation of biochemical markers expression and abnormal mechanical property in preterm membrane. Annual ISTM symposium. Staffordshire, UK, May 2017.

Sudeshna Bhunia, Shaughn O'Brien, Pensee Wu, Ying Yang, Investigation of the Mechanism behind Preterm Premature Rupture of Membrane (PPROM) Annual ISTM symposium. Staffordshire, UK, May 2018.

Conference presentation (Poster)

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Sudeshna Bhunia, Shaughn O'Brien, Pensee Wu, Ying Yang. Preterm Premature Rupture of Membrane (PPROM). Health Research Conference, Staffordshire University. October, 2016.

Sudeshna Bhunia, Shaughn O'Brien, Pensee Wu, Ying Yang. Investigation of Mechanism underlying Preterm Premature Rupture of Membrane (PPROM). Bell Blair Research society and Royal College of Obstetricians and Gyanaccologists, London, UK, 2nd March 2017.

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Chapter 1

Introduction

1.1 Background:

Human fetal membranes originate from fetus and act as barrier between placental and maternal sections (Menon et al., 2016). The placenta is a temporary organ that develops during pregnancy soon after implantation of the blastocyst to the uterine wall. It links to the fetus and the mother via the umbilical cord, thus allowing transportation of nutrients and waste to and from the fetus, in addition to gas exchange. During the placenta's development, it forms an inner layer of membrane called amnion and outer layer of membrane called chorion, which surrounds the fetus (Figure 1.1). These membranes grow and expand along with the embryo during pregnancy and the fetus surrounds by a clear amniotic fluid in uterus. Amniotic fluid acts as a cushion and provides protection to baby (Perry et al., 1998).

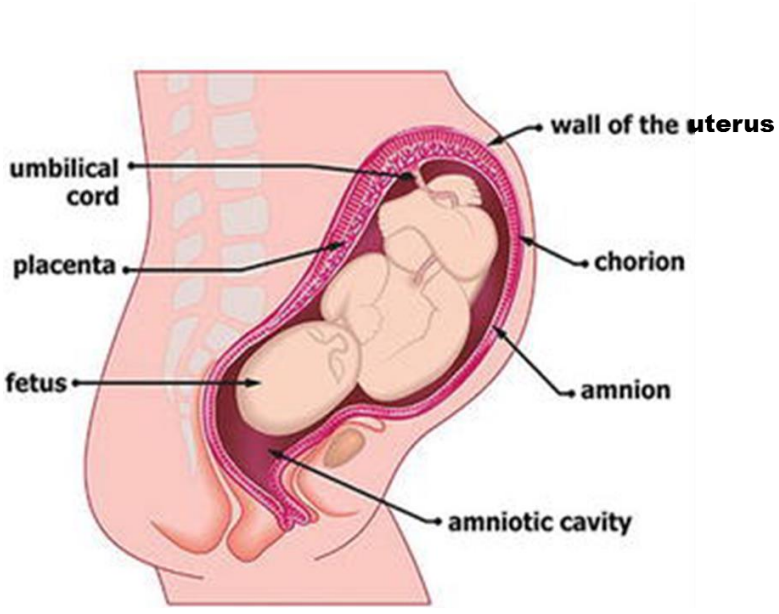

Figure 1.1: Human fetus in womb. – Schematic diagram of a human fetus in the womb, including the placenta and fetal membrane orientation. (Das, 2018)

Amniorrhexis or rupture of the Fetal membranes (FM) is a fundamental stage in normal vaginal delivery and it is a physiological membrane rupture. When the membrane ruptures between 38-42 weeks of gestation, it is considered as full term pregnancy. If membrane ruptures at or after 37 weeks without labour, it is referred as premature rupture of membrane or PROM. It is a pathological rupture. This occurs in approximately 6-19% of all pregnancies. Occasionally, the membrane ruptures before 37 weeks (24-37 weeks) at which point, the fetus is not considered as mature, which is then defined as preterm premature rupture of membrane (PPROM). Its incidence is approximately 2% of all pregnancies and is responsible for almost 40% of all preterm births (Mercer et al., 2003, NICE guideline, 2015). Nearly 35% of the world's 3.1 million neonatal deaths take place because of preterm birth complications each year. Presently, preterm birth related complications are the second biggest cause of death in under 5-year-old children after pneumonia (Liu et al., 2010, Blencowe et al., 2012). Though it is commonly seen in lower socioeconomic groups, it is a major health issue across all socioeconomic classes worldwide. PPROM is one of the leading causes of premature birth resulting in increased perinatal and neonatal mortality and morbidity. Additionally, it can affect neurodevelopmental functioning leading to cerebral palsy, learning difficulties, visual disorders and other long-term neurological complications in adulthood (Blencowe et al., 2012). The primary concerns from the neonatal perspective as a result of PPROM are umbilical cord compression, cord prolapse, malpresentation, and stillbirth. Lung disorders, in particular, respiratory distress syndrome (RDS) is one of the serious complications commonly found in babies after PPROM. Also, other complications such as pulmonary hypoplasia, skeletal deformities have been reported in PPROM babies who have survived. Furthermore, the above mentioned morbidity factors could be directly or

indirectly affect psychological and financial aspects of an individual and society likewise (Goodwin et al., 2010, Desai et al., 2012).

Although there are treatment options available to deal with the complications of PPROM, unfortunately, no effective predictive or preventative treatment for PPROM has been identified to date. The main reason for this is a poor understanding of the mechanism of PPROM. There have been numerous studies attempting to explain this phenomenon, but none have been successful so far. Traditionally, mechanical stress secondary to contractions has been attributed to cause membrane rupture. However, only ~10% full term and approximately 40% of preterm membranes rupture after contraction during deliveries (Perry et al., 1998). So, physical stress cannot be the only cause for membrane ruptures. FM tissue ruptures in a unique way which is a programmed physiological process where biochemical factors involve weakening the membranes (Joyce et al., 2009, Burzle et al., 2014). Many recent studies have focused on the biochemical factors such as ECM protein components, matrix degrading enzymes, programmed cell death of the fetal membrane and also other factors including endocrine pathway, infection, and membrane stretching. Despite this, many questions remain unanswered regarding the associated mechanisms relating to PPROM. To reduce the global burden of premature birth, defining the related risk factors and understanding the pathophysiology of PPROM are crucial.

1.2 The structure and function of the fetal membranes

Human fetal membranes (FM) or chorioamnion membrane is derived from fetal tissue. This membrane supports the developing fetus and forms the amnion cavity. Chorioamnion membrane consists of 2 layers-the amniotic membrane (AM) and the chorionic membrane (CM), coupled by an extracellular matrix (Menon et al., 2004). Chorion and amnion, the fetal membranes are not attached initially. Along with the developing fetus, the placenta and membranes collectively grow and eventually start fusing to form chorioamnion. Deformation of the fetal membrane is certain during gestation as a result of both maternal and fetal movements and also because of amniotic fluid pressure. Generally, for most part of the pregnancy, the fetal membrane's integrity does not differ. However, at the end of gestation period, fetal membrane rupture initiates the process of normal vaginal delivery (Perry et al., 1998).

1.2.1 Human fetal membrane formation and development

Perinatal development occurs in the uterine tube and it starts with formation of the transparent, tough, elastic and polysaccharide coated (zona pellucida) fertilized egg, called a zygote (Perry, 1981). After conception, mitotic cell divisions take place in the zygote and form a solid mass of undifferentiated (totipotent) cells, known as morula. Soon after (end of day 5 of conception) the egg reaches the uterine lumen, this morula transforms into two layered 'hollow ball of cells', called blastocysts. The cavities of blastocysts are referred as blastocoele or blastocystic cavity (Perry, 1981, Felomena et al., 2015). The inner cell mass accumulates on one side of the ectodermic wall, which is also known as embryoblast giving rise to embryo. The trophoblastic cells which lie at the periphery of the blastocysts form extraembryonic structure and the outer layers of placenta. Following fertilization, after 6-8 days, blastocyst gets attached to the endometrium (connective tissue of uterus) and this process is known as implantation

which is considered as the initiation of pregnancy (Figure 1.2) (Joyce, 2009). Development of the fertilised egg to a blastocyst is via cell division (Figure 1.3) (Niakan et al., 2012).

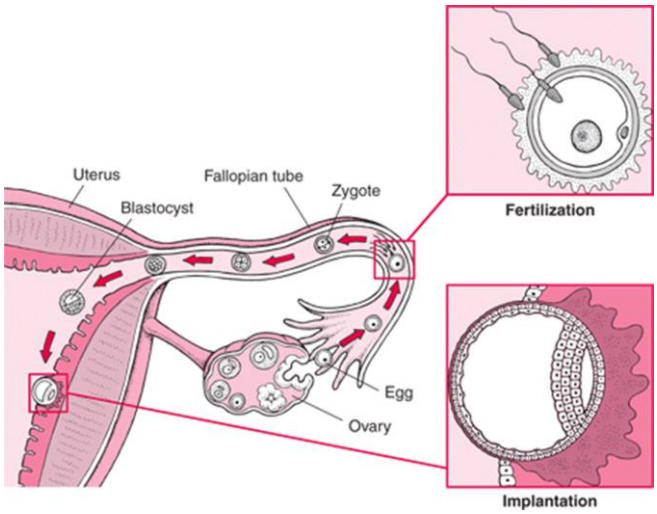

Figure 1.2: Schematic representation of egg fertilization to blastocyst stage and implantation. (Artal et al., 2019)

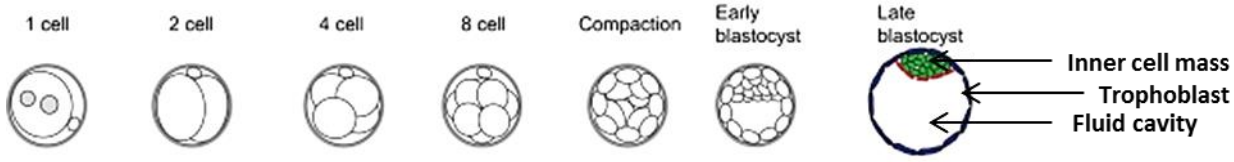

Figure 1.3: Development of the fertilised egg to a blastocyst via cell division.

(Niakan et al., 2012)

After implantation, the embryoblast forms an embryonic disc composed of two types of cells-epiblast and hypoblast. From epiblast, amniotic epithelium forms and amnioblasts (amniogenic cells) gets separated from it to form a thin membrane called amnion. Within a week, the amniotic cavity is enclosed by the amnion. The implantation process continues up to 14 days (Moore et al., 2013, Burzle et al., 2015). Exocoelomic membrane forms from hypoblasts which covers the cavity of blastocysts and interior part of

cryotropoblasts. The primary yolk sac or primary umbilical vesicle soon originates from exocoelomic membrane and cavity. Now, the outer cell layers of primary yolk sac form a bundle of connective tissue named extraembryonic mesoderm. Trophoblast cell layers are the leading cells to develop the chorionic sac and villi (Figure 1.4).

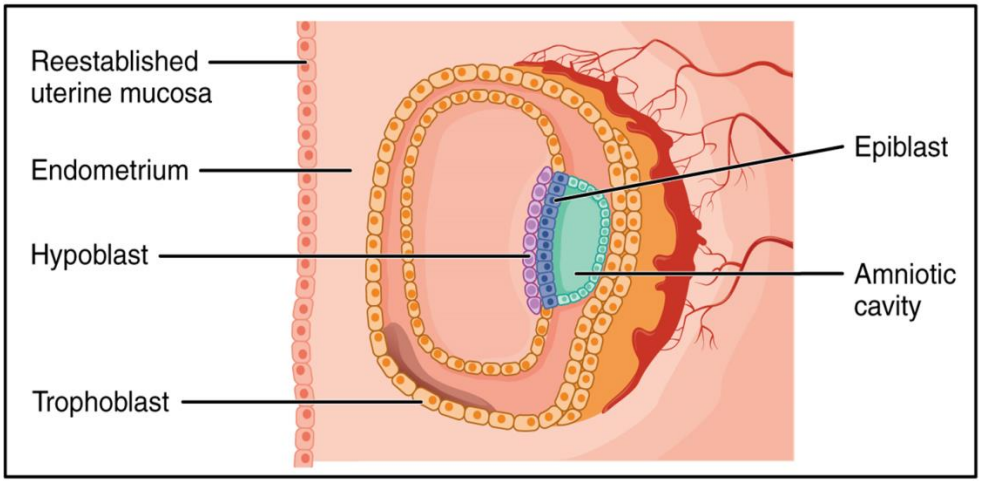

Figure 1.4: Three primary germ layers forms during the initial 2 weeks. (opentextbc.ca, 2019).

Gastrulation is one of the important phases during embryonic development which involves the formation and construction of the organs and body parts of the fetus. In this process, the embryo is restructured from a single layered blastula to multi layered gastrula. The three primary germ layers of the embryo are ectoderm, mesoderm and endoderm. Ectoderm forms the brain, nervous system, cornea, eye lenses and inner ear. The skeletal systems, muscles, heart and circulatory system are derived from the mesoderm. In addition, the digestive system, liver and pulmonary organs are derived from the endoderm, the inner most germ layer (Figure 1.5) (Carlson, 2004, Moore et al., 2013, Burzle et al., 2015)

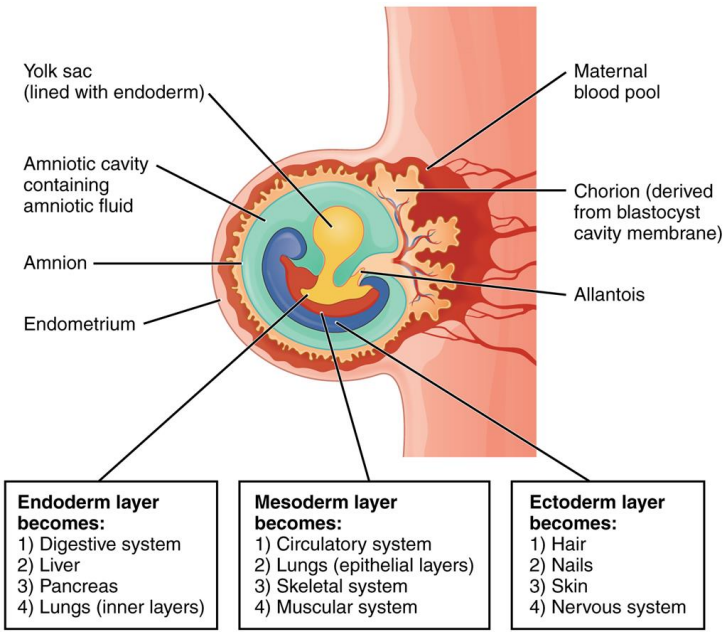

Figure 1.5: Germ layers fate into embryo. -- Embryonic cells from three primary layers of germ cells. (Opentextbc.Ca, 2019)

The ectoderm, mesoderm and endoderm start to migrate after gastrulation phase and differentiate into cell lineage. These three lineages give rise to different organs and organ systems of the fetus (Benirschke et al., 2012). Within 12 weeks of pregnancy almost all the organs are formed with the exception of brain and spinal cord whose growth is initiated and development progresses throughout the pregnancy.

In 6-8 weeks of pregnancy gestational sac starts expanding and starts suppressing the placental villi. Eventually with low blood supply the placental villi degenerates and another membrane forms-the chorion. As placenta expands, the placental villi attaches onto the uterus wall which is connected with decidua basalis, brunches and retrenches and soon expands into a 'treelike' organization. This chorionic sac eventually begins to build up the fetal part of the placental membrane (Figure 1.6) (Moore et al., 2013, Brown et al., 2016). With the advancing gestational age, amnion cavity expands and by 17-20 weeks amion and chorion roughly fuse with each other and form the fetal membrane (Ilancheran et al., 2009, Perinni, 2015). Chorion is the fetal part of the placenta and is

securely placed on to the endometrium layer called decidua basalis by the cytotropoblastic shell (Figure 1.6) (Moore et al., 2013).

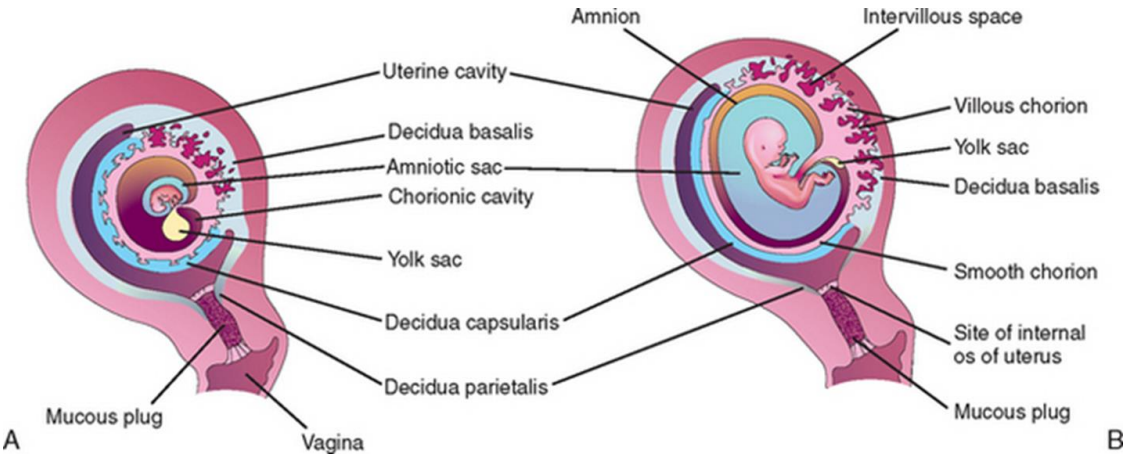

Figure 1.6: Image of fetal membrane formation. A) The amniotic sac expands and B) chorioamnion forms after fusing amnion and chorion in about 17-20 weeks of gestation (Buerzle et al., 2014).

1.2.2 Amniotic membrane (AM) & Chorionic membrane (CM):

The inner layer of fetal membrane is amnion and this functions as a shell for the amniotic fluid. It is thin, transparent, strong and viscoelastic in nature. Amnion is a multilayer tissue membrane composed of epithelial cells and connective tissues. The chorion is the thicker outer layer of the FM and is comparatively more cellular and less compact than the amnion. It is attached to the thick decidua. Until the first trimester of the pregnancy, amnion and chorion exists separately by exocelomic cavity. Then, both the layers are fused passively and form an intact fetal membrane (Benirschke et al., 2012).

The aneuronal and avascular amnion contains five separate sublayers. (I) The innermost layer which is in direct contact with the amniotic fluid is the epithelial layer (II) adjacent to this is the basement membrane, (III) next to that is compact layer, (IV)followed is

Fibroblast layer and (v) the last amniotic layer is spongy layer (Parry et al., 1998). (Figure 1.7)

The epithelial layer:

The epithelial layer of AM mainly consists of amniotic epithelial cells. Single layered epithelial cells are cuboidal and covered by many microvilli (Bourne et al., 1966). Collagen type III and IV are the main composition of this epithelial layer along with some non-collagenous protein substrates such as laminin and fibronectin (Menon et al., 2004, Parry et al., 1998).

The basement membrane:

The basement membrane is predominantly composed by collagen type IV along with collagen type III and V. This tiny amniotic layer is connected to the epithelial layer and the epithelial cells are firmly adhering on it (Figure 1.7). Other extracellular matrix proteins, such as laminin, fibronectin and elastin are also present in this layer (Figure 1.8).

The compact layer:

The compact layer next to the basement layer is the core support structure of the amnion. This acellular layer contains fibronectin and shows complicated fibrillary network arrangement. This layer is made of interstitial collagen type I and III with a sparse amount of collagen V and VI which provides maximal tensile strength throughout the pregnancy (Menon et al., 2004, Parry et al., 1998).

The fibroblast layer:

The fourth layer is a thick fibroblast layer which has a rich source of mesenchymal cells and macrophages. This layer consists of loosely arranged fibroblasts and collagen type I,

III and VI. High quantity of fibronectin and laminin are present in this layer (Parry et al., 1998).

The spongy layer:

The last amnion layer is a spongy layer which is intermediating between the chorion and amnion and contains collagen type I, III, and IV together with a good amount of proteoglycans and glycoproteins (Parry et al., 1998). Proteoglycans in addition with collagen generate tensile strength and play an important role to provide mechanical support of FM (Meinert et al., 2001).

Chorion is made of four layers and is in direct contact with the maternal decidua: (I) cellular layer (II) reticular layer, (III) the basement membrane and (IV) Trophoblast layer.

The Cellular layer:

This thin layer is found in fetal membrane only during early pregnancies (Bourne et al., 1966). A thin trophoblast cell layer has been detected here (Gupta et al., 2015).

The reticular layer:

This layer contains different types of collagens and proteoglycans. (Figure 1.7) This thin layer of chorion is mainly composed of fibroblast cells and macrophages. Loosely arranged network of collagen types I, III, IV, V and VI are present.

The basement layer:

This layer mainly consists of collagen type IV anchoring into the cytotrophoblast.

The Trophoblast later:

Trophoblast layer situates adjacent to the maternal decidua. This layer constitutes various layers of trophoblast cells. These cells appear in various morphology from round,

small to large, and polygonal with various degrees of proliferation and differentiation. However; they are arranged in a loose network (Parry et al., 1998).

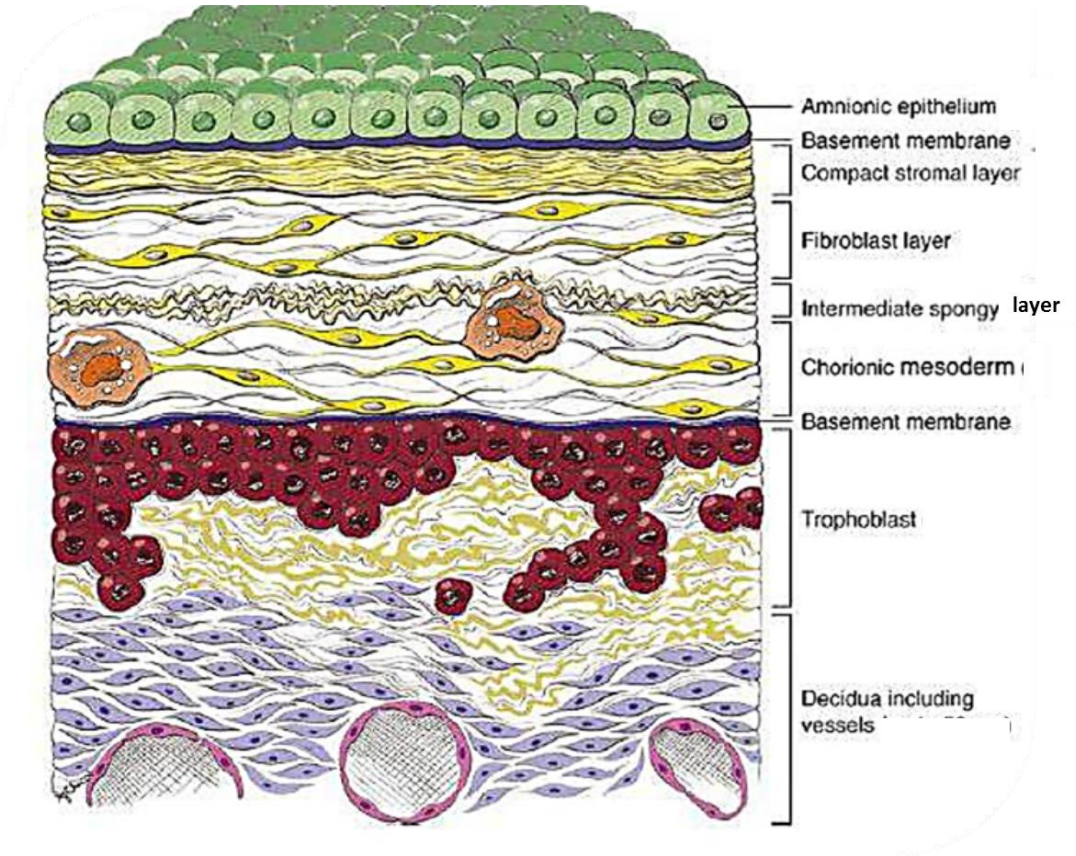

Figure 1.7: Schematic diagram of human fetal membrane showing the cellular sub-layers of amnion and chorion, (Baergan, 2011)

1.2.2.1 Extracellular matrix (ECM) composition:

The proteins of ECM are synthesised by both the amnion and chorion cells. The integrity and strength of the fetal membrane is derived from these extra cellular proteins. Though the main structural protein present in fetal membrane is collagen, other non-collagenous components such as fibronectin, elastin, laminin, decorin, proteoglycan, integrin, and nidogen also exist throughout the layers of chorioamnion membrane (Menon et al., 2004).

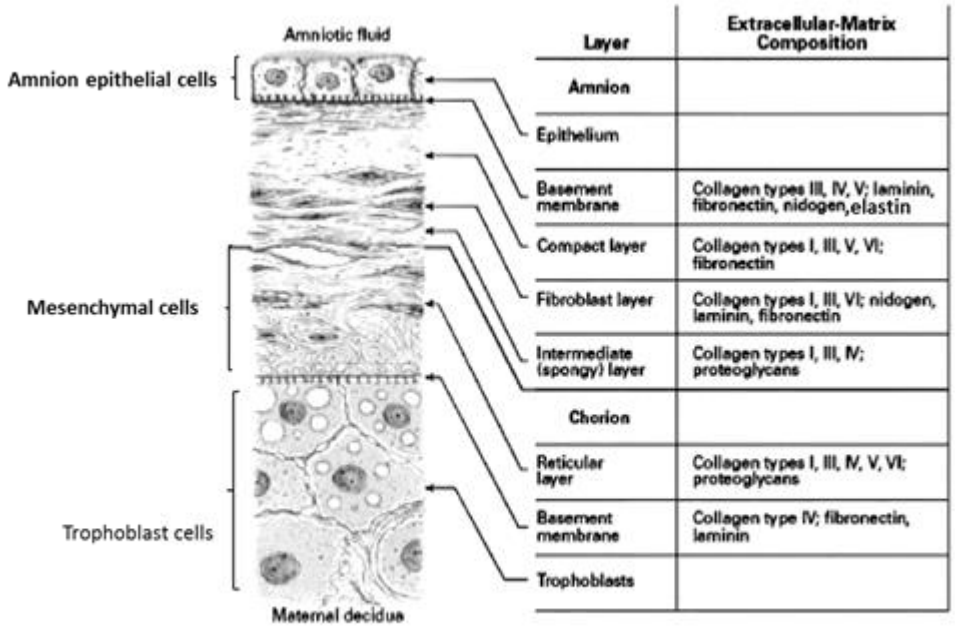

Figure 1.8: Schematic representation of FM cross section, showing AM and CM layers and the extracellular matrix composition, (Young, 2012)

1.2.2.1.1 Collagen:

Collagens are triple helical structures and are major proteins of the fetal membrane. The different types of the collagen components synthesised by amniotic epithelial and mesenchymal cells of amnion and chorion are crosslinked to each other and build a wide collagen network which play a major role in maintaining the mechanical integrity of the fetal membrane (Hampson et al., 1997). These collagens have a high turnover in a continuous process during pregnancy with raising volume and mechanical integrity to maintain the gestation. (Kanayama et al., 1985).

The alignment of the collagen fibres in the fetal membrane extracellular matrix is particularly of interest. Type I and III collagen fibres are widely banded which are cross-linked with the help of Type V and VI collagen filaments. These networks of filaments are connected to the basement membrane with the help of type IV collagen fibres. The

mechanical integrity of the chorioamnion is believed to depend on this network (Malak et al., 1994).

The collagenous structure of the FM section was clearly displayed under nonlinear leaser scanning microscopy (Figure 1.9). The microstructural distributions of collagen in different layers of the FM were shown. Under the amniotic epithelium, homogenous compact layer of collagen was visible. Collagen fibres accumulation in fibroblast layer and reticular layer also has been identified. Chorionic layer was mainly composed by trophoblast cell layers. Collagen in fibroblast and spongy layer, displayed a non-uniformed altered structure. Reticular layer also showed similar alternation, whereas collagenous structure of the compact area seemed consistent (Mauri et al., 2013)

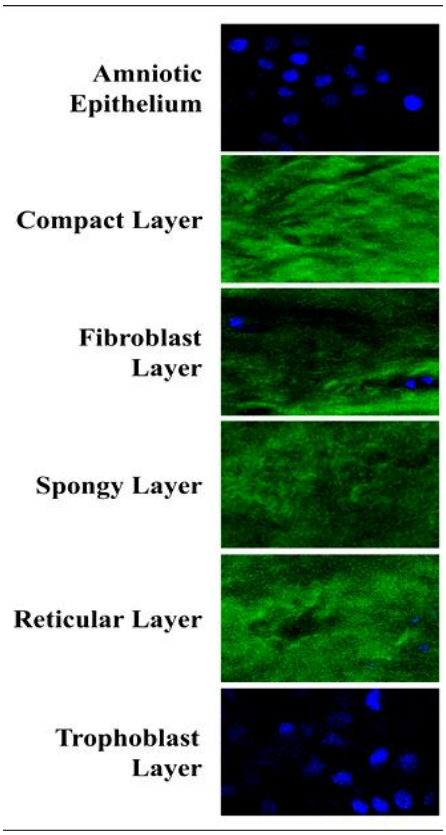

Figure 1.9: 2D image of collagenous microstructure of FM, representing collagen structure of each layer. (Mauri et al., 2013).

1.2.2.1.2 Fibronectin:

Fibronectin is another primary component of the fetal membrane. Fibronectin is used as a marker in clinical studies to identify preterm delivery. Generally, towards end of gestational term, in the lower uterine segment, the chorion separates from the decidua. This separation is thought to be due to splitting off of fibronectin binding from the extracellular matrix. The detached fibronectin is then released through the cervical or vaginal exudation. Hence, fibronectin levels (Fn) in these exudations are used as markers to determine the chances of preterm delivery (Lockwood et al., 1991, Greenwood et al., 1998). It is also believed that either mechanical or enzymatic breakdown of the fibronectin or both are involved in the delinking between fibronectin and extracellular matrix (Greenwood et al., 1998).

1.2.2.1.3 Elastin:

The fetal membrane is a visco-elastic tissue since it shows both reversible and creeping element with deformation. The cross linking of the elastin components expressed in elastic fibres provide the elasticity to the membranes (Greenwood et al., 1998). Mesenchymal and reticular layers of amnion as well as cytotrophoblasts of the chorion express abundant amount of micro fibril. These micro fibrils crosslink with elastin and provide elasticity of fetal membranes (Malak et al., 1994). Tropoelastin, the soluble precursor of elastin were found to present in human AM and CM and insoluble elastin also were detected from human fetal tissue. Biochemical and histopathologic study was the evidence of multiple layers of very tiny elastic fibres deposition by human FM (Hieber et al., 1997).

1.2.2.1.4 Proteoglycan:

Proteoglycan is another important component which plays an important role in providing the mechanical strength of fetal membranes (Meinert et al., 2001). Proteoglycans consists of core protein domain 'O-linked' with glycosaminoglycan (GAGs). Proteoglycans are classified into two groups, large and small proteoglycans (Goodship et al., 2008). Biglycan is a small leucine-rich proteoglycan which is mostly present in peri-cellular matrix of the FM binding to the collagen fibers and other growth factors (Kalamajski et al., 2010, Meinert et al., 2001). Another small proteoglycan, decorin which is of similar homology with biglycan, binds to collagen I and III and takes part in collagen fibril production (Strauss et al., 2013). Therefore, it facilitates the collagen fibre network which gives mechanical stability to the amniotic membrane. Along with the supporting role in maintaining the structural integrity of fetal membrane, proteoglycans also participate in fetal membrane remodelling process through binding of growth factors

(Meinert et al., 2014, Meinert et al., 2001).

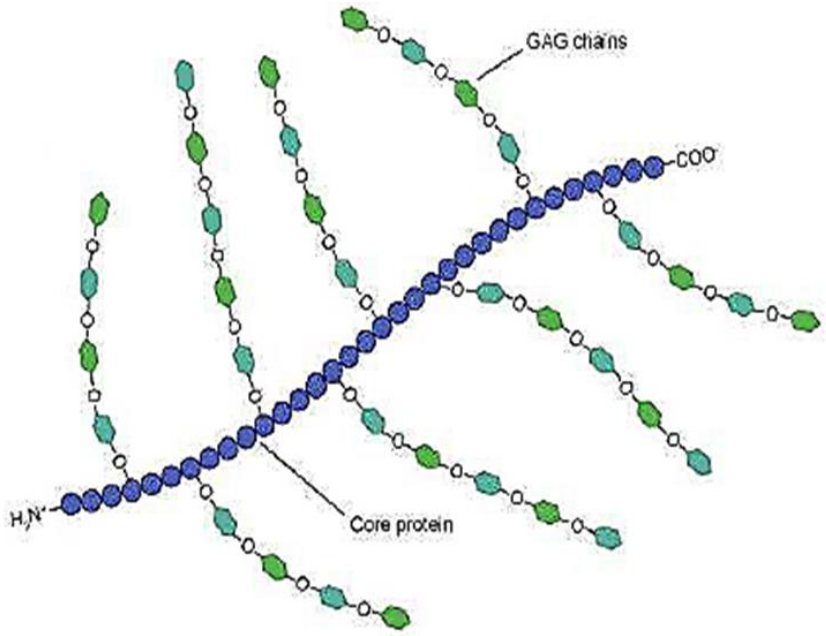

Figure 1.10: Schematic drawing of proteoglycan structure (Haller et al., 2013)

1.2.2.2 Zone of altered morphology (ZAM):

The morphology of chorioamnion constantly changes towards the end of the pregnancy in the preparation for delivery. This is known as “zone of altered morphology” (ZAM). It could be either in the form of degradation in the basement membrane of the amnion, stromal layer swelling, shrinking of the trophoblast layers, distortion of extracellular matrix or overall reduction in the thickness of the membrane (Kanayama et al., 1985, Perrini 2016). A study by Malak and Bell observed some morphological changes in certain areas of the fetal membrane.The architectural support was disrupted in these areas; hence they turn into structurally weak areas (Malak et al., 1998).An area of altered morphology in the fetal membrane was found overlying the cervix during the final stage of the gestation (Malak & Bell, 1994, McParland et al., 2003). This area was detected to have a possible association with ruptured site in spontaneous full term birth (McLaren et al., 1999). It has been suggested that either reduced connective tissue thickness or decreased strength (El Khwad et al., 2005) of this ’weak zone’ of fetal membrane are programmed. This ‘pre-weakening’ or ‘programmed weakening’ of the fetal membrane on otherwise normal area is linked to membrane rupture at term (Chai et al., 2012, Lim et al., 2016). However, the mechanical failure and the mechanisms *in vivo* are yet to be explored.

1.3 Mechanical properties of the fetal membranes:

The mechanical properties of a fetal membrane are dependent on the arrangement of the layers as well as the collagen distribution and its orientation. Comparing to other collagenous tissues, chorioamnion undergoes much higher physiological stress. Also, it is programmed to rupture after a certain point of time (Perrini, 2016). The tensile strength

of the membrane is not distributed equally. The area of the membrane nearer the cervix is exposed to a different mechanical condition than other areas away from the cervix. There is a difference in mechanical properties of artificially ruptured membranes (including caesarean section) and the spontaneous ruptured membrane (Moore et al., 2006, Lannon et al., 2014). Furthermore, there are variations with amnion and chorion alone as well as when they are fused together. Though amnion is much thinner (20% of whole membrane thickness) than chorion, it has been found to be far stronger in comparison (Arikat et al., 2006).The mechanical strength of the amnion also differs during stages of pregnancy and particularly towards labour and the mechanical strength of fetal membrane is decreased with increasing gestational age (Oyen et al., 2006) Evidently, the mechanical behaviour of this membrane is very complex and fluctuates with many factors.

There have been extreme variations in reporting the mechanical properties of amnion and chorion. Helmeig et al., stated that the tightly fused layers-amnion and chorion, during normal delivery, were loosely attached with each other. They showed that chorion ruptured at low force with some deformation while the amnion needed high rupture strength (Helmig et al., 1993). In a separate study with amnion and chorion alone, Oyen.et.al noted that amnion was stronger but showed less deformation compared to the chorion (Oyen et al., 2006). Both these studies used uniaxial tensile technique. Lavery and group from their series of studies by using inflation test have reported on the viscoelastic property of fetal membrane for the first time. They also noted varying strengths in different areas from a single membrane and that the chorion was weaker than amnion. Furthermore, they found variability in viscoelastic property between the term and the preterm membrane. Their investigation also included the time dependent creeping and relaxation response of the membrane (Lavery et al., 1977, 1979, 1982). In a time dependent study, Mauri et al., investigated the microstructural response of amnion. They

found reversible short term deformation of the membrane as a result of water outflow from the collagenous network (Mauri et al., 2015). Some studies postulated that amnion was affected more during labour compared to chorion, whilst at the same time; it was thought that amnion was stronger and less likely to undergo deformation than chorion (Helmig, 1993, Oyen, 2006, Ariket, 2006).

All these studies clearly indicate that amnion is mechanically stronger than chorion, but reports on rupture strength of the amnion and chorion have been inconsistent in the literature. In terms of rupture sequence, few studies stated amnion ruptures first (Hemlig 1993, Schober et al., 1994, Greenwood et al., 1998) while few other studies reported chorion ruptures first (Artal et al., 1976, Laveryet al., 1979, Arikat et al., 2006). Perrini et al., observed this in detail and described that predominantly amnion ruptured earlier and did so faster in the peripheral areas of the sample than in the centre. However, on the other hand, in chorion sample, the centre area ruptures sooner than the periphery (Pereini et al., 2013). Nevertheless, Arikat et al., found the membranes were stronger, tougher and showed increased deformability when they were intact compared to amnion or chorion alone. They prepared a video where they used this method and documented the sequence of fetal membrane rupture *in vitro*. They also concluded that chorion separated from amnion prior to the rupture event and chorion ruptured before amnion (Arikat et al., 2006).

The diversity of FM in mechanical behaviours could possibly be explained due to testing methods and the experiment set up. In addition, small sample size and failure to study other factors involved in rupture simultaneously could be other causes for the differences. FM is a complex tissue; the mechanical behaviour of FM varies depending on several factors, such as gestation, mode of deliveries, areas of the same membrane, separated amnion and chorion, freshness of the tissue and many others. Therefore, the

measurements of the mechanical properties of FM can give good comparison of the specific FM groups within an individual study, but not across different studies. Table 1.1 lists the mechanical properties, UST, puncture force and modules of FM by tensile test and puncture test (Oxlund et al., 1990, OYEN et al., 2004, Chowdhury et al., 2014).

(I) Tensile test data from Oxlund et al., 1990

Membrane name	Maximum strength (N/mm^2)	Tensile modulus (N/mm^2)
Fresh chorioamniotic membrane (CA)	1.03±0.09	15.0±0.9
Fresh chorionic membrane (CM)	0.62±0.06	10.9±0.8
Amniotic membrane(AM)	3.94±0.24	47.3±3.0

(II) Puncture force at failure from OYEN et al., 2004

Membrane name	Unlabored term (N)	Laboured term (N)	Preterm (N)
Chorioamnion (CA)	4.26 ± 1.09	4.04 ±1.52	5.09 ± 1.30
Amnion (AM)	3.18 ± 0.37	2.10 ± 0.681	4.30 ± 0.71
Chorion (CM)	1.78 ± 0.49	1.72 ± 0.44	1.19 ± 0.00

(III) Tensile test data from Chowdhury et al, 2014

Membrane name	Ultimate tensile stress (MPa)	Tangent modulus (MPa)
Amniotic membrane from cervix area (CAM‖)	2.28± 0.04	4.9
Amniotic membrane from placental area (PAM‖)	4.52±0.6	7.5

Table 1.1: Mechanical properties of FM and FM layers from different measurement protocols and different research groups. (I) The tensile strength and elastic modulus values of fresh and separate chorionic membrane, amniotic membrane and FM membrane (oxlund et al.,1990). (II) Puncture force at failure point for FM layers with different mode of deliveries and gestation. (Oyen et al., 2004). (III) Tensile ultimate strength and modulus of FM taken from different locations (near cervix (CAM) and near placenta (PAM) with parallel oriented collagen fiber alignment (Chowdhury et al., 2014).

These observations suggest that further investigations are needed to understand the fetal membrane rupture properties in comparison with full term membrane. Nevertheless, these findings would help to further enhance the understanding of mechanisms in PPROM.

1.4 Proposed causes for preterm rupture of fetal membranes:

Although the epidemiology of PPROM and its related complications are well defined, the underlying mechanism is poorly understood. Numerous factors have been proposed with numerous ongoing studies to uncover the aetiology behind PPROM. It is proposed that mechanical factors, biochemical factors (such as infection and inflammation) and collagen deficiencies may be mainly associated with PPROM.

1.4.1 Models to determine mechanical properties on the FM:

In the past many decades, researchers have focused on the mechanical properties of fetal membrane to investigate the mechanism leading to early rupture of membranes. Studies that have investigated the mechanical behaviour of fetal membranes have used different test methods such as uniaxial tensile test, puncture test, and inflation test. These will be further detailed later. Before establishing reasons for membrane failure, it is necessary to understand the mechanical behaviours of the chorioamnion tissue from full term labour.

In the early 70s, Artal and group performed a tensile test with compact chorioamnion samples collected from normal delivery and caesarean section with or without labour. They noticed a wide variation among the preterm and the term ruptured membranes. They also found the area of the membranes near the ruptured line was weaker compared to the other areas which were far from the ruptured line of the same fetal membranes. However, the rupture strength of the non-premature ruptured membranes was not different from the premature ruptured membranes (Artal et al., 1976, Moore et al.,

2006). While some studies reported that the increasing contraction during the process of labour may result in a mechanically weaker membrane (Lockwood 1991, Helmig, 1993). Helmeig et al., also found that the membranes ruptured more rapidly in those from normal deliveries than from C-section deliveries (Helmig 1993, Moore 2006). Puncture test data also showed considerably higher mechanical strength in preterm membranes than the full term ones (Schober et al., 1994, Oyen et al., 2006). Also, full term membrane from vaginal deliveries exhibited less rupture strength compared to the full term membranes from C-sections (Oyen et al., 2006). This gives an indication that the effect of labour can change the mechanical properties of the membrane. However, according to other studies there was little difference in rupture strength between term and preterm membranes (Artal 1976, Greenwood, 1998). Toppozada et al., described that membrane weakness was a result of repetitive stretching in their in vitro study (Toppozada et al., 1970). Pandy et al., reported that repeated stretching required greater mechanical strength to rupture the fetal membranes (Pandey et al., 2007).

Although, it has been more than 100 years since researchers first studied the mechanical behaviour of fetal membrane specially it links to the mechanism of PPROM, the existing data still failed to provide clear understanding of it. That is mostly because of the complicate biomechanical changes in the entire uterine environment during pregnancy, including effect of the pressure, membrane thickness and contraction (Verbruggen et al., 2017). Consequently, some studies in recent times with hypothesis that biochemical or biological factors could be involved in the pathogenesis of PPROM.

1.4.2 Microstructural alteration:

A few studies have attempted to investigate the alteration of microstructure of fetal membranes. These suggest that the microstructural framework of the collagen network could affect the mechanical properties of fetal membranes (Greenwood et al., 1998) as

synthesis and disruption of extracellular membrane's components are believed to be responsible for the mechanical strength of chorioamnions. Consequently, the altered collagen structure, content or activity is thought to be involved in early membrane rupture (Perry et al., 1998).

Previously, some studies have already investigated the connection of collagen network alternation and PPROM. Canzoneri et al., described that the cellular layer of chorion was thinner when collected from the patients with PPROM compared to the preterm labour or term (Canzoneri et al., 2013). Skinner and group noted progressive reduction of collagen content in amniotic membrane during 32 to 40 weeks of gestation periods (Skinner et al., 1981). Kanayama et al., found a decreased level of type III collagen in amniotic membrane from the premature samples (Kanayama et al., 1985). In another study, few years later, Al-Zaid et.al demonstrated that there was no type V collagen in prematurely ruptured membrane (Al-Zaid, 1988). However, Victoria et.al in their study did not notice any significant changes of type V collagen in preterm samples. They thought that discrepancy was due to the smaller sample size in the previous study and they argued that small sample sizes or small rupture samples were enough to give a clear explanation on the change of extra cellular matrix composition or collagen metabolism in preterm rupture membrane prior delivery (Hampson et al., 1997).

Vadillo and group commented on decreased level of collagen synthesis and increased level of its activity as well as solubility in preterm amniotic membrane. Though they have not reported any significant difference in any collagen content, the association between collagen metabolism and PPROM was clearly mentioned (Vadillo et al., 1990). As the structure of the collagen fibres, their arrangements, and the other matrix component indicate the mechanical integrity of fetal membrane. Vadillo et al. predicted that PPROM can be the most common result of weakness of collagen matrix due to any condition.

Structural abnormalities and matrix weakening may happen due to the interference of collagen metabolism (Petraglia et al., 2007). For example, in patients with connective tissue disorder such as Ehlers-Danlos syndrome associated with abnormal collagen structure, the fetal membrane has a higher risk of PPROM (Vadillo et al., 1990). On the other hand, Evaldson and group disputed any contribution of collagen content on PPROM as they did not observe any difference in the amount of collagen between preterm membranes and spontaneous term ones (Evaldson et al., 1987). Similarly, MacDermott and Landon also did not notice any difference in collagen content of amnion which can lead to PPROM. Therefore, they hypothesized that PPROM was not associated with reduced collagen content and rather it occurred because of localized membrane weakness (MacDermott et al., 2000).

Stuart et.al also investigated the cross linking of the collagen in premature fetal membranes and found no variation between the PPROM sample and their control group with spontaneous full term labour. They concluded that collagen fibre arrangement has no involvement with the PPROM mechanism, but, the variation in the crosslink was involved in the formation of the 'rupture-initiation site' (Stuart et al., 2005). Buerzle et al., have confirmed that any reduction in collagen content or variation in cross-linking is the cause of fetal membrane weakness and reported a possible connection between reduced resistance of collagen fibre framework and PROM (Buerzle et al., 2014). In a recent study, Meinert et al., found an involvement of proteoglycans in the etiology of premature delivery. Proteoglycan binds to collagen I and III and facilitates the collagen fibre network which gives a mechanical stability to amniotic membranes (Meinert et al., 2014). Therefore, weakening of fetal membrane ECM due to collagen degradation is another proposed factor involved with the mechanism behind PPROM- either in form of variation in the localized membrane thickness or in reduction of the collagen contents.

Results of previous studies contradict with each other and this could be explained by their inability to focus on the morphological structural change of the ruptured site. In a specific area of the rupture site or "along the line of rupture" a unique morphological change has been observed. This area or ZAM exhibits decreasing structural strength of the tissue with increasing gestation period and this included changes in membrane thickness as well as tissue organization and alternation of collagen types. This structural tissue remodelling has been found more extensively in preterm births (Malak et al., 1994 McLaren et al., 1999). Puncture test result showed much lower ruptured strength in the tissue collected from ZAM and its surrounding area than the rest of the areas (El Khwad, 2005). However, Buerzle et al., did not notice any difference in rupture strength between the two areas when using the inflation method (Buerzle et al., 2014). Calvin and Oyen used different test methods and concluded that tissue thickness decreases the rupture strength without any other interference. Therefore, membrane thickness which relate to collagen orientation and denseness is one important factor which could explain PPROM (Calvin et al., 2007).

Another reason for differences in opinion on the morphological changes that have not been studied well could be the involvement of endogenous factors. These factors are responsible for collagen degradation or exogenous factors such as bacterial infection and host inflammatory response which activates the degradation of the membrane collagen.

1.4.3 Matrix degrading enzymes and their inhibitors:

Internal bonding of the connective tissues of chorioamnion and extracellular matrix are protected from external environment. A group of specific proteases known as matrix metalloproteinases (MMPs) take part to weaken this bonding and degrade the membranes. MMPs have ability to degrade any collagen and non-collagenous component with which ECM is made of. (Vadillo et al., 2005)

MMPs are zinc-dependent enzymes and are the subfamily of metalloproteinases, called matrixins. There are several different MMP proteins that are present in humans. However, from literature, it is not entirely clear about the total number of proteins that exist in MMP family. Some groups have described 23 or 24 whereas some other group suggested 25 and others state 28 MMPs. The correct number is yet to be found (Yonemoto et al., 2006, Menon et al., 2007, Weiss et al., 2007). These MMPs are found to be inhibited by specific tissue inhibitors and at present, four tissue inhibitors of MMPs (TIMPs) have been discovered. They are TIMP-1, TIMP-2, TIMP-3 and TIMP-4. The activity of MMPs is controlled by the equilibrium of the metalloproteinase activators and inhibitors. (Weiss et al., 2007)

Based on the structures and functions, MMPs are split into five different groups. These are collagenases, gelatinases, stromelysins, membrane type and heterogeneous group. (Weiss et al., 2007) The first 3 groups are believed to be involved in chorioamniotic rupture. However, within fetal membrane, specific role of some of the MMPs has not been revealed very clearly so far. MMP 1, MMP 8 and MMP 13 belong to interstitial collagenases group that regulates the degradation of collagen types I, II and III. MMP 2 and MMP 9 (gelatinases A and B, respectively) are gelatinases which acts on collagen type IV and V as well as on non-collagen like elastin, proteoglycan and fibronectin degradation. MMP 3 is one of the stromelysin which is expressed in labour. (Vadillo et al., 2007)

Notably, these enzymes are not secreted in their active form. Other factors or signals are needed to activate them and most importantly they can be inhibited by TIMPs. This activation process of the MMPs is complex and takes place in a stepwise manner. Current investigators assume that the mechanism of pre labour rupture of fetal membranes is due to interplay of MMP actions. However, MMPs are not only involved in abnormal degradation of chorioamnion, but they also have an essential role in normal growth and

remodelling of fetal membrane during pregnancy by breaking down the ECM components. They also help in tissue weakening and rupture during full term labour. (Vadillo et al., 2005, Menon et al., 2007)

1.4.3.1 Role of MMPs in normal term labour:

Normal term labour is characterized with few progressive events including cervical softening and dilation, fetal membrane weakness, contractions of uterine wall, and at the end, fetal membrane rupture. All through the gestation up until the membrane rupture, MMPs play a major role (Weiss et al., 2007). Towards the delivery period, the equilibrium between activated MMPs and TIMPs alters and expresses the other way. Hence, collagen network of extracellular matrix of fetal membrane begins to degrade (Parry, 1998). Riley et al., observed low level secretion of the TIMPs in amniotic fluid and significantly high level expression in chorion laeve decidua parietalis and placenta during onset labour. Chorion and decidua parietalis are found to be a main source of all the four TIMPs releasing in the amniotic fluid (Riely et al., 1999).

According to literature, MMP 2, 9, 7, 8 and 13 have a role in rupturing membrane in normal labour. An increased level of MMP 2 and MMP 9 production has been observed prior to uterine contraction. An intense change in MMP and TIMP ratio i.e. higher level of MMP 2 and 9 and reducing level of TIMP-1 occurs during contraction (Ulug et al., 2001). Goldman and colleagues have demonstrated that the MMP 2 and MMP 9 are secreted in higher concentrations in chorioamnion membrane and amniotic fluid after contraction starts. Also, MMP 2 was found to be more active in decidua whereas in amnion it was MMP 9. Notably, in the chorion both the MMPs were silent during labour. During post onset labour, the concentration of MMP 9 superseded MMP 2 in the amniotic fluid and the concentration of TIMP-1 decreased (Goldman et al., 2007).

According to Otega et al., levels of MMP 9 were not very obvious before the labour. It was augmented dramatically later once the contraction began. But MMP 2 was found in both the occasions (Otega et al., 1995). They have also reported that MMP 1 gene expression was high before the onset of labour but after term labour the concentration of TIMP 1 was increased. (Otega et al., 1995, Greenwood et al., 1995) Greenwood and group in their study showed a significantly high level of MMP 3 and MMP 9 during active labour. On the other hand, MMP 1 and MMP 2 level were marginally increased during pre-contraction (Greenwood et al., 1995).

MMP 7 was also identified at early gestation and during term. During the second trimester of pregnancy, Maymon and group detected MMP 7 gene expression in amniotic fluid and they found that the level of it was significantly higher during the third trimester. However, little information exists on the activation route of MMP 7 in amniotic fluid. (Maymon et al., 2000) Expression of MMP 13 also found in fetal membrane and amniotic fluid but the role of it was not clear yet (Fortunato et al., 2003).

Matrix MMPs are produced by a number of cells which hydrolyses one or more components of ECM and the MMPs then act on collagen degradation. At the same time, specific TIMPs are produced to inhibit the function of these enzymes. The triple helix of collagen type I and III are cleaved by collagenases (MMP 1 and MMP 8). Subsequently, collagen breaks down further by the action of gelatinases (MMP 2 and MMP 9). TIMP-1 inhibits the activity of MMP 1, MMP 8 and MMP 9. TIMP-2 binds to both dormant and activated MMP 2. Expressions of TIMP-3 and TIMP-4 are not very noticeable in tissue, although recently they have been found to play the similar inhibitory role on MMPs like TIMP-1 (Parry et al., 1998).

TIMPs are produced in chorioamnion during pre and post labour. Generally, during most period of pregnancy, the fetal membrane integrity does not differ. The main reason could

be due to increased expression of TIMPs and reasonably reduced concentration of MMPs (Ortega et al., 1990).

Ortega et al., elucidated that the MMP 9 activity increased in amnion and chorion with labour while the concentration of TIMP-1 declined dramatically (Ortega et al., 2005). Other studies demonstrated that the membranes from both caesarean section and spontaneous labour after full term showed increased MMP1 activity before labour. During labour, MMP 9 and MMP 3 expression increased whereas the TIMP-1 showed high level of concentration after labour (Greenwood et al., 1995). Menon and Fortunato described that decreased concentration of the TIMP-1 and increased activity of the MMP 9 was capable to weaken the tensile strength of chorioamnion (Menon et al., 2007).

In summary, the collagen degradation within the fetal membrane is a controlled sequence of events during labour. The ratio of MMP and TIMP is maintained inversely in a progressive manner throughout the gestation and this leads to fetal membrane rupture or delivery.

1.4.3.2 The role of MMPs in preterm membrane:

The pattern of MMPs and TIMPs balance and activation suggested that an interruption in their ratio may lead to collagen degradation of the membrane's ECM untimely and may cause PPROM. Therefore, the investigators are now interested to study the involvement of MMPs in PPROM. In general, the hypothesis concerning the association of MMPs with PPROM is that MMPs are activated inappropriately as a result of inflammation in extra cellular matrix. The MMP 1, MMP 2, MMP 3, MMP 8, MMP 9 and also MMP 7 have been identified in the amnion and choriodecidua as well as increased levels of these MMPs were detected in amniotic fluid when PPROM occurred (Menon et al., 2004).

Higher levels of MMP 3 have been recognised in amniotic fluid from PPROM patients compared to term ones. In the same manner, MMP 2 and MMP 14 were also found in amniotic fluid and amniotic membrane also supporting their influence in early membrane rupture (Fortunato et al., 2001). Yonemoto and colleagues identified the association of MMP 2 with fetal membrane rupture. Besides that, they found increased activity of the MMP 2 and MMP 9 only in amnion and not in chorion with the labour. Therefore, they proposed that the whole event such as expression, activation and secretion of MMPs from any particular site of the tissue may regulate the mechanism of membrane rupture (Yonemoto et al., 2006).

Xu and co-workers revealed that the distribution and expression of the MMP 2 and MMP 9 is cell specific. Their study exhibited both MMP 2 and MMP 9 was produced by trophoblast cells in chorion, while MMP 9 was secreted by epithelial cells and MMP 2 by mesenchymal cells in amnion. They also observed a remarkably high level of MMP 9 expression in pre-ruptured membrane but did not notice any significant difference in MMP-2 (Xu et al., 2002).

A study by Athayde et al., noticed higher concentration of MMP 9 in patients with PPROM compared to intact membranes of term delivery (Athayde et al., 1998). Another study done by Romero and group also declared that the PPROM was associated with an elevated concentration of active MMP 9, in contrast, they found decreased level of MMP 2 in the same condition. (Romero et al., 2000) Based on all these outcomes, Xu et.al proposed the ratio of MMP 9 and MMP 2 may facilitate FM rupture untimely (Xu et al., 2002).

With the increased level of MMPs, the involvement of decreased levels of TIMPs were investigated and compared in preterm gestations with the term ones. Clark et.al stated that TIMP-1 levels remain same until 37 weeks when compared to a non-pregnant level,

but during the final weeks of pregnancy, its level slightly increases; however, it decreased during labour (Clark et al., 1994). Similarly, Ortega and his co-workers showed reduced level of TIMP-1 in amniotic fluid with progressing gestation (Ortega et al., 1995). Tency et.al found decreased level of TIMP-1 and TIMP-2 in preterm gestation than the term samples from any labouring status (Tency et al., 2012). In few studies, (Riley 1999, Tency, 2012) TIMP-4 expressions during preterm have been studied. During the first trimester, TIMP-4 was found to present in low level in amniotic fluid and with advancing gestation it increased significantly (Riley et al., 1999). However, there is no data found about TIMP-4 association to PPROM. Generally, there was no change in serum TIMP-4 concentration exhibited in both preterm and term labour (Tency et al., 2012).

MMP 8 concentration was not only found to be high in amniotic fluid in spontaneous labour but was also proved to be associated with PPROM (Maymon et al., 2000). With this perspective, a rapid bedside test has been designed recently to detect the increased MMP 8 concentration in amniotic fluid. This test has a good specificity in ruling out an impending preterm delivery (Nien et al., 2006). Tency and his team have observed the elevated concentration of MMP 9 in maternal serum during preterm labour and they also observed that the level was significantly higher than labour at term. They have also investigated the MMP 3 level and could not detect any change (Tency et al., 2012). Another study done by Park et al., demonstrated the increased level of MMP 3 is associated with the preterm labour (Park et al., 2003).

Therefore, it was suggested that the pathogenesis of the PPROM is an underlying imbalance in the ratio of the MMPs and TIMPs concentration. The increased ratio of MMP 9: TIMP-1 and MMP 9: TIMP-2 has been noticed in preterm labour (this ratio suggested higher MMP 9 and lower TIMP-1 & 2). There was no specific correlation found between MMP 2 and the PPROM. MMP 9 was found to be present in infected membrane but was

not present in non-ruptured one. Also, TIMP-1 level was dramatically low and TIMP-2 concentration was decreased in the preterm membranes (Tency et al., 2012).

Overall, there is a general consensus regarding the role of MMPs and TIMPs in initiating normal rupture of membrane but in case of PPROM, the cause of untimely expression of MMPs and in particular involvement of specific MMPs remains to be determined. Nevertheless, Maymon et al., reported the concentration of MMP 1 was high in affected (PPROM) amniotic fluid either with or without infection. But the MMP 1 expression was significantly low in amniotic fluid of the control (Maymon et al., 2000). In a different study, Maymon et al., claimed there was an association of MMP 7 with PPROM. MMP 7 concentration was increased in the context of intra-amniotic infection. (Maymon et al., 2000) Therefore, there is a possibility that MMPs can also be produced with the absence of any infection or inflammation. Other factors like hormones, myoblast and fibroblast differentiation, and genetic factors (e.g. SERPIHN1 gene) can activate the MMPs untimely (Greenwood et al., 1995).

	MMPs	TIMPs	citation
Full term	Increased level of MMP 1,MMP 2 ,MMP 9,MMP 3, MMP 7,MMP 13 AM- increased level of MMP-1,2,9 CM- increased level of MMP-2,9 Ruptured site-Increased level of MMP-2,9	Reduced level of TIMP-1 &TIMP-2 AM-Reduced level of TIMP-1,2 CM-Reduced level of TIMP-1,2	Ulug et al., 2001) Otega et al.,1995,Greenwood et al., 1995, Maymon et al., 2000, Fortunato et al., 2003. Goldman et al., 2007. Ortega et al., 2005.
PROM	Increased MMP 2,MMP 14,MMP 8,MMP 9 AM-Increased MMP2,9,8,14 CM-Increased MMP-9	Reduced level of TIMP-1,TIMP-2 & TIMP-4 AM-Reduced level of TIMP-1,2 CM-Reduced level of TIMP-1,2,4	Tency et al., 2012, Clark et al., 1994, Fortunato et al., 2001, Yonemoto et al., 2006 Xu et al., 2002
PPROM	Increased level of MMP 1,MMP 3,MMP 9,MMP8 AM-Increased MMP-1,2,9 CM-Increased MMP-9	Reduced level of TIMP-1,TIMP-2 & TIMP-4 AM-Reduced TIMP-1,2 CM-Reduced TIMP-1,2	Athayde et al., 1998, Romero et al., 2000, Maymon et al., 2000

Table 1.2: MMP/TIMP level found in full term and preterm (PROM & PPROM) membranes (Based on multiple literatures).

1.4.4 Role of progesterone hormone and its receptors:

Hormones such as progesterone and β-estradiol have been found to be responsible for high TIMP and low MMP concentrations. In addition, they could influence collagenase production during pregnancy and inhibit the interleukin activity (Sato et al., 1991, Parry et al., 1998). Greenwood et.al proposed that the decidual hormone called relaxin is one of the candidates likely to induce the MMP 1 and MMP 3 secretion. This could be responsible for the fetal membrane weakness and rupture (Greenwood et al., 1995). Although, these hormones are involved in reproductive process, it is yet to be found in more detail about their role in the rupture of fetal membrane. Before pregnancy and during early pregnancy, progesterone is mainly produced by the corpus luteum of the ovary. In mid to late pregnancy, it is produced in large amounts by the placenta and plays a vital role in pregnancy, especially to develop a healthy fetus and maintain and continue a healthy gestation (livestrong.com). Progesterone is a steroid hormone and it does play a vital role in development and maintenance of human pregnancy (Conneely et al., 2002). It is synthesised from the endogenous steroid called pregnenolone. Syncytiotropoblasts of placenta and trophoblasts of chorion layer secrete the progesterone hormone in the initial phase of pregnancy. In quiescent state of pregnancy, progesterone can help to relax the myometrial activities blocking the contraction-association proteins (CAP) activations. A steady concentration of the progesterone hormone is sustained until the onset of labour (Goldman et al., 2007). A steep fall of progesterone level is known to occur during the onset labour (Smith et al., 2002).

There are very few studies explaining the association of low progesterone and PPROM. Nevertheless, various clinical trials have supported the hypothesis of reduction in preterm delivery with progesterone supplement and some data also suggest that it may help to prolong delivery in women with PPROM. Meis and colleagues reported that weekly injection of progesterone was able to reduce the risk of recurrent preterm delivery (Meis et al., 2003) but, they did not mention whether the preterm delivery was spontaneous or it occurred after PPROM. Another placebo controlled study showed progesterone administered as vaginal suppository reduced the risk of preterm birth (Fonseca et al., 2003, Norwitz et al., 2011). Luo and group found progesterone to inhibit apoptosis of fetal membrane cells by blocking TNF-alpha during pregnancy. They suggested that this could be one of the strategies in preventing preterm birth and PPROM (Luo et al., 2010). On the other hand, O'Brien et al., found little difference in delaying preterm birth between women with PPROM who were on vaginal progesterone and the control group (Briery et al., 2011). More research is needed to explore the role of progesterone in inducing membrane rupture.

PR-A, PR-B and PR-C are the three isomorphs of progesterone receptor found to exist in human reproductive tissues (Graham et al., 2002, Goldman et al., 2007). PR-A and PR-B are mainly known to mediate the effect of progesterone in human and both are transcribed from single gene (Kaster et al., 1990). Structurally, both these proteins are very similar. The only difference is PR-B contains one extra amino acid sequence at the end of N terminal than the PR-A protein (Wen et al., 1994). Functionally, PR-A plays an active role in addition with PR-B in regulation of nuclear receptors such as glucocorticoid, oestrogen and androgen activities. Transcription is enhanced by PR-B and inhibited by PR-A through obstructing the function of PR-B (Li et al., 2003).

Although the role of progesterone receptor in establishing pregnancy and maintaining total gestation is well known, there are very limited studies that have investigated its expression during pregnancy. Goldman et al., found good expressions of PRs in amnion and decidua before contraction begins and it rapidly decreased after contraction commenced and dominant isomorph PR-C was also found to exist in amnion although its role in fetal membrane mechanism is not clear yet (Goldman et al., 2005).

1.4.5 Risk factors for preterm birth:

1.4.5.1 Infection:

Clinical or subclinical intrauterine infection is considered as one of the main etiological factors of PPROM pathogenesis and plays a key role in neonatal morbidity (Simhan et al., 2005). According to some studies, fetal membrane rupture due to intrauterine infection may be the result of various mechanisms where a variety of bacteria and microorganisms triggers ECM degradation. An enzyme called protease secreted by them weakens the fetal membrane by damaging its collagen framework (McGregor et al., 1993, Draper et al., 1995).

Bacteria from vagina can pass through cervical canal and reach the fetal membrane in an ascending fashion which then infects the amniotic fluid and fetal blood. Another route of infection could be microorganisms from maternal blood entering the fetal membrane. Also, it could be iatrogenic during a procedure or may be through the opposite direction through the fallopian tubes. However, the most common way is the ascending direction and mostly occurs in second semester (Goldenberg et al., 2008).

For a long time, researchers argued whether intrauterine infection was responsible directly to trigger PPROM or whether it was a result of it (Parry, 1998). Few studies have confirmed the presence of microorganisms in vaginal flora immediately after membrane

rupture. They also described that the bacterial infection is one the obvious risk factor associated with the pathogenesis of preterm birth (McDonald et al., 1994, Carey et al., 2005). Goldenberg and colleagues discovered a 3-fold higher risk of premature labour (less than 30 weeks) with bacterial infection compared to the spontaneous labour (Goldenberg et al., 2000).

Chorioamnionitis-an inflammatory process was found to be associated with a high risk of preterm birth and major neonatal complications. A major study led by Alexander and group, found increased rate of PPROM among women with chorioamnionitis as well as a various neonatal morbidities such as sepsis, respiratory problems, and seizure within 24 hours of birth (Alexander et al., 1998). Bacteria are known to activate the production of matrix-degrading enzyme and bacterial toxins may be the source of membrane rupture prematurely. McGregor et.al reported a downfall in tensile strength and elasticity of the fetal membrane after coming in to contact with the bacterial collagenase and collagenase producing microorganisms' *ex-vivo*. They also suggested that these factors lead towards early membrane rupture. They also noticed no significant effect in the control group where there was no exposure to the bacterial toxins (McGregor et al., 1990, Menon et al., 2007).

Although they used a model study, McGregor and colleagues showed that the quantity of bacterial end products found in the amniotic fluid was not enough to create a toxic effect ex vivo. Antibiotic therapy was found to be successful in minimising this effect; however, it was still not able to limit the risk of PPROM (McGregor et al., 1993). Recent studies hypothesized that the bacterial infection was not directly associated with premature labour and PPROM, but, it acted as an initiator while the main contributory factor was believed to be inflammation process (Menon et al., 2007).

Inflammatory response as a result of bacterial infection could possibly be a pathophysiological factor which might be directly or indirectly associated with preterm premature rupture membrane. Following infection, neutrophils and macrophages were recruited to the infected site and these cells secrete cytokines, matrix metallo-proteinases (MMPs) and prostaglandins, which initiates inflammatory response. Cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alfa influences MMPs expression (Parry, 1998). An animal model study reported that increased production of TNF-α, PGE2, IL-6, IL-8 and CCL2 in amniotic fluid can lead to preterm birth (Waldorf et al., 2015). Waldorf and colleagues found TNF-α or thrombin exposure to fetal membrane caused membrane weakness by increased levels of MMPs. Infection/inflammation is one of the known associations of PPROM (Kumar et al., 2014). TNF-α and IL-1β were shown to enhance the MMP 9 level and PGE2 production in chorioamnion, which triggered to apoptosis leading to PPROM (Ferrand et al., 2002, Barrett et al., 2019).

1.4.5.2 Apoptosis:

Apoptosis or programmed cell death is a normal process associated with ECM remodelling. Amnion epithelial cells undergo the programmed cell death towards the active onset of labour. This apoptosis seems to trigger the beginning of degradation of ECM during labour (Menon et al., 2004). Early death of epithelial cells can lead to PPROM, which is mainly initiated by infection. Cytokines and TNF-alfa play a major role in premature apoptosis, hence, resulting in PPROM (Lannon et al., 2014). Leppert et.al noticed many apoptotic cell deaths in amnion and chorion of PPROM membranes. They found them predominantly near the ruptured areas and few around other parts of the membrane. They have also reported that in membranes affected with chorioamnionitis, granulocytes were found attached to the dead amniotic epithelial cells. So, immune response could accelerate the apoptosis in the fetal membrane (Leppert et al., 1996).

Oxidative stress upregulated by reactive oxygen species initiates apoptosis in the chorioamnion tissue increasing collagen degradation thus causing FM weakening and rupture (Polettini et al., 2018, Barrett et al., 2017).

1.4.5.3 Membrane stretch:

Membrane stretch is noted to be associated with upregulation of pro-inflammatory cytokines (interleukins) and proteolytic enzymes in amniotic fluid and FM with pathological failure of fetal membranes (Fortunato et al., 1997, Scott et al., 2017, Polettini et al., 2018). In different studies, investigators have assumed that the shear force and membrane stretch induced by the uterine contraction during labour could be associated with PPROM. Fetal membranes are subjected to continuous tension and stretching all through the gestation. The maximum stretching occurs during term and it reaches to peak during uterine contractions (Maradny et al., 1996). Hence, it is believed that repetitive stretching during the end of gestation and contractions facilitates FM weakness progressively in normal labour. According to Moore et al, as shown in Figure 1.9, the fetal membrane weakening and rupture occurred through two biological processes. Firstly, the alteration in physical properties of fetal membrane through apoptosis and programmed biochemical changes and secondly, through physical property changes via stretch force which then induces further apoptosis and other biochemical changes. (Figure 1.9) They also mentioned that the fetal membrane strength could be restored and weaken membrane could be reserved by the help of biochemical or physical agents (Moore et al., 2006).

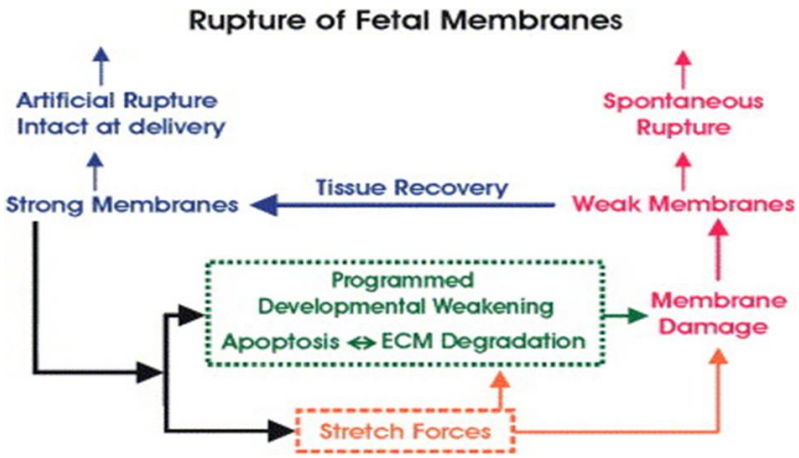

Figure 1.11: Diagram of Possible pathways of stretch related membrane weakness. (Images has been taken from Moore et al., 2006) Fetal membrane weakness and rupture can happen due to programmed biochemical pathway. Also, another factor to consider is the utero stretch force which damages the fetal membrane by inducing the transcription factors and activating the proteins that promotes apoptosis.

There are no specific stretches patterns that have been confirmed to be associated with fetal membrane rupture. However, numerous *in vitro* studies have investigated the effects of the force generated from repetitive stretch regulated by mechanotransduction pathways leading to FM rupture (Mohan et al., 2007, Pandey et al., 2007, Chowdury et al., 2014).

Mechanotransduction is the process where cell senses external or physical forces and converts the mechanical stimuli into biochemical signals which control the specific biological responses (Paluch et al., 2015). Signalling triggered by mechanotransduction plays a major role in tissue remodelling and performing functions of human fetal membrane. Different type of expression pattern of Integrin subunits and extracellular matrix ligands of fetal membrane cells have been identified (Divers et al., 1995, Kendal-Wright., 2007). Calcium channel is an important mechanotransduction (Singh et al., 1986). Calcium flux can trigger multiple biochemical synthesis including cyclooxygenase2 (COX2)

(Kusama et al., 2015). Nifedipine is a type 2 calcium channel blocker that inhibits the inward flow of calcium across the L‐type slow channels of cellular membranes (Young et al., 1993, Smith et al., 2000). Currently nifedipine is used in the prevention of preterm delivery. Fetal membrane stretching was found to induce production of other inflammatory markers like, prostaglandin E2, interleukin-8 and cyclooxygenase2 (Cox-2) (Yoshida et al., 2002, Maehara et al., 1996, Mohan et al., 2007, Wright et al., 2007, Chowdury et al., 2014). Furthermore, prostaglandin upregulated the MMP 1 and MMP 3 secretion by fibroblasts and reduced the formation of membrane collagen. (Maradny et al., 1996, Parry, 1998) Interleukin-8 of chorioamnion was found to increase collagenase activity. Hence, these biochemical changes which increases the risk of preterm membrane rupture appears to result from membrane stretching (Parry et al., 1998). However, some studies ruled out any association of stretching to fetal membrane weakness and rupture (Pandey et al., 2007). It has been reported uterine over distention is associated with preterm pregnancies in women twin or more fetus. The proposed mechanism was linked with inflammation and the inflammatory events, which aggravated when mechanical stress was high and did lead to preterm rupture (Waldorf et al., 2015). Overall, biochemical pathways and stretch possibly could lead to weakening of the fetal membrane, further investigation is necessary. Barrett (2018) proposed a pathway which demonstrated how stretch activation of protein expression in amnion side and led to ECM degradation and membrane weakening, eventually FM rupture. Stretch induced calcium channel upgrading can combine the proposed pathway (Figure 1.12).

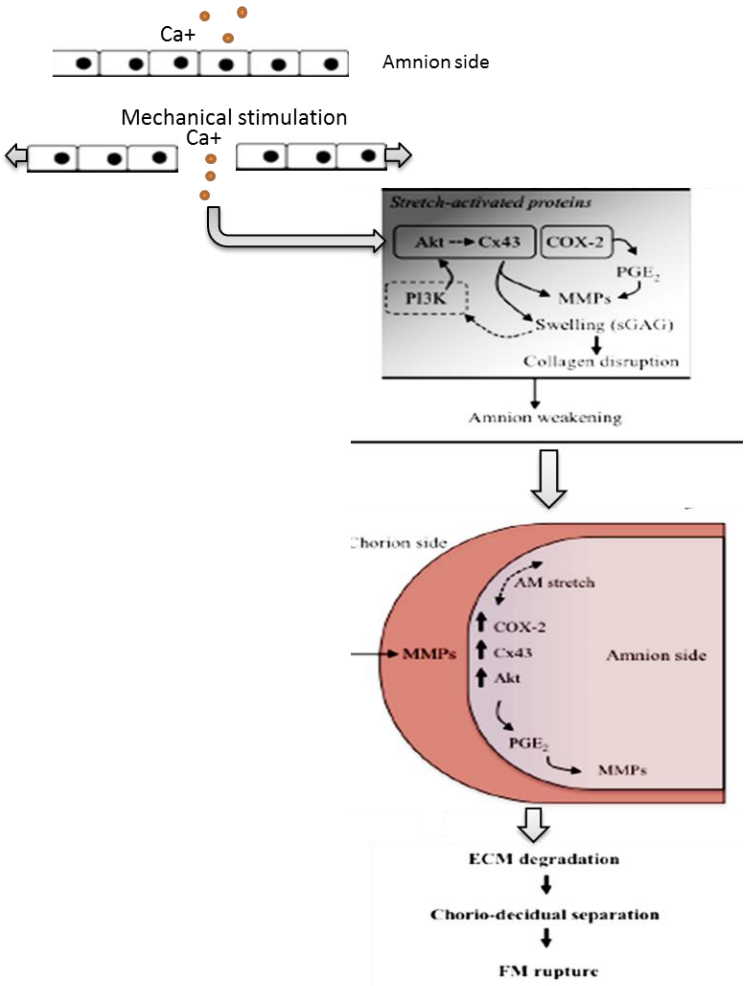

Figure 1.12: Diagram of stretch activated potential mechanotransduction mechanisms leading to membrane rupture. Fetal membrane stretching activated Calcium channel. Calcium flux then triggers the Cx43, COX-2 upregulation, then pro-inflammatory activation. Elevated MMPs and PGEs could be cause of collagen disruption. All these events together may lead to fetal membrane rupture via degradation of ECM and amnion-chorion separation. (Barrett, 2018 modified image)

1.4.5.4 Maternal smoking:

Maternal cigarette smoking during pregnancy is another well-known risk factor for preterm birth and PPROM. It is not only associated with maternal and neonatal morbidity but is also associated with stillbirth. (Shah et al., 2000, England et al., 2013) Recently, few

epidemiological studies have investigated the connection between maternal smoking and preterm and early preterm birth (Cnattingiuset al., 1999, Burguet et al., 2004). A significant association has been observed. England et al studied nearly 18,000 women in Canada and found that heavy smoking (10 cigarette/day) significantly increased the risk of PPROM (England et al., 2013). Another controlled trial review concluded that maternal smoking increased the risks of pregnancy related complications, low birthweights and premature births. They also reported that the intervention during pregnancy not only helped in reducing preterm birth but was also effective in controlling some percentage of women to stop smoking during their pregnancies. (Lumley et al., 2009) On the other hand, some other studies have not found any apparent relationship between smoking and preterm birth (Peacock et al., 1995, Ancel et al., 2000). Although negative effect of maternal smoking on pregnancy and its association with new-born complications including preterm birth has been known for long, the mechanism involved is still not clear.

There are a number of toxic chemical compounds present in cigarette such as nicotine, carbon-monoxide (CO) and various metals. Some of them are biologically toxic agents. Of these agents the one responsible most for the adverse effect of maternal smoking is yet to be identified. There are only a handful studies that have explored the association between PPROM and maternal smoking (EKWO et al., 1993, Nabet et al., 2007, England et al., 2013). However, according to Mercer et.al and Dekker et.al maternal smoking was not a significant factor of PPROM risk (Mercer et al., 2000, Dekker et al., 2012). Therefore, though the harmful effects of maternal smoking towards preterm birth and PPROM are recognized, there is room for further investigation and also to look at the mechanism behind this association.

1.4.5.5 Diabetes:

Diabetes mellitus or DM is one of the metabolic disorders characterised with persistent high sugar levels in blood stream. An imbalance in metabolism of carbohydrates, fatty acids and proteins occurs when body fails to produce absolutely no or relatively low insulin due to DM. Diabetes is one of the most common medical conditions worldwide. (Mozurkewich et al.,1999, Kari et al., 2017). Gestational diabetes or GDM is a type of diabetes which appears during pregnancy. Generally, it develops during second or third trimester of gestation (Joseph et al., 2018). The prevalence rate of diabetes (DM and GDM) during pregnancy is increasing. According to one recent survey, approximately 17% of all pregnancies worldwide were recognised to be complicated with hyperglycaemia and in most of cases it was GDM (Goldenberg et al., 2016). Diabetes during pregnancy has several maternal, fetal and neonatal complications including risk of pre-eclampsia and caesarean section delivery and some neonatal complications such as shoulder dystocia, macrosomia, respiratory problem, jaundice and infection. (Suhonenet al., 1993, Ray et al., 2001, Lao et al., 2003, Kari et al., 2017). Previous studies have reported direct association of maternal diabetes and preterm deliveries as well as PPROM and related complications (Kock et al., 2010, Riyami et al., 2013). One British survey in 1982 reported that diabetes or gestational diabetes is a major contributor for preterm delivery. They noticed 50% of the preterm deliveries (<37 weeks) were complicated by diabetes and 22% of them were due to gestational diabetes. (Beard et al., 1982). However, their observations were debatable and DM considered as an independent risk factor in literature as some studies have not found any significant risks of preterm births or PPROMs in diabetes women (Lao et al., 2003, Nordin et al., 2006). Therefore, it is still not clear whether diabetes has any association with preterm labour or PPROM. GDM is linked with lifestyle and other factors including low income, race, maternal age, family history of DM, etc (Moosazadeh et al., 2016). Obesity and infection are the two key consequences associated with maternal hyperglycaemia which possibly lead to fetal and neonatal complications through

premature deliveries (Ray et al., 2001, Kari et al., 2017). However, the effect of diabetes during pregnancy on PPROM or preterm birth is not clear yet. Nevertheless, it is clear that treating and or preventing maternal hyperglycaemia can be beneficial both medically and economically. There are not many studies investigating the association of hyperglycaemia with PPROM.

1.4.6 Other potential risk factors and association:

Maternal age is believed to be associated with maternal complications including preterm birth. Advancing maternal age is attributed to increased risk of premature birth. However, there is no consistent evidence (Fuchs et al., 2018).

Placental abruption related vaginal bleeding is one of the strong risk factor of preterm birth. Any type of vaginal discharge during pregnancy was identified as a potential risk for PPROM. Patients with abnormal vaginal discharges during pregnancy showed almost 3-fold increased risk of PROM (Tarek et al., 2012). This might be due to bacterial infection or short cervix (Assefa et al., 2018).

Shorter cervical length is a potential risk factor for preterm delivery and PROM. Ultrasound and digital examination of cervix have proved that shorter cervix brings greater risk of preterm deliveries (Andrews et al., 2000, Goldberg et al., 2008). The association of PROM with past history of cervical operation and cervical incompetence has been observed (Kaye et al., 2003).

Previous history of PROM is known to associate strongly as a risk factor further preterm labour and spontaneous preterm rupture of the membranes. Recurrent PPROM also inversely proportioned with the gestational age with increased chance of another PPROM (Romero et al., 2007). The mechanism of this association is unclear but other intermediate factors like diabetes, hypertension, and obesity are more likely to be

involved (Goldberg et al., 2008). Also, previous history of abortion (Assefa et al., 2018) and multiple gestations (Meis et al., 1998) is believed to be a risk factor for preterm birth and PROM.

Nutrition as a factor appears to have a role in preterm birth. Women with normal ranges of concentration of minerals like iron, zinc and floride in serum have less chance of preterm deliveries than the women with low concentrations of serum minerals (Tamura et al., 1996). The possible pathology of this could be the decreased volume of blood and blood flow in malnourished mothers. Another possible mechanism could be the maternal infection which develops because of the low concentration of vitamins and minerals in the maternal body (Neggers et al., 2003, Goldberg et al., 2008). The risk of preterm birth is also higher in obese or women with high BMI mostly as it can develop pre-eclampsia and diabetes in mother (Goldberg et al., 2008).

Other risk factors including maternal race and lower socio economic status have been associated with preterm births. In United Kingdom and United States, Afro Caribbean or African American mothers are known to have higher risk of premature deliveries and PROM than women of white race (Haug et al., 1993, Goldberg et al., 2005). Even the rates of very preterm births are also much higher in black women than other groups of women (Goldberg et al., 2005). That may be because bacterial vaginosis is more common in black women compared to the white women (Fiscellaet al., 1996). Low social economic and educational status is also considered as an associated risk factor of preterm birth (Smith et la, 2007, Brett et al., 1997). Single marital status and stressful long hours at work are also accounted to increased risk of preterm birth. However, the mechanisms behind the maternal demography related risk factors on shortening gestational age or preterm rupture of the membranes is not clear (Goldberg et al., 2008). The relation between these factors and PPROM has no or conflicting evidences in literature.

Hypertension is one of the factors known to have a significant link with the preterm birth and other complications. Chronic hypertension was found to increase the risk of preeclampsia and preterm preeclampsia (Catov et al., 2007). So, it can be explained that the association of hypertension with the increase risk of pre labour might be related to preeclampsia, vascular damage and higher BMI (Catov et al., 2008).

Maternal anaemia is considered as a potential risk factor of premature deliveries. That could happen through various pathways like maternal infection because of iron deficiency, hypoxia due to low haemoglobin level and hypoxia induced by oxidative stress (Falkenberg et al., 1999, Zhang et al., 2009). Anaemia in early pregnancy showed a significant risk of PPROM (Zhang et al., 2009).

Oxidative stress (OS) is another possible factor which is associated in PPROM pathogenesis. Premature membrane damage by oxidative stress can be caused by various ways including collagen weakening, MMP 9 introduction, DNA break down, and catalytic enzyme secretion. [77] Towards term, because of augmented physiological stress and increased metabolic activities, oxidative stress starts building up and triggers membrane weakness. [78] However, further research is required to determine the role of these factors.

1.5 Treatment options:

There is no active preventative strategy for PPROM yet. Generally, after PPROM, preterm labour starts almost immediately. In rare cases, it could be delayed up to few days. The available treatments are mainly aided to slow down the preterm labour or speed up fetal lung maturity before term. One of the standard treatments is taking corticosteroid (healthlinkbc.ca).

1.5.1 Dexamethasone:

Dexamethasone is a corticosteroid and an anti-inflammatory drug which is commonly used for anticipatory preterm birth between 24 and 34 weeks of gestation. If fetal membrane is not intact, then treatment with dexamethasone is recommended between 24 and 32 weeks (Briggs et al., 2006). It is effective to reduce the respiratory problems in new born (Crowley, 1995) and preventing other neonatal mortality and morbidity. Pattinson et al., have done one randomised trial with PPROM patients who received dexamethasone. They found reduced or no risk of infection in mothers or babies. They reported a good trend of perinatal health improvement (Pattinson et al., 1999). One dexiprom study group has noted a clear benefit of using dexamethasone in PPROM women for their new-borns (Pattinson et al., 1999). Although there are some concerns about using dexamethasone, according to Ogueh et al., it can affect significantly on placental endocrine concentration which is a risk (Ogueh et al., 1999). Crowley et al have reported risk of increasing infection, neonatal adrenal suppression, pulmonary distress and imbalance of blood glucose control. (Crowley, 1995) Although some studies have shown positive result on prevention of preterm birth complications in neonatal health, there was no evidence of the effect of dexamethasone on risk for preterm deliveries or PPROM. Therefore, further studies are required to clarify and understand the efficacy of dexamethasone and its working mechanisms.

1.5.2 Calcium channel blocker:

Calcium channel blocker is widely used for treatments of patients with hypertension and angina pectoris. Calcium channel blocker is commonly used for 'pregnancy induced hypertensive disorder' and recently gaining interest in preterm labour managements. Nifedipine is a dihydropyridine based L-type calcium channel blocker found to prolong the pregnancy and minimise the neonatal respiratory problems (Papatsonis et al., 2001) in a randomised trial. Many current meta-analysis studies proposed that calcium channel

blocker as tocolytic therapy of preterm birth would be efficient (Lamont et al., 2019, Ali et al., 2019). However, more studies are required to certify the conclusions. Also, the efficacy on PPROM has not been investigated yet.

1.5.3 Progesterone supplements:

In 2011, the FDA has approved progesterone as a supplementation for women at risk of preterm birth due to PPROM and it has been shown in some but not all studies to decrease premature birth (fda.gov, 2011).

1.5.4 Other options:

Other interventions such as use of antibiotics, hydration, tocolytic therapy have found to delay the delivery for few hours but not more than 48 hours to prevent preterm delivery (norwitz et.al 2011). Added options which included in treatment management are, close observation with general measures, antibiotics to help treating the infection in amniotic fluid, if there is an evidence of infection, another option is expediting labour and delivery to get rid of the risk of infection(healthlinkbc.ca).

1.6 Mechanical test methods:

Knowledge of mechanical characterisations of the connective tissue is vital to understand their functional and structural influences. In vivo performed mechanical testing methods for soft human tissues carries limitations of instrumentation and illustration. Ex vivo studies are relevant. For in vitro study ideal design of equipment is necessary to enable tissue specific characterisation.

Based on the literature, four types of mechanical tests have been performed so far to describe the membrane strength ex vivo. They are a) Uniaxial tensile test, b) Planner

biaxial tensile test c) puncture test and d) inflation or burst test. These methods have some advantages and disadvantages to study PPROM.

1.6.1 Uniaxial tensile test:

The uniaxial tensile test is simple and straightforward. In this test, rectangular membrane strips with specific measurement were placed inside the grips and were pulled uniformly till it ruptured (Figure 1.13) (Moore et al., 2006). The uniaxial test is simple to perform but it fails to mimic the loading condition of the fetal membrane *in-vivo*. The membrane inside is biaxial loaded by the force of amniotic fluid.

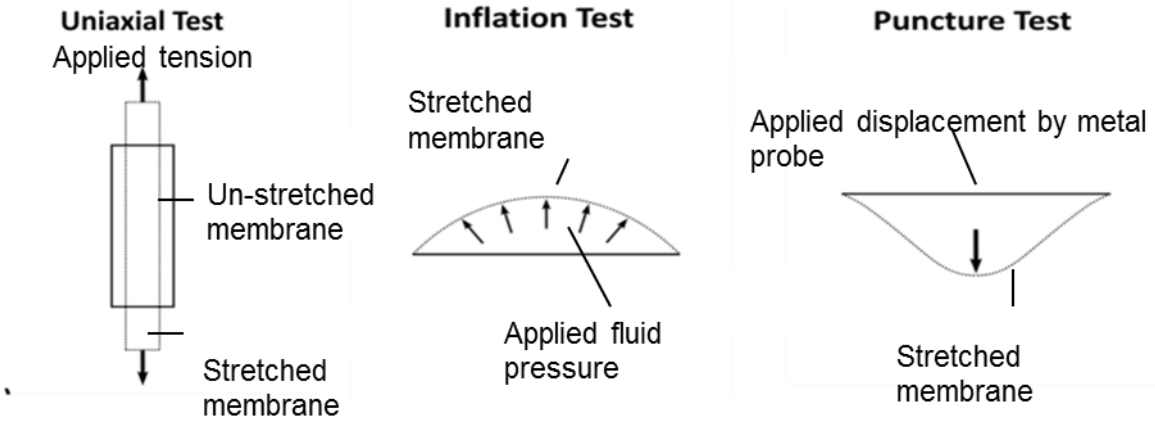

Figure 1.13: Schematic images of different types of mechanical tests for fetal membrane study. (Perrrini et al., 2016, modified)

1.6.2 Planner bi-axial tensile test:

This test is done using samples in square shape and mechanically pulled from four sides with equal stress. However, not many studies used this test method on the chorioamnion membrane. Joyce et.al used this method to explore the reason behind mechanical failure

of the fetal membrane. They studied the membrane stiffness and tension specially. They have reported that this method is suitable to illustrate the sub-failure of the membrane "forces below that which induces rupture" (Joyce et al., 2009). This method is not very suitable to test fetal membrane as it is very thin and slippery.

1.6.3 Burst test:

The biaxial burst test or inflation test is very close to mimicking the membrane physiology in terms of the mechanical deformation and fluid pressure in vivo. A piece of membrane is placed in an upright manner and is inflated with air or any fluid pressure from other side until it bursts (Figure 1.13). Though the inflation test is very close to the physiological environment of the body, a drawback of the inflation test is the size of the apparatus which is big and therefore a larger membrane sample is required for testing. Hence, sometimes it fails to give a convincing outcome (Moore et al., 2006).

1.6.4 Puncture test:

The puncture test overcomes this problem, where the membrane piece needed can be small in size and it is comparatively simple to use. In this method, circular small pieces of membrane are held tightly. A sharp metal probe is used to puncture in the middle of the membrane with a functional load until the membrane ruptures (Figure 1.13) (Mauri et al., 2015). However, the force is very locally applied to the membrane. Most recent studies have used this method. Recently, Bürzle et al., analysed the deformation behaviour and rupture sequences of the FM tissue by using puncture testing. This method is able to provide the biaxial loading condition (Bürzle et al., 2014).

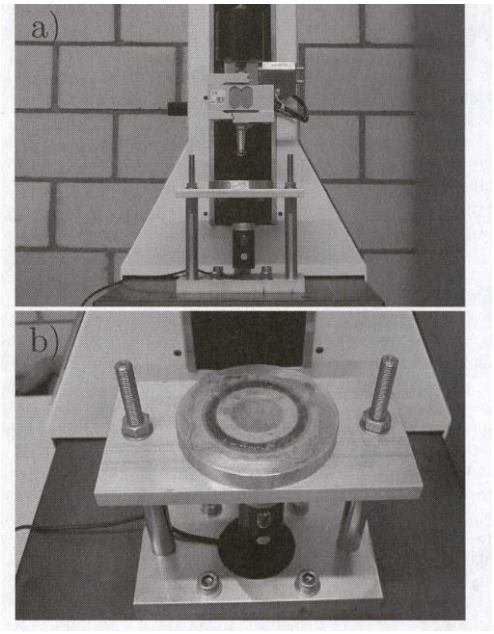

Figure 1.14: Puncture test set up. (a) The whole set up (b) sample fixation. (Bürzle et al., 2014)

One of the arguments against this method was the direction of the load as the applied force to puncture the tissue deforms in the opposite direction in comparison to in vivo condition. However, Schober et al., clarified that confusion. They tested the strength and the deformation of the tissue with fixed diameter and used probes with different sizes. According to their study, data from puncture test were equivalent to the data from burst test when ratios of needle diameter to the diameter of the membrane fragment were adjusted (Moore et al., 2006, Schober et al., 1994). Despite this, locally applied force to deform the sample failed to match the in vivo loading force.

Further on, similar experimental test models with minor modifications were developed for studying the mechanical properties of fetal membrane or the effect of mechanical force leading to FM rupture. Of note, all of these were based on one or other principles of the above methods (Pandey et al., 2007, Buerzle et al., 2014, Chowdhury et al., 2014, Perrini et al., 2015).

1.7 Methods for characterisation of connective tissue:

The collagen arrangement in the connective tissue determines the mechanical properties of any biological tissue including fetal membranes. Thus, alteration in distribution of connective tissue can disrupt the normal function of the tissue. Methods have been developed and being updated to characterise the quantity, crosslinking and arrangement of collagen fibres.

1.7.1 Polarised light microscopy:

Polarised light microscopy technique has been used to identify an unknown material since the last century. This technique is also widely used for imaging the collagen fibre alignments in the tissues. An electromagnetic light wave passes through polarising filter of the microscopy vibrating at right angle to the direction, strikes the surface of the sample and the birefringence of the sample is measured (Frendsen et al., 2016, Sally et al., 2008). This technique is quick and easy to observe the optical properties of the sample. It is highly sensitive and can produce high contrast images. Another advantage of using this technique is its cost-effectiveness. This optical imaging technique is the first established technique which was used to characterise the alignment of collagen architecture of human cartilage (Benninghoff et al., 1925). Since then, to investigate the collagen structure of the tissue, polarised light microscopy has been widely used (Sally et al., 2008). Linearly used polarised light has drawback to visualise all the parts of collagen fibres whereas circularly polarised light eliminates that problem and offers better images of all parts of collagen fibres (Rich et al., 2005). Although the circular and linear polarised light ability to determine the different characteristics of the collagen structure in both orientation and molecular level is performed easily through the examination of polarised light interaction, in most cases it caters to qualitative based result which can also be semi quantified (Vidal et la, 2003). Advance level of quantitative polarised light microscopy can

be done but it requires a complex computer based calibrations which could be an issue (Xia et al., 2001, Bi et al., 2005). Therefore, the disadvantage using this technique may be the error in interpretation of low resolution and magnification light produced qualitative or semi quantification images.

The polarised light microscopy with the combination of picrosirius red staining can improve the quality of the images and can provide enhanced assessment of collagen content (Whittaker et al., 1994). Sirius red dye dissolved in saturated picric acid has a unique anisotropic molecular organisation like collagen, so birefringence of collagen is enhanced and view becomes bright under polarised light and thus, both thin and thick fibres can be identified easily (Puchtler et al., 1973, Whittaker et al., 1994). So, for analysing the collagen structure of biological tissues, polarised light microscopy combined with picrosirius red staining serves a powerful tool.

1.7.2 Quantification of collagen:

The orientation and distribution of collagen in tissues are altered under certain situations like abnormal pathology, damage or accident which can change the biological function of a particular tissue. Quantification of collagen level and collagen distribution has a potential role in identifying the problem in timely fashion (Coentro, et al., 2017). Determination of collagen in the tissue is not easy as the extraction procedures are limited. Commonly used method for collagen quantification is measuring hydroxyproline after hydrolysis of the tissue via HPLC. However, this method is time consuming, labourious and needs a special set up for acid removal. Hydroxyproline based collagen assay is simple and convenient method for collagen quantification (Hanemaaijir et al., 2011).

Hydroxyproline is a non-photogenic amino acid which is exclusively present in collagen (Cissell et al., 2017). It is synthesised during collagen biosynthesis via post-translational hydroxylation of another collagen specific amino acids called proline. Hydroxyproline concentration is associated with the collagen metabolism hence physiological and pathological changes of the tissue. Therefore it is an important indicator to find out any disorder or disease related to collagen modification like PPROM. Thus, measurement of hydroxyproline by assaying it for collagen quantification is one of the useful methods to investigate collagen malfunction of the tissue (Qiu et al., 2014).

Another approach is Picrosirius red assay. Sirius red dye specifically binds the soluble amnio acid residues of collagen fibres and does not bind with the non-collagenous proteins in most of the cases (Cissell et al., 2017). Bright field microscope displays red stained collagen fibres on pale yellow background. The intensity of the staining reveals the quantity of the collagen present in the biological tissue.

1.8 Aims and objectives of the project:

The aim of this thesis is to gain better understanding of the mechanisms of preterm birth and PPROM. New and bio mimic imaging techniques which can measure fetal membranes mechanical properties and their response to the external force have been explored and adopted. The associated biochemical molecules expression and alteration have been measured alongside the known risk factor analysis. We hypothesize that the PPROM membranes exhibited higher heterogeneity in mechanical properties and expressed different level of selected biochemical molecules in comparison to full term. In addition, the sensitivity of the changing biochemical molecule expression in response to external force in full term and PPROM membranes was quite distinct. The thesis is structured with four experimental chapters aimed to reveal the heterogeneity in mechanical properties and expression level of selected biochemical molecules in full term and PPROM

membrane, as well as the sensitivity of the changing biochemical molecules triggered by external force.

Chapter 3: Heterogeneity of biomechanical properties of fetal membranes from full term and PPROM

Aim- we aimed to obtain better understanding of the membranes' mechanical properties of fetal membrane which were unable to gain using other experimental approaches. We studied the mechanical properties between the sublayers of fetal membrane and full term and preterm deliveries membranes.

Hypothesis- There are heterogenic mechanical properties in different area of fetal membranes, especially preterm membranes.

Objective- This chapter developed and adopted two new biomimic imaging techniques to study the heterogeneity of mechanical properties between the sublayers of fetal membrane and full term and preterm deliveries membranes.

Outcome- The ball indentation test and OCE/OCT techniques have been established and applied successfully. This study has clearly shown a large difference in the mechanical properties between ruptured and non-ruptured sites of preterm membranes, as demonstrated by creep, modulus and thickness analysis.

Chapter 4: Expression level of key biochemical markers in fetal membranes from full term and PPROM

Aim- We aimed to explore the correlations between key biochemical molecules expression level to mechanical and microstructural properties of fetal membranes in full term and preterm rupture.

Hypothesis- There are correlations of biochemical expression level of FM from full term and PPROM, and different locations.

Objective-This chapter looked at expression level of key biochemical markers as well as collagen and sGAG concentration in the different areas of membranes and between full term and preterm ruptured membranes.

Outcome- This chapter has clearly demonstrated that there was bigger heterogeneous expression pattern between rupture and non-rupture sites in preterm membranes than in full term and preterm. This was mirrored well with the biomechanical property in full term and preterm membranes measured in chapter 3.

Chapter 5: Induction of expression change of biochemical molecules in membranes by external force and maternal risk factors

Aim- The aim of this study was to reveal the sensitivity of the changing biochemical molecule expression level in response to the external force (mainly fetus movement) between full term and PPROM membranes. Further aim of this chapter was to explore the risk factors like maternal smoking and maternal hyperglycaemia ex vivo. Additionally, we aimed to verify the efficiency of dexamethasone for controlling the biochemical upregulation.

Hypothesis- The fetus originated force upregulates inflammatory cytokines, MMPs and fibronectin through mechanotransduction, resulting in membrane weakness. Full term and PPROM membranes may respond differently. The effect of risk factors in the presence of the external force could be a triggering factor for preterm rupture too.

Objective-This chapter developed a new experimental set up mimicking external force exerting to membranes, which induced the expression level change of key biochemical molecules in the membranes. To verify the mechanotransduction role, calcium channel

blocker was applied to abolish the influence of external force. We also examined the effect of risks factors in the presence of force by the help of the new set up.

Outcome- In this chapter, an in vitro loading model for the study of biochemical behaviours of FM in the presence of external force was successfully established. This revealed that force originating either from the baby or fetal fluid can accelerate the production of matrix degradation enzymes. The downstream expressions of biomarkers by calcium channel blocker suggested that the calcium signalling pathway may be the point of integration of mechanotransduction in FM cells. In addition, maternal smoking correlated with membrane weakness as it responded to elevated biomarkers. Diabetes did not show direct correlation under the influence of external force, but diabetic maternal membrane can be a risk for PROM. Finally, decreased expressions of membrane weakening biomarkers under the influence of dexamethasone revealed its potentially beneficial effects to reduce the risk of FM rupture

Chapter 6: Correlation of known potential risk factors with PPROM

Aim- This chapter aimed to correlate the known risk factors of preterm birth and PPROM to the collected FM samples.

Hypothesis- There may be correlations of clinical history of the samples and risk factors in preterm rupture of the membrane.

Objective- The selected risk factors include the mode of delivery, maternal smoking, and previous history of preterm delivery, maternal age, mode of delivery and diabetes during pregnancy was studied via case histories of the samples.

Outcome- There were potential relationships between risk factors and preterm birth. This chapter has definitely helped in better understanding of pathology of PPROM and preterm birth

Chapter 2

Material and method

I. Materials:

Materials that were used for this research are listed in table 2.1:

Materials	Catalogue number	Suppliers
1,9-Dimethyl methylene blue (DMMB)	341088	Sigma- Aldrich ,UK
4',6-diamidino-2-phenylindole (DAPI)	D9542	Sigma-Aldrich ,UK
4-20% Tris-HEPES Protein gels	NH21-420	Novex-Life Technologies, UK
4-20% Tris-HEPES Protein gels	NH31-420	Novex-Life Technologies, UK
4-20% SERVAGel™ TG PRiME	43277.01	SERVA,UK
Acetic Acid	537020	Sigma-Aldrich,UK
Acetone, for analysis	A/0600/PC21	Fisher chemical, UK
Anti -GAPDH antibody	Ab8245	Abcam
Anti-Goat IgG, HRP- linked antibody	7078P2	Cell Signalling Technology ,UK
Anti-mouse IgG, HRP- linked antibody	7076P2	Cell Signalling Technology ,UK
Anti-Rabbit IgG, HRP-linked antibody	7074P2	Cell Signalling Technology, UK
Ascorbic Acid	A8960	Sigma-Aldrich ,UK
Bicinchoninic acid assay (BCA protein assay) Kit	23225	Thermo-Fisher-Scientific ,UK
Bovine Serum Albumin (BSA)	A2153-50G	Sigma-Aldrich, UK
BupH™ Tris-HEPES-SDS Running Buffer	28398	Thermo-Fisher-Sceintific,UK
Chloroform	288306	Sigma-Aldrich, UK
Anti-Collagen I antibody (Rabbit monoclonal IgG)	ab138492	Abcam
Anti-Decorin antibody(Goat polyclonal)	ab189364	Abcam
Anti-Biglycan antibody (Goat polyclonal)	ab58562	Abcam
COL3A1 Antibody (mouse monoclonal antibody)	sc-271249	Santa Cruz Biotechnology
Dexamethasone	D4902	Sigma-Aldrich, UK

Dimethyl Sulfoxide (DMSO)	D2650	Sigma-Aldrich,UK
Dulbecco's Modified Eagle Medium(DMEM)	BE12-707F	Lonza,UK
Donkey anti-rabbit polyclonal Antibody	Sc2089	Santa Cruz Biotechnology

DPX Mountant	44581	Sigma-Aldrich, UK
Ethanol	E0650/17	Thermo- Fisher Scientific, UK
Ethylenediaminetetraacetic acid(EDTA)	E6758	Sigma-Aldrich, UK
Eosin		Sigma-Aldrich, UK
Formaldehyde solution	F8775	Sigma-Aldrich, UK
Fibronectin Antibody	sc-69681	Santa Cruz Biotechnology
Goat anti-mouse monoclonal antibody	Sc-16516	Santa-Cruz Biotechnology,UK
Goat anti-mouse IgG, poly HRP secondary antibody, HRP	32230	Thermo-Fisher Scientific.UK
Goat anti-rabbit IgG secondary antibody	A-11037	Thermo-Fisher Scientific, UK
Halt™ Protease Inhibitor Cocktail (100X)	78429	Thermo-Fisher Scientific, UK
Haematoxylin		Sigma-Aldrich, UK
Histo-Clear	NAT1334	Scientific Labouratory Supplies
Hydrochloric acid (HCL)	H1758	Sigma-Aldrich, UK
Hydroxyproline Assay Kit	MAK008	Sigma-Aldrich, UK
Live/Dead assay kit	L3224	Thermo-Fisher Scientific, UK
Industrial Methylated Spirit (IMS)	I 99050	Genta Medical,UK
IL-1β Antibody (rabbit polyclonal IgG)	sc-7884	Santa Cruz Biotechnology
IL-6 Antibody(mouse monoclonal IgG)	sc-32296	Santa Cruz Biotechnology
Methanol	322415	Sigma-Aldrich,UK
MMP-9 Antibody(Goat polyclonal antibody)	sc-6840	Santa Cruz Biotechnology
MMP-13 Antibody (mouse monoclonal IgG)	sc-515284	Santa Cruz Biotechnology
Rabbit anti-mouse IgG B	Ab8517	abcam
N,N dimethyl formaldehyde	227056	Sigma Aldrich, UK

Nicotine	36733	Sigma Aldrich, UK
Nifedipine	N7634	Sigma Aldrich, UK
NOVEX sharp pre-stained protein ladder	LC 5800	Thermo-Fisher Scientific, UK
Novex™ Tris-Glycine SDS Running Buffer (10X)	LC2675	Thermo-Fisher Scientific, UK
NuPAGE™ MES SDS Running Buffer (20X)	NP002	Thermo-Fisher Scientific, UK
Optimal Cutting Temperature (OCT) compound	AGR 1180	Agar Scientific ,UK
PageRuler™ Unstained Broad Range Protein Ladder	4360954	Invitrogen, UK
Papain	P4762	Sigma Aldrich, UK
Paraformaldehyde	158127	Sigma Aldrich, UK
Phosphate Buffer Saline (PBS)	BE17-516F	Lonza, UK
Pierce BCA protein assay kit	23221	Thermo-Fisher Scientific, UK
Pierce™ 20X TBS Twin Buffer	28360	Thermo-Fisher Scientific, UK
Pierce™ ECL Western Blotting Substrate	32109	Thermo-Fisher Scientific, UK
Poly-L-Lysine coated slides	P0425	Sigma Aldrich ,UK
PR Antibody (rabbit polyclonal IgG)	sc-539	Santa Cruz Biotechnology
Radio immunoprecipitation assay buffer (RIPA)	89900	Thermo-Fisher Scientific, UK
Sodium Chloride (NaCL)	433209	Sigma Aldrich , UK
Sirius Red (Direct Red 80)	365548	Sigma Aldrich , UK
Toluidine blue	T3260	Sigma Aldrich , UK
Tris Buffered Saline with tween (TBST-10X)	SRE0031	Sigma Aldrich , UK
Xylenes	534056	Sigma Aldrich , UK

Table 2.1- List of names, catalogue numbers and supplier's names of materials used in this project

II. Methods:

2.1 Ethical Approval and sample collection:

Ethical approval of this study protocol was obtained from both national (National Research Ethics Committee, London) and local (University Hospital of North Midlands) authorities. All fetal membrane samples were collected according to the ethical committee guidelines of Research Ethics Committee (Reference 14/WM/1169). The study forms and R&D information sheets were designed by me and approved from clinicians at UHNM Trust. Written and oral consents were taken from each patient before collecting the sample by clinicians. The proforma for sample collection includes age, type of labour and other background factors including previous history of preterm, smoking, diabetes, hypertension etc. Of note, there was no provision for history of infection and chromosomal abnormalities. All samples were from singleton pregnancies. The potential risk factors were studied and associated with preterm premature rupture. Total 42 placentas were collected from patients of preterm (33-36 weeks) and full term (37-39 weeks) with normal vaginal delivery (full term- 12 samples and preterm-10 samples) and caesarean section (full term-10 samples and preterm-10 samples).

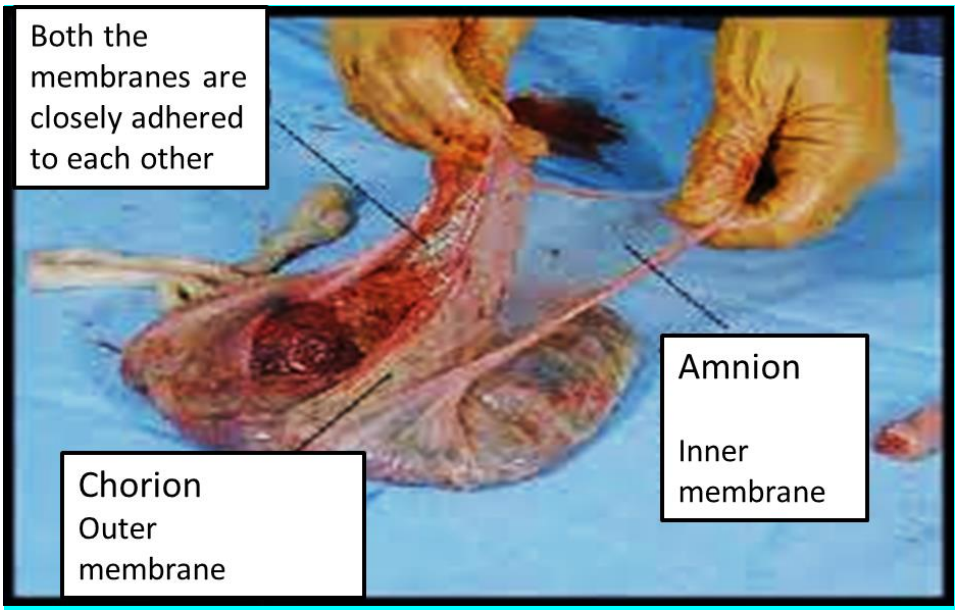

Figure 2.1: Whole placenta with fetal membrane. Intact membrane and two separated layers- Amnion and Chorion. Amnion is the inner layer and Chorion is outer layer. (Image was taken from is.muni.cz, 2016)

Total fetal membrane samples – 42 (From 42 different mothers)

Full term samples (37-39 weeks)- 22

Pre-term samples (33-36 weeks)- 20

Mode of delivery	Full term	Preterm	Experiments
Normal vaginal delivery	12	10	Mechanical study (chapter3) and biochemical study (chapter4)
Caesarean section	10	10	In vitro Model study (Chapter 5)

Table 2.2 : Detail of overall collected FM samples

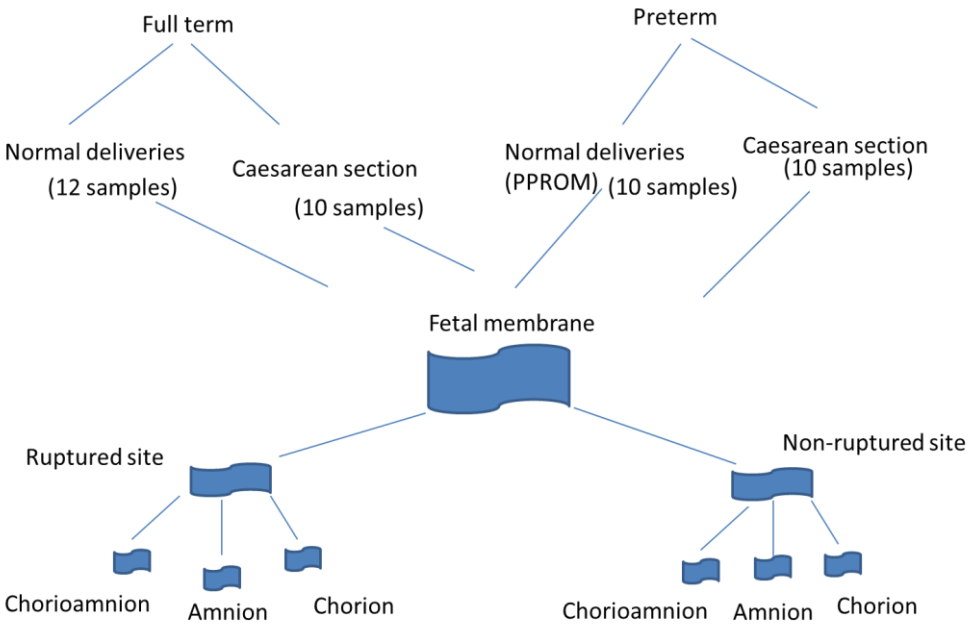

Figure 2.2: Detail of the types of the received FM samples and the subgroups of the membranes studied for different experiments.

Fetal membrane samples from the edge of the rupture area or tear zone and membrane samples near placental area were collected (Figure 2.4). Chorion and amnion from those two areas were separated manually. The samples for mechanical test were washed and kept in PBS (keeping moisture of the samples) as the test was performed within few hours of delivery and the rest of the samples were stored in -80°C for further analysis. Fetal membranes from the edge of the tear areas overlying the cervix (which was implicated as weak zone and ruptured area for normal deliveries) denoted as ruptured and membranes far from tear zone (6-10 cm from rupture area depending on the size of placenta and the ruptured area), denoted as non-ruptured were dissected out. The chorion and amnion were separated manually for tests (Figure 2.1). Collected sample groups from preterm and full term fetal membranes, different areas of the membranes and their different layers respectively were demosnatrted in Figure 2.2.

During the initial stages of sampling, membrane samples were obtained from three different areas--edge, middle, and centre (Figure 2.3). Later on, it was confirmed that the samples were collected from areas far from rupture site, either middle or centre areas presented similar outcome. Hence, we optimised the protocol to two areas, ruptured site (edge) and non-ruptured sites (centre) only.

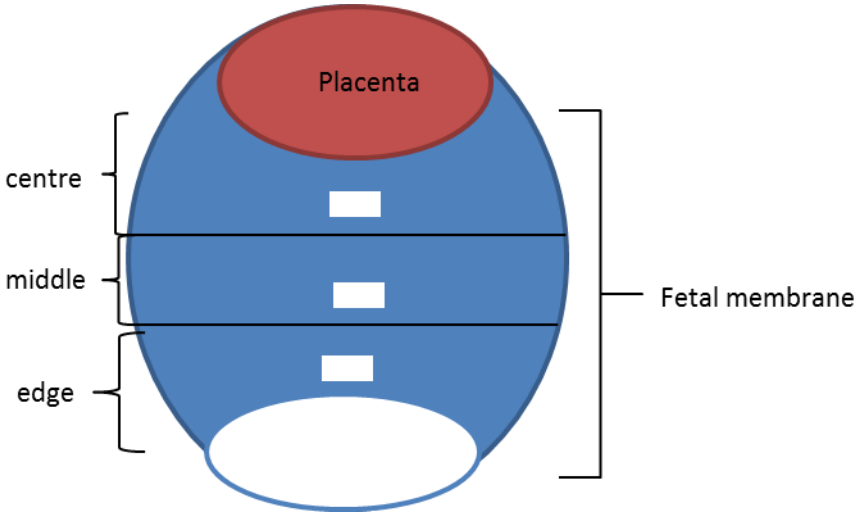

Figure 2.3: Schematic diagram showing three different areas of the fetal membrane- edge, middle and centre. Showing the locations of the initially collected samples.

Normally, the distance between these two sites were maintained. However, as the tear zone was not always at the same place on all the fetal membrane samples and the membrane surrounding the placenta was not symmetrical and furthermore the size of placenta was not always the same, it was difficult to follow any one pattern of pre-determined area. Hence, the test was repeated with triplet samples from each category of the two areas –ruptured and non-ruptured sites, as shown in figure 2.4.

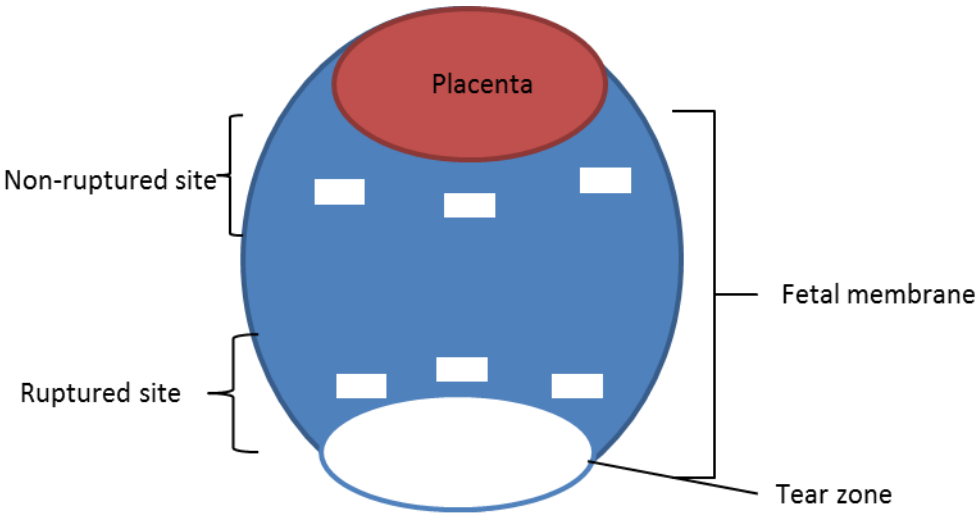

Figure 2.4: Schematic drawing to demonstrate the physical locations of defined rupture and non-ruptured zones relative to placenta for collection of membrane samples.

2.2 Biomechanical study:

2.2.1 Ball indentation mechanical test:

Ball indentation method enables mechanical characterisation of fetal membrane samples in vitro. The method and techniques involved are discussed in detail in chapter 3 (section 3.4.2).

2.2.2 Thickness measurement by Optical Coherence Tomography (OCT):

Membrane microstructure and cross sectional thickness were measured by an OCT device (Telesto II, Thorlab, USA) with the centre wavelength at 1300 nm providing around 2 mm imaging penetration. OCT is an optical imaging technique, which is able to provide a high resolution imaging and useful for non-invasive cross sectional assessment of tissues. This imaging technology performs in similar way as ultrasound imaging does, except near-infrared light is transmitted through the samples instead of sound. OCT is a powerful tool in biological research and clinical applications. Two-dimensional images are produced by

the backscattered or back reflected light intensity (Fujimoto et.al, 2000). OCT is a beneficial tool for determining the thickness of the tissue. Prior to mechanical testing, chorioamnion samples from ruptured sites and non-ruptured sites, from full term and preterm pregnancies were imaged by OCT to record the thickness of the membranes. A small piece of freshly dissected membrane was placed inside a covered round small Petri dish immersed in small amount of PBS to avoid drying. OCT images of the samples across selected areas were obtained.

2.2.3 Optical Coherence Elastography (OCE):

The OCE technique examines the stiffness of the membranes without requiring clamping, which eliminates artefacts. The current OCE setting uses phase sensitive optical coherence tomography (PhS-OCT) combined with shear or surface acoustic wave (SAW) methods. The modulated acoustic radiation force (ARF) to the samples induced phase change due to the vibration, and the PhS-OCT was utilized to record the phase change of tested samples. The underlying working principle to obtain the modulus is that when ARF is applied to a two-layered sample, constant stress fields can be assumed to apply to both layers. Thus, under the same stress ($\sigma1 \approx \sigma2$) in a heterogeneous material, the ratio of Young's modulus in layers 1 and 2 is equal to the inverse ratio of strain $\varepsilon1$ and $\varepsilon2$. The local strain is determined from detected tissue displacement caused by the vibration amplitude which can be calculated by the fast Fourier transform (FFT) of the phase difference [FFT ($\Delta\varphi$)]. Therefore, using compound samples with the test sample overlying a reference agar layer with known mechanical properties, the Young's modulus of the test sample can be quantified by the following equation:

$$\frac{E_1}{E_2} = \frac{\frac{\sigma_1}{\varepsilon_1}}{\frac{\sigma_2}{\varepsilon_2}} = \frac{\sigma_1 \varepsilon_2}{\sigma_2 \varepsilon_1} \approx \frac{\varepsilon_2}{\varepsilon_1}.$$

Membranes from two different areas (ruptured and non-ruptured) of full term and preterm with similar delivery modes were imaged and compared by OCE. OCE measures the mechanical property of tissues like Young's modulus as well the thickness of biological tissue. The ultra-high resolution imaging capacity of OCE is beneficial to distinguish the abnormal changes of tissue properties from the normal ones (Huang et.al, 2015). The basic principle behind it is measurement of vibration amplitude from stimulated target tissue section. This part of the study was collaborated with Prof. Huang and team from Dundee University. Quantitative measurement of the mechanical properties of all types and subtypes of membranes was conducted using a novel OCE system in University of Dundee. Device setup was the same as described previously (Guan et.al, 2013). The OCE system consists of two main parts: vibration stimulation and signal detection. A total number of 12 samples from normal vaginal deliveries (6 from full term and 6 from preterm x 3 replicates) were studied. Before the scanning procedure, each tissue sample was placed on 2% agar phantom with a thickness of ~5 mm which was used as a modulus reference. Agar powder was dissolved in water and heated to 29 degree to prepare a homogenous solution. Then agar blocks were obtained after cooling down the solution properly at room temperature. Three B-scans were taken from each ruptured and non-ruptured samples of both full term and preterm membranes. Scanning time was approximately 2 minutes per sample. OCE images were collected.

2.3 Histology:

2.3.1 Histological section:

Entire chorioamnion membranes from both the sites including ruptured and non-ruptured sites were sectioned. Intact pieces of fetal membranes from both sites were taken from both full term and preterm samples. 4-6μm thick tissue sections were

collected on Poly-L-lysine coated slides (Sigma-Aldrich,UK). Sections were prepared via two different methods; paraffin embedding and cryosection.

2.3.1.1 Paraffin embedded sectioning:

Most samples were fixed and dehydrated manually while some of them were fixed and dehydrated by using the tissue processer (Leica). Samples were fixed with 4% formalin in a separate PTFE capped small glass bottles. Sufficient amount of formalin was added in the bottle to cover well the membrane pieces. Samples were fixed at room temperature for overnight. Once the samples were fixed dehydration process started with the ethanol in ascending manner to displace the water. Membrane pieces were transferred onto the 70% of ethanol concentration twice for 1 hour each. Following that, they were transferred to the 80%, 90% and 100% of ethanol concentration for an hour each and subsequently to three changes of xylene or histo-clear (1 hour x3) in room temperature. Following that, the samples were dipped into the melted (~ 60°C) paraffin wax for 1 hour (x2) and the samples were prepared for embedding in the wax, Embedding was performed by placing the wax infiltrated samples in the metal cascades at desired orientation (sideways, so that the sections would be cut over amnion and chorion layers) and subsequently melted wax was added and covered by a plastic cassette on the top. Then, the cascades were kept in 0°C cold platform and the paraffin embedded fetal membrane blocks were obtained after few hours once the paraffin was solidified totally. 4-6µm tissue sections were cut by using the microtome (Bright, UK) and the ribbon of the sample sections were placed directly onto the warm water bath. Water bath was turned on beforehand with fresh dH_2O at 35-37°C. Floated sections were collected onto the poly-l-lysine coated slides. Rest of the blocks were stored in room temperature for sectioning later.

2.3.1.2 Cryosection:

Cryosectioning of the fetal membranes was done by Cryostat (Leica Biosystems). Fresh

frozen samples were cut into approximately 2-3 cm diameter pieces. Then pieces were placed individually in a suitable tissue mould inside the cryostat after confirmation of their intact anatomical orientation. An optimal cutting temperature (OCT) compound (Agar scientific, UK) was added to it immediately for embedding the tissue. OCT compound freezes and solidifies in -20°C. OCT mounted sample blocks were then transferred on to the metal grids and were sectioned in 4-6 µm thicknesses by ultra cryotome of cryostat. Sections were collected onto poly-L-lysine coated slides and kept in room temperature for about a minute until the OCT compound melted. The sections were then fixed by paraformaldehyde directly before proceeding for staining. The extra slides and OCT containing sample blocks were stored in -80°C.

2.3.2 Haematoxylin and Eosin (H&E) staining:

Paraffin sections were air dried and baked in 45°C for 2 hours. Then, the samples were deparaffinised by putting them in xylene or histo clear for 10 minutes thrice. Rehydration was carried forward after that by 100% ethanol for 3 minutes twice and then 90%, 80% and 70% ethanol 1minute each. Finally, the slides were rinsed in distilled water before proceeding with the staining procedure. Sample sections were stained with Wiegert's Haematoxylin (table 2.1) for 5 mins. Then, samples were stained with Eosinophilic Eosin (table 2.1) for 2-3 mins after washing thoroughly with running tap water. Next, samples were washed by 90% and 100% of ethanol and slides were cleaned with histo-clear (table 2.1). After drying the slides, xylene based mounting medium (Distyrene, tricresyl phosphate plasticiser and xylene, DPX from Fisher Scientific, UK) was used for mounting them.

2.3.3 Sirius Red staining:

For preparation of picrosirius red stain solution. 0.5 g Sirius red (table 2.1) was mixed in 500 ml aqueous solution of picric acid. Fixed frozen section slides were hydrated and

stained with picrosirius red solution. After 1 hour of incubation with Sirius red, slides were washed with two changes of acidified water (5 ml acetic acid+1lit water). Then, they were dehydrated by three changes of 100% of ethanol. Finally, sample slides were mounted by DPX medium after being cleared with xylene as described in 2.3.2.

2.3.4 Toluidine blue staining:

Toluidine blue solution was prepared with 5 ml of toluidine blue stock solution (1 g Toluidine blue O (table 2.1) dissolved in 100 ml of 70% ethanol) mixed with 45ml of 1% sodium chloride (0.5 g sodium chloride dissolved into 50 ml distilled water, pH 2.0-2.5). The working solutions were always prepared freshly before use and maintained at pH 2.5. Deparaffinised sections or frozen sections were hydrated by distilled water and toluidine blue solution was added onto the slides for 10-15 mins followed by three times washing with the distilled water. Dehydration of the samples were performed with two changes of 95% and 100% alcohol each and mounted with resinous mounting medium after clearing with histo-clear.

2.4 Immunochemistry:

Frozen cryosections (4-6 µm) of the samples were fixed in 4% paraformaldehyde for 10 mins. After washing with PBS, 5% bovine serum albumin (BSA) was added to all the sections for 60 mins to block the nonspecific binding. Then, following the recommended dilution concentrations from the suppliers, the sections were incubated with primary antibodies (1:100 dilutions in 1% BSA) for overnight at 4°C (all tested proteins). After washing with PBS, secondary antibody (dilution 1:200 in BSA) was added and incubated for 1 hour in room temperature. Subsequently, the slides were mounted with 4.6-diamidino-2-phenylindole (DAPI) coated mounting medium after proper wash with PBS. The tested primary antibodies included MMP 9 (table 2.1) MMP 13 (table 2.1) Fibronectin

IL-1β (table 2.1) IL-6 and progesterone (table 2.1) FITC conjugated secondary antibodies (table 2.1) were used to detect the expressions of above markers in membranes. Collagen-II and collagen-III (table 2.1) were used for detecting the collagen distribution on the membranes. 3-4 sections of every sample were tested along with negative control (without using primary antibodies). Optimization of the antibody concentration was obtained from supplier provided database and recommended literatures.

2.5 Biochemical assays:

2.5.1 DMMB assay:

DMMB or 1, 9-dimethlymethylene blue is widely used for sulphated GAG (glycosaminoglycan) quantification. A pre-established protocol was followed (Farndale et al., 1986) to evaluate the total amount of GAG production in fetal membrane samples. Same amount of tissue from full term and preterm membranes, ruptured and non-ruptured sites were digested by papain solutions (table 2.1). Papain solution was prepared by dissolving 125 µg/mL of papain in 0.1 M.

Sodium phosphate, 5 mM EDTA, and 5 mM cysteine-HCl. PH was maintained at 6.5. Once samples were digested thoroughly, 100ul of digested samples were transferred to the 96 well plate and 200ul of DMMB solution was added to each well. DMMB reagent solution was prepared by dissolving 4mg of DMMB in 250ml of distilled water containing 0.75g glycine, 0.58g sodium chloride (NaCl) and 2.08ml of 0.1M hydrochloric acid (HCL). 100ul Standard solution of bovine tracheal chondroitin sulphate in dH2O (0-40 µg/ml) also was placed in 96 well plate and 200 µl of DMMB solution was added in the same plate with the samples to get a standard curve (Figure 2.5). Standard curves were generated to determine the R^2 values, which was greater than 0.95. The absorbance was read by using a plate reader (Synergy II Biotech) at 530nm immediately.

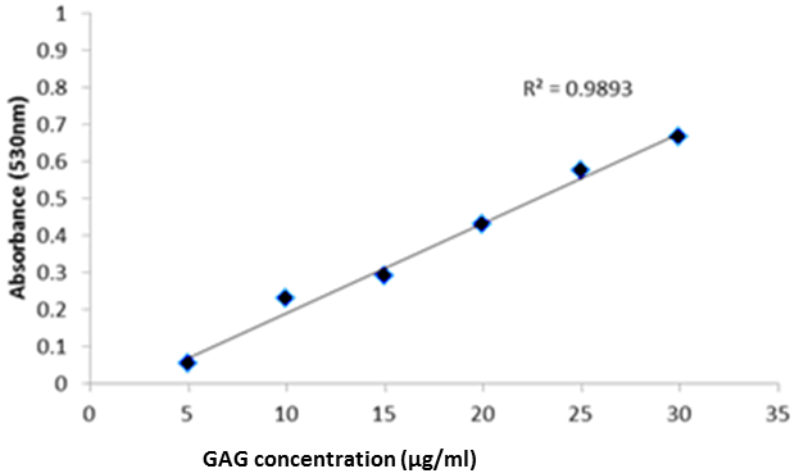

Figure 2.5: Standard curve performed for DMMB assay showing the linear relationship absorbance at 530 nm of bovine tracheal chondroitin sulphate standards.

2.5.2 Collagen assay:

Collagen content of the fetal membranes was quantified by Hydroxyproline assay. Hydroxyproline, the non proteogenic amnioacid is present in insoluble collagen which determines the collagen level of the tissue (Biovision). This colorimetric assay kit from Sigma-Aldrich (St.Louis, MO) was used for both the sites of full term and preterm chorioamnion membranes. Homogenised 10 mg of each tissue samples were weighted and placed into the PTFE topped glass vials. Each of the samples was combined with 100 µl of concentrated HCL (37%, 12M) and 100 µl of deionized water and hydrolysed overnight (12-18 hours) at 110°c oven. Dry hydrolysates were diluted with distilled water and brought into same volume. Then, 20 µl of these were transferred to the 96 well plates. Hydroxyproline standard was prepared from ranging 0- 10 µL of the 0.1 mg/mL by diluting in distilled water. These standard solutions were also placed into the 96 well plates and put this in 60° oven for drying. After that 6 µl of freshly made concentrated Chloramine T and 94 µl of oxidation buffer mixture was added to each well and incubated

for 5 minutes in room temperature followed by adding 100 µl of the diluted DMAB reagent (provided in the kit). Then, the plate was incubated in 60° again for 90 minutes. The absorbance at 560 nm was read with a spectrophotometer (BioTek Synergy II) for samples and standard.

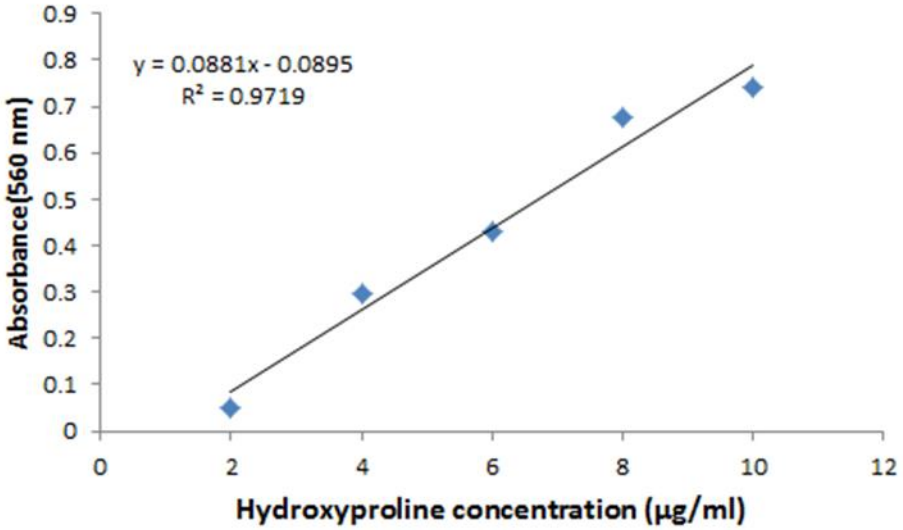

Figure 2.6: The standard curve for the hydroxyproline assay.

2.6 Western blotting:

2.6.1 Preparation of tissue lysates:

Similar sizes of tissue pieces from all types of membranes were washed with PBS and then homogenised manually or by a tissue homogeniser. Mixture of 100 µl RIPA lysis buffer (table 2.1) and protease inhibitor cocktail was used for extracting the protein. Samples were digested for an hour placed in ice and after that centrifuged in 4°C at 15000 g for 10 mins. Supernatant or extracted protein was pipetted out carefully and immediately.

2.6.2 Estimation of protein concentration:

Extracted protein concentration was measured via bicinchoninic acid (BCA) protein analysis method. 4% copper (II) sulphate pentahydrate solution was added to the BCA

solution to make BCA regent by 1:50 ratio. Bovine serum albumin (BSA) solution was prepared to use as a standard. It was prepared in different concentration between 0-1 mg/ml. In a 96 well plate, 10 µl of each standard solutions and 5 µl of all sample solutions and their replicates were placed. 100 µl of BCA regent was added to each well with sample standard solutions. Then, the plate was incubated for 30 minutes at 37°C. Synergy II BioTek plate reader was used to read the solutions at 570 wavelengths. A protein standard curve was prepared with the BSA standard readings. Protein concentration of each individual lysate was estimated by the help of calibration curve.

2.6.3 Western blot analysis:

Prior to loading protein to the PAGE gel, protein samples were denatured. 5 µL of a mixture consisted of 150 µL of 1X NuPAGE sample and 7.5 µL of β- mercaptoethanol were added to each 30 µg of protein sample lysate and heated for 15 minutes at 80°C in a thermal cycler (OmniGene, United Kingdom). Optimal antibody concentration was determined based on the manufacturer recommended data sheet.

30 µg proteins loaded to per well of a NuPAGE 4 -20 % Tris-HEPES gel. 5 µl NOVEX® sharp pre-stained protein ladder was loaded in one well. 1X BupH™ Tris-HEPES-SDS running buffer was used to run the loaded gel under a voltage of 70 V for 90 minutes and subsequently blotted to an Amersham Hybond P 0.45 PVDF membrane with the 1X Novex® Tris-Glycine SDS transfer buffer at 30v for 1 hour.

The obtained blot was washed three times by 1x TBST (Tris-buffered saline, 0.1% Tween 20) and blocked with 5% of semi skimmed milk solution at room temperature for 1 hour. Each blot was incubated overnight at 4°C with primary antibody (1:500) after blocking with 5% semi skimmed milk in PBS-T buffer (PBS with 0.2% Tween 20) for an hour. Next day, after briefly washing with TBST buffer, the membranes were incubated with

horseradish peroxidase conjugated secondary antibodies (1:1000) for 60 mins. The membranes were reblotted with anti-GAPDH antibody (1:5000) as the loading control. The same antibodies were used here as in above immunostaining assay.

Protein concentration was determined by Bicinchonic Acid (BCA) assay kit. Bovine serum albumin (BSA) protein solutions were used as the standard to generate a standard curve (Figure 2.7), ranging from 0 – 2 mg/ml.

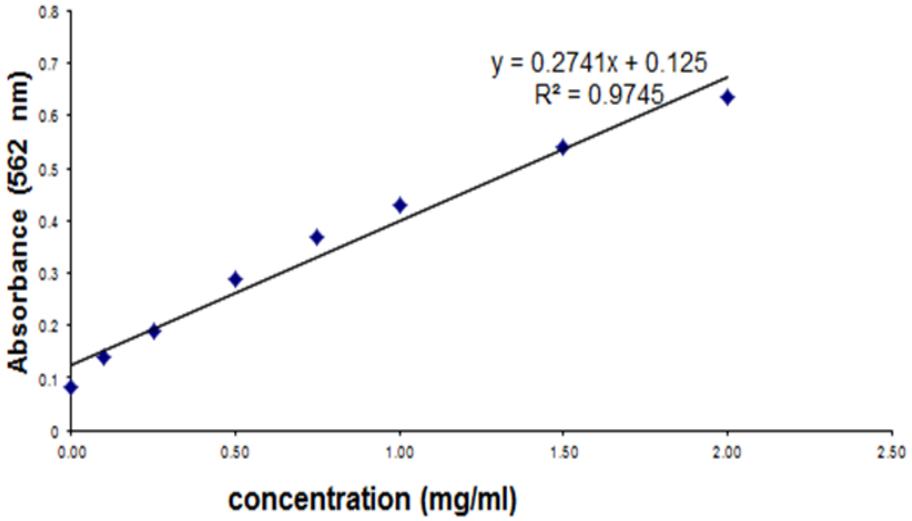

Figure 2.7: The BSA standard curve showing relationship between BSA standard and absorbance.

2.7 Cell and tissue viability test:

To assess the cell viability of the membranes before doing any biochemical experiment live/dead test was performed by using LIVE/DEAD double staining kit (Thermo- Fisher Scientific, UK). This assay kit is capable of providing real-time detections of live and dead cells or parameter of cell viability with the help of two different fluorescence coloured dyes. Calcein-AM ester dye labelled the cells green in colour, indicating live cells and

Propidium Iodide dye stained cell's nucleus into red, indicating dead cells. Fetal membrane sample pieces were exposed to the 10 µM Calcein-AM and 1 µM Propidium Iodide solutions after washing with PBS in a 24 well plates and incubated in dark condition with 5% CO_2 and at 37°C for 20 min. Following that, samples were washed twice with PBS and imaged under a Confocal Laser Scanning Microscope (CLSM, Olympus Fluoview FV 1200 with Fluoview software 4.1 version).

2.8 Semi-quantification technique of staining intensity:

Immunostaining and western blotting results were quantified by ImageJ software (1.4.3.67 version). 3-5 random areas from the image and an area with no staining were selected as background. Then, the integrated intensity via ImageJ was measured. The staining intensity was acquired after subtracting the background intensity from the sample integrated intensity. All the immunostainings images were taken using confocal microscopy under same setting and same exposure time. Therefore, the fluorescence intensities of all the samples and all the antibodies were directly comparable

2.9 Statistical analysis:

A minimum of 3 individual donor membranes were used for each experiment and each group. For each group of all the experiments, 3-6 membrane samples were used as replicates. The two-way analysis of variance (ANOVA) was performed to examine data on mechanical testing (ball indentation test) of chorioamnion, amniotic and chorionic membranes in normal and caesarean section samples, in full term and preterm samples and in ruptured and non-ruptured groups. These tests used 6 individual donor membranes. The same was applied to the data of biochemical factors expression in different groups before and after loading stimulation. A level of 5% was considered significant in all cases. Turkey's test was used for post hoc analysis. Statistical significance

was expressed at p-value < 0.05. In the graphs, statistical significance is indicated at three levels such as *p< 0.05, **p< 0.01 and ***p< 0.001. All the statistical analyses were performed using Graph pad Prism 6 software. For each analysis, all data were expressed as mean ± standard deviation (SD) and tested for normal distribution and homogeneity variance. All the statistical analyses were performed using Graph pad Prism 6 software.

CHAPTER 3

Heterogeneity of mechanical properties of fetal membranes from full term and PPROM

3.1 Introduction:

Fetal membrane (FM) supports the fetus throughout the gestation. Amnion, the inner layer of the human FM is thin, strong and viscoelastic in nature. Chorion, the outer layer of FM is thicker and more cellular (Benirschke et al., 2012). These two layers together undergo high physiological stress towards the failure threshold (Oyen et al., 2006). Heterogeneity of the mechanical property of the FM in full term has been studied before (Malak et al., 1994, Moor et al., 2006) but preterm membrane has not previously been studied in detail. Although the mechanical properties of human FM have been studied for a while, the outcome is still controversial. The reason could be due to, different methods being used to assess membrane strength *in vitro,* such as the uniaxial tensile test, biaxial tensile test, puncture test and inflation test or burst test. These methods have some advantages and disadvantages.

The mechanical properties when tested with the amnion and chorion alone exhibited conflicting results. Oyen et al found amnion was stronger than the chorion (Oyen et al., 2006) and Helmeig et al., noted amnion had higher rupture strength while chorion needed less force to rupture. (Helmeig et al., 1993) Also, some studies have reported amnion ruptured before chorion (Helmeig et al., 1993, Schober et al., 1994, Greenwood et al., 1998), whereas the other studies have indicated that chorion ruptured earlier than the amnion (Lavery et al., 1979, Arikat et al., 2006). These variations could be due to consequence of different testing methods used and the experimental set up (Perrini et al., 2015). The uniaxial tensile test is simple and easy to perform but does not mimic the loading condition in vivo. The bi-axial tensile test is not very suitable for chorioamnion membranes as it is very thin and slippery. The burst test or inflation test could potentially mimic the in vivo condition; however, this test requires a larger sample size of membrane, sometimes the whole membrane, which might be an issue to achieve a convincing result. The puncture test is the most commonly used method in the recent time. The method is simple and requires smaller sample size. Despite this advantage, the locally applied force to

deform the sample fails to match the in vivo loading condition (Buerzle et al., 2014). In summary, the conflicting results regarding the mechanical strength of FM is possibly the result of using inconsistent testing methods. The establishment of a convenient and biocompatible protocol for mechanical assessment is required to study mechanical behaviour of the FM, in order to a better understanding of preterm rupture of FM.

An ideal FM rupture method mechanically should have a set up that can mimic the tissue physiology closely, i.e. causing membrane deformation radially. FM is viscoelastic in nature. The two layers of FM (amnion and chorion) have different mechanical behaviour too. Considering the vital role of supporting fetus played by FMs, it is obvious that there are consistent pressure from fetus and amniotic fluid transferred to the FMs. Therefore, a mechanical test to evaluate and study the membrane deformation over time under constant load (weight) (creep behaviour) in FMs, amnions and chorions separately is required.

Ball indentation method established in our lab is a non-destructive method which can assess creep property of biological soft tissues *in vitro* (Ahearne et al., 2005). The deformation of the test sample over time can be imaged in real-time. This method incorporates the advantages of the bi-axial and asymmetric stress distribution in the deformed samples, which mimic the fetal tissue environment *in vivo*. This method is applicable for membranes either permeable or semi permeable. In addition, it has accurate resolution of 10 μN and 10 μm for force and displacement respectively (Ahearne at al., 2005). The viscoelastic properties of the FMs can be analysed by the creep behaviour and ball indentation method would be ideal for that. The load from the ball deforms the membrane samples, and by measuring its central displacement against time we will be able to determine some mechanical properties of FMs.

3.2 Objective:

The aim of this chapter was to develop new protocols which mimicked the *in vivo* conditions to measure and analyse the mechanical properties of membranes (from both full term and preterm PROM deliveries, from different locations and different layers). We aimed to characterise the heterogeneity between mechanical property such as the deformation/Creep property, elasticity, and thickness of both types of membranes from ruptured and non-ruptured sites. This enables a better understanding of mechanical properties of FM especially preterm membrane.

3.3 Material and methods:

3.3.1 FM sample collection:

FM samples were obtained and prepared as previously described. (Chapter 2, section 2.1). Full term membranes from normal vaginal deliveries and preterm membranes from spontaneous PPROM deliveries were taken for biomechanical tests. Mechanical properties including instant deformation and creep up to 12 hour under constant ball indentation were studied with intact FM from both areas (ruptured and non-ruptured sites) and their sublayers (amnion and chorion) by ball indentation test method. OCE and OCT analysis were performed on intact FM samples from both the sites (ruptured and non-ruptured) and both the types (full term and preterm).

3.3.2 Ball indentation techniques:

The Ball indentation test is a new and convenient technique to study mechanical characterisation of soft tissues. The main advantage of this method is that, it is able to mimic biological conditions with minimum damage to the tissue (Ahearne et al., 2005). For FMs, the ball indentation method mimics the in vivo loading and the load direction clearly. The mechanical test or indentation test was conducted using a previously established protocol in our lab. Tests were done on fresh membranes after few hours of delivery after washing with PBS. Pieces (approx. 20-30 mm) of intact FM, chorioamnion (CA), amnion (A) and chorion (C) from the rupture sites, which was considered as tear zone and from non-rupture sites (6-10 cm), which was far from the rupture

area depending on the size of placenta and the ruptured areas, were collected. The samples were then placed between two transparent plastic rings (20 mm) separately and held between two parallel flat metal plates with a circular opening and tightened by a number of metal screws. (Figure 3.1 A) The whole assembly (Figure 3.1 B) was then placed into a square Petri dish containing PBS to provide humidity.

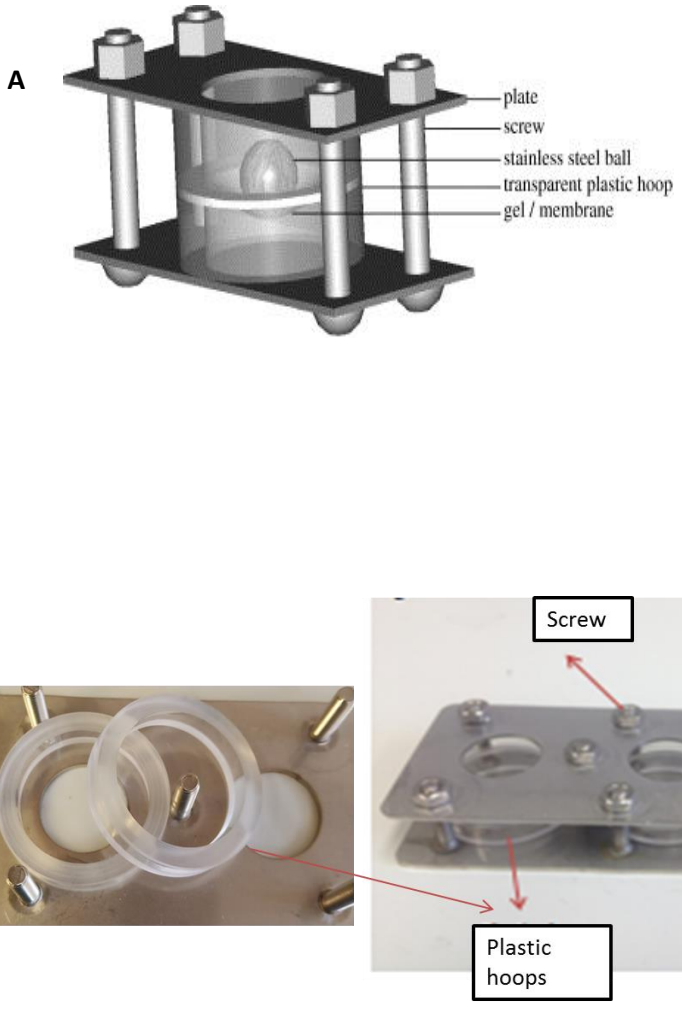

Figure 3.1 Tissue sample holder of ball indentation instrument. (A) Schematic drawing to show the individual sample holder of ball indentation set up. (B) Image of the sample holder for

creeping tests. FM samples are mounted between two plastic O shaped rings, secured by the metal plates and screws. This sample holder can hold 3 samples at a time.

The assembly in the Petri dish was positioned on a translation stage. (Figure 3.2 d) and a ball (0.071 gm, 2 mm diameter) was placed onto the central of the membrane to induce the deformation of the membranes.

The images of deformation were taken using a long focal distance microscope, connected to a Charge Coupled Device or CCD camera (XC-ST50CE, Sony, Japan) and computer (Figure 3.2 D). Images of indented samples were recorded in different time points (Time 0, 4-6 & 12-14 hours) under room temperature. The entire indentation procedure of the membrane samples took place in the incubator (at 37◦C and 5% CO_2) with the given time intervals. The images were recorded by image acquisition software called Labview program (National Instruments, UK). Central displacements of the deformed samples were measured. Unlike tensile test, the ball indentation technique cannot measure TM (tensile modulus) and UTS (ultimate tensile stress). The magnitude of the force was determined by the ball weight which is constant. The appropriate ball weight was selected to trigger the deformation of FM, and ensured it was not too heavy to go through the membrane instantly, and then creep was recorded.

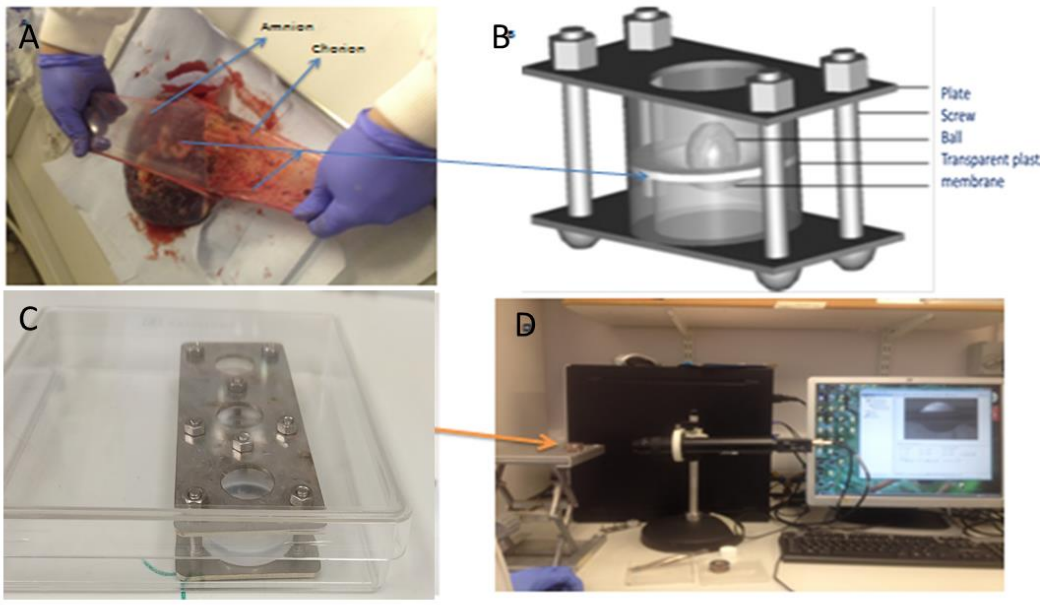

Figure 3.2: The set-up of the mechanical assessment --ball indentation test.

(A) Preparations of amnion and chorion membranes separated manually. (B) The membranes placed between two transparent plastic hoops. (C) Multiple membranes (amnion alone, chorion alone and fetal membrane) assembled in the holder and placed in a petri dish. (D) The deformations were recorded by using CCD camera.

3.3.3 Optical coherence elastography (OCE):

Mechanical properties like Young's modulus of the FMs were also assessed using OCE. Intact membranes from both full term and preterm deliveries and ruptured and non-ruptured sites were assessed (n=4x3) through OCE as described in detail in chapter 2, section 2.2.2.

3.3.4 Optical Coherence Tomography:

Membrane thickness was measured using OCT. Intact membranes from full term and preterm and both ruptured and non-ruptured sites were studied. The protocol was described in detail before in chapter 2, section 2.2.1.

3.4 Result:

Mechanical study results showed different properties between full term and preterm PROM membranes. Most of the preterm samples were collected from normal vaginal delivery and they were compared to full term membranes.

3.4.1 Overall physical features of the samples:

The physical observation and examination of the ruptured and non-ruptured sites of collected FM samples from full term and preterm deliveries were conducted first. FM samples from preterm normal deliveries were less delicate than the full term samples. When full term normal delivery samples were compared with PPROM delivery samples, most of the full term samples were found to damage easily. The preterm placentas were much smaller in size than the full term ones. The amnion and chorion in preterm membranes were intact and not easily separable; also in non-ruptured sites amnions and chorions were firmly attached. But in normal full term samples, amnions and chorions were very loosely attached and in ruptured site mostly were found to detach from each other already.

3.4.2 Ball indentation test result/ Creep behaviour:

The mechanical test/Ball indentation test took place with fresh tissues immediately after collecting the samples. Tests were performed with 12 different samples including 6 full term samples and 6 preterm samples. Two different sites (near ruptured and non-ruptured sites) of each sample were taken with 3 replicates for each category. In total, 36 separated and 36 intact membranes were examined. Figure 3.3 demonstrates the locations of FM; umbilical cord, ruptured areas and non-ruptured areas in a collected placenta before separation and taking FM for assessment.

As previously mentioned in Chapter 2, a few samples were initially taken and tested from three different sites across the membranes (covering almost whole FM). Towards the placenta sites was denoted as centre, ruptured site or tear zone was denoted as edge and those between these two

areas which were named as middle zone. Centre sites of membranes showed low displacement and other two areas showed approximately similar displacement at giving loading (the same weight ball) (Figure 3.4). This ensured that mechanical testing for two different areas such as near tear zone and non-ruptured zone will give a reliable outcome. Therefore, the following tests were continued with samples from two different sites only-centre zone which was non-ruptured site and the edges or ruptured sites. Membrane, when deformed more or showed higher creep, represented mechanically weak and when deformed less indicated strong membrane. FM deformed gradually with time because of the ball triggered creep. From the camera images, it was obvious that the chorions displayed maximum displacement (creep), so, chorion was mechanically weaker than amnion alone or chorioamnion.

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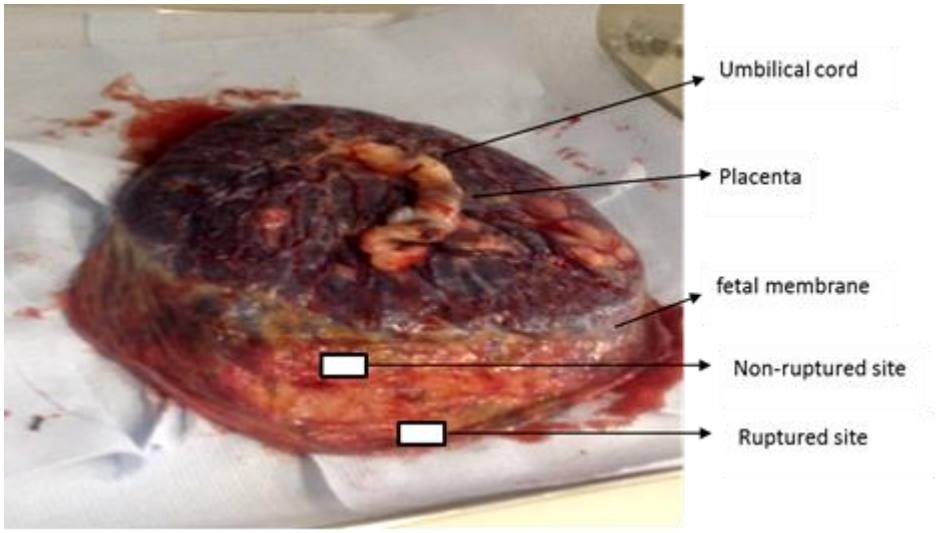

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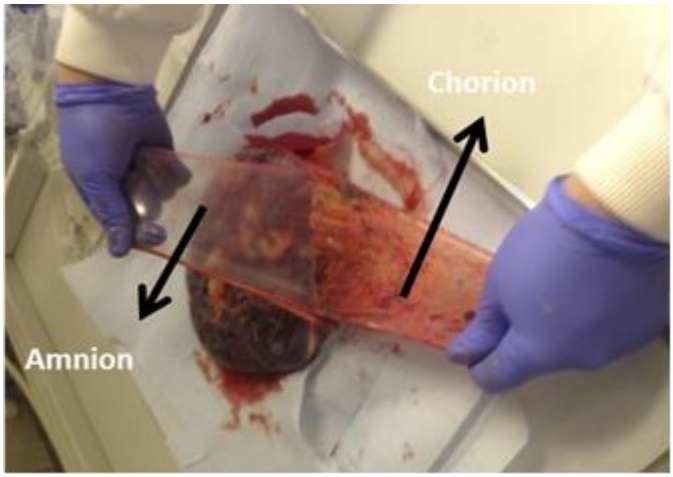

Figure 3.3 Image of the whole placenta with FM collected immediately after delivery. (A) The tear zone considered as rupture area and areas away from the rupture site or around the placental line considered as non-ruptured site.(B) Amnion and chorion manually separated.

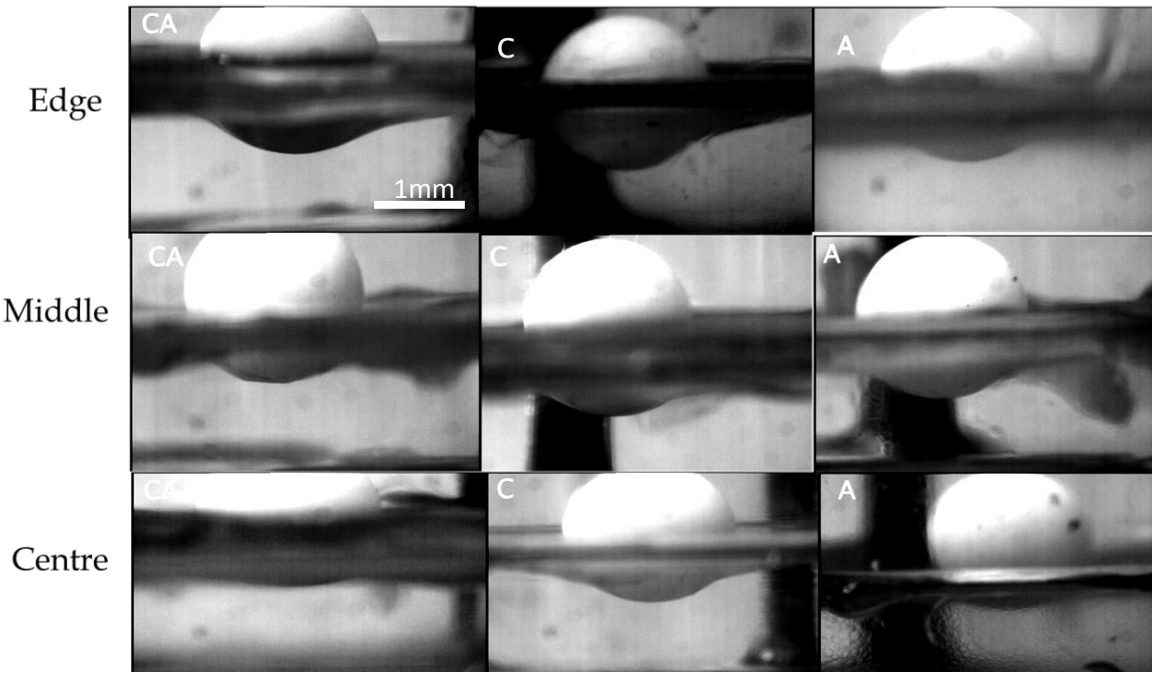

Figure3.4: Ball indentation test images. Membranes from three different areas –edge, middle, and centre. Membranes isolated away from centre showed less deformation than the middle and peripheral or edge areas. Chorion showed the most deformation. Scale bar = 1 mm.

The ball indentation test aimed to simulate pressures within the pregnant uterus (e.g. fetal weight and amniotic fluid) on the FM, which enabled study of creep property of the membrane conveniently. We were able to demonstrate significant creep difference not only between the membranes from full term and preterm deliveries, but also within the same membrane in the ruptured and non-ruptured regions, as well as between amnion, chorion and chorioamnion layers. Among the layers, greatest deformation was noted in chorion, in comparison to the amnions and chorioamnions.

3.4.2.1 Ruptured site showing high deformation:

Using the ball indentation technique, deformation of the FM of ruptured sites was indented for different time durations using the constant weight (0.071 gm) by a ball. The creep/deformations were monitored. The deformations of the membranes were imaged at different durations (time 0, after 4 hours and after 12 hours). The image of the FM samples from ruptured sites showed high deformation in a short span of time. (Figure 3.5 A) This pattern of creeping was found in all types of ruptured site of membranes. In many occasions, the chorions of the ruptured sites from full term spontaneous birth specially, was found to rupture during the end of 12-14 hours incubation while chorioamnions and amnions had still not ruptured, although deformation was increased. (Figure 3.5 B).

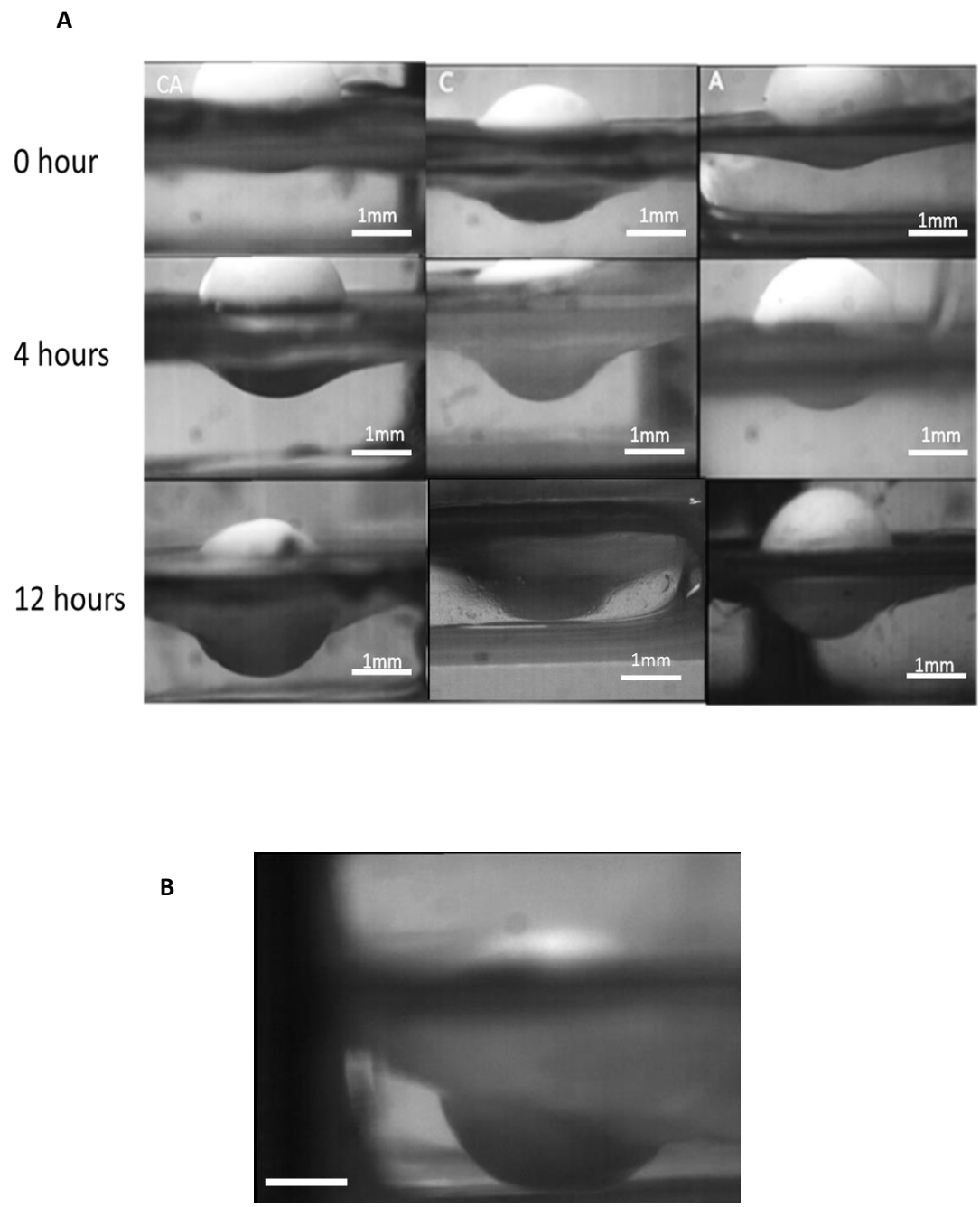

Figure3.5: Deformation images of membranes from the ruptured site of fetal membrane. (A) Membrane deformation over the time. Chorion had the most deformation and ruptured rapidly. CA-chorioamnion, C-chorion and A-amnion. (B) Ruptured chorion after overnight incubation. Images were from full term ruptured sites (n=6 donors). Scale bar = 1 mm.

The quantified result of Figure 3.5 showed significant deformation between amnion and chorioamnion also amnion and chorion over time during the ball indentation tests (Figure 3.6).

Amnion and chorioamnion membrane deformed gradually with the time. Chorion deformation after 3-4 hours suddenly went high (Figure 3.6).

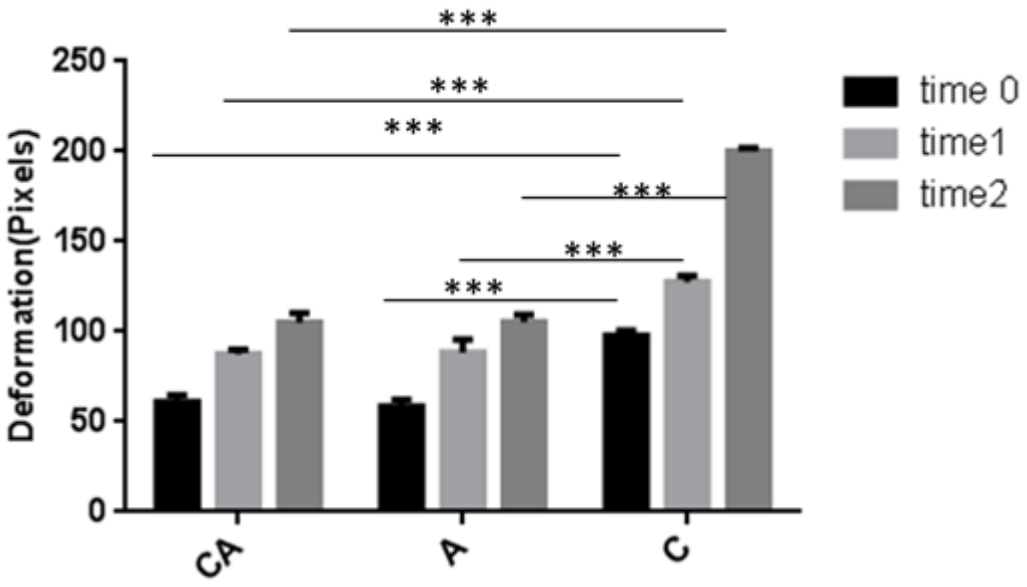

Figure 3.6 Quantification of deformation over time. (A) Full term ruptured site showed maximum deformation in chorion. (B) Over time, the least deformation occurred in chorioamnion and the most in chorion (time 0 – 0 hr, time 1 – 4 hrs, time 2 – 12 hrs). Data are presented as mean ± SD. ***P< 0.001. (6 donor membranes with 3 replicates for each test group). CA-chorioamnion, C-chorion and A-amnion.

3.4.2.2 Non-ruptured sites deforming less than ruptured sites:

In a similar way, the ball indentation technique was used for the assessment of non-ruptured sites of membranes with chorioamnions, chorions and amnions. Deformation patterns were same as

ruptured sites. Chorions deformed more over time than amnions and chorioamnions and this was alike in either full term or preterm membranes.

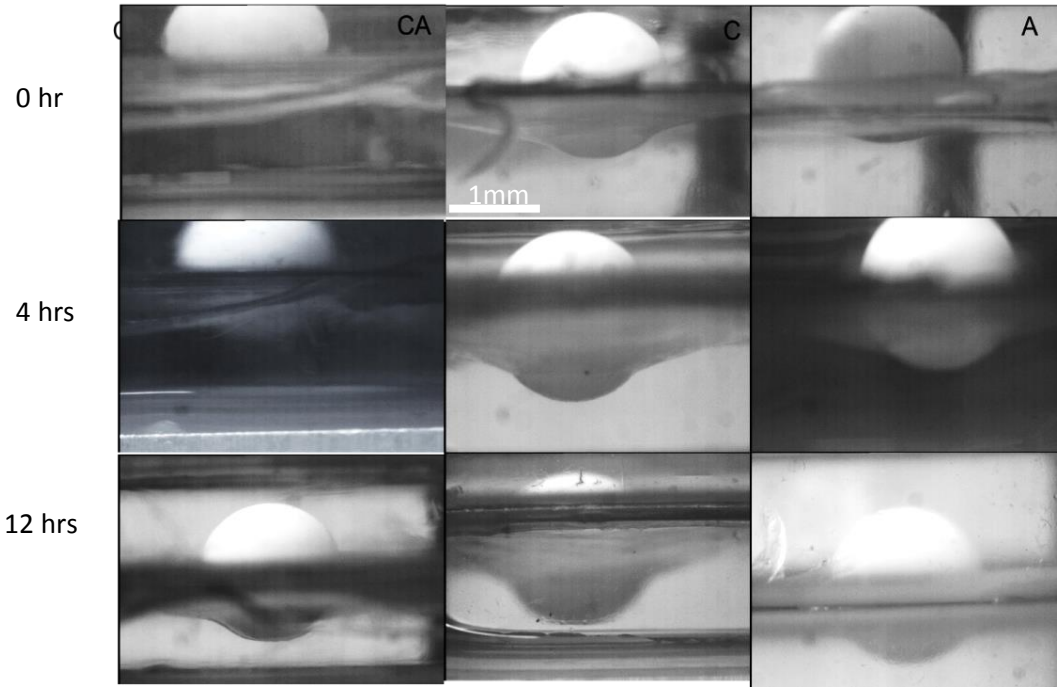

Figure 3.7: Ball indentation test result of the non-ruptured site fetal membrane. Full term non ruptured sites of the membranes deformed more with time. Chorion had the most deformation and ruptured rapidly. CA-chorioamnion, C-chorion alone and A-amnion alone. Scale bar = 1mm (6 donor membranes with 3 replicates for test group).

A clear difference was found in creeping properties when the ball indentation test results were compared between the ruptured sites and the non-rupture sites of the membranes. Membranes showed less creeping over time and higher rupture thresholds than ruptured sites of membranes. The deformation rates were singnificatly lower in non-ruptured sites of the membranes compared to the ruptured sites of the same membranes (Figure 3.8).

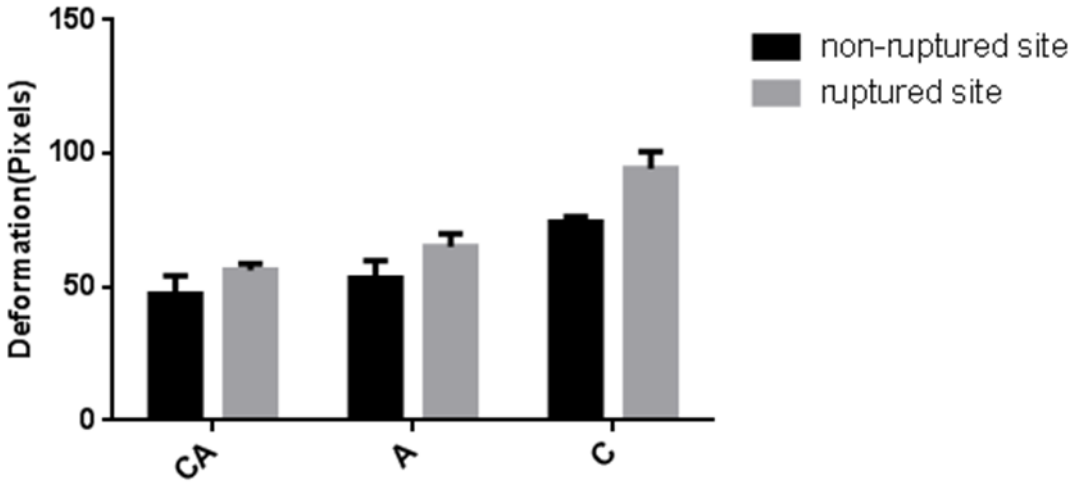

Figure 3.8: Quantification of displacement from the ball indentation test for the deformed values of membranes near ruptured and non-ruptured sites. Data showing that the ruptured sites of membranes deformed more in comparision to the non-ruptured sites of membranes (full term after 3-4 hours of ball indentation). Data are presented as mean ± SD . (n=4 donor samples with 3 replicates for each test group) **P< 0.01 and ***P< 0.001. CA-chorioamnion, C-chorion alone and A-amnion alone.

3.4.2.3 Full term vs preterm:

The deformation patterns were similar in preterm membranes too. Chorion was the weakest and membranes from non-ruptured sites were stronger in comparison to the ruptured sites. However, an interesting difference was noticed when they were compared with the full term membranes. As preterm samples were mostly collected from the normal vaginal deliveries, it was justifiable to perform tests and compare the results between normal full term and normal preterm. Lower deformations were always observed in non-ruptured site of the membranes as compared with the ruptured site of the membranes. A significant big difference in creeping behaviours was noticed between ruptured site and non-ruptured site of preterm membranes. Compared to the full term membranes, this difference of deformation in preterm PROM samples was much higher. Ruptured

site exhibited similar or higher deformation in preterm samples when compared to their counterpart full term samples; but non-ruptured sites of the membranes in preterm showed lower deformation than their counterpart full term samples. A large bio-mechanical heterogeneity in preterm membrane was observed (Table 3.1).

	CA			C			A		
	NR	R	Difference	NR	R	Difference	NR	R	Difference
full term	80	94	14± 3	97	110	13±2	80	95	15±2.2
Preterm	71	110	39±2	88	135	47±2.5	71	103	32±3

Table 3.1: The average displacement values of membranes from both non-ruptured (NR) and ruptured (R) sites (full term and preterm, after 3-4 hours of indentation). CA-chorioamnion, A-amnion and C-chorion. Data are presented as mean ± SD (n=4 donor membranes).

Non-ruptured sites of preterm membranes deformed less than the full term non ruptured membranes whereas in ruptured sites more deformations were found in preterm membranes compared to the full term membranes. (Figure 3.9)

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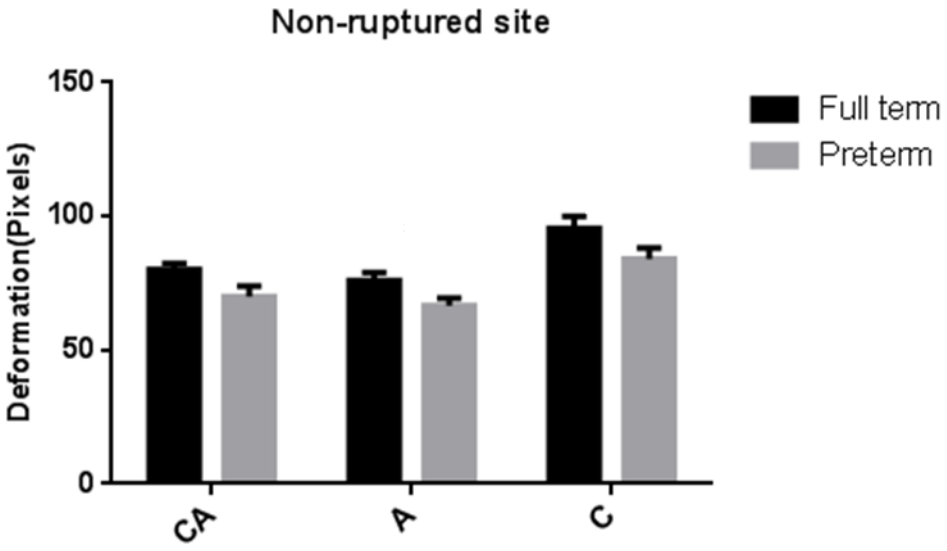

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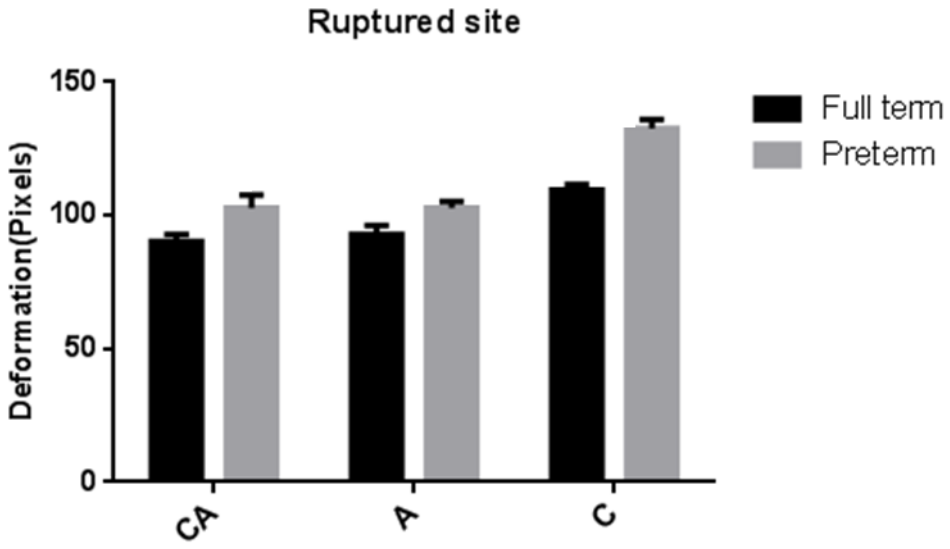

Figure 3.9: The acquired displacement value of the membranes near ruptured and non-ruptured sites between preterm and full term samples from ball indentation test.

Representative data showing displacement after 3-4 hours of loading in non-ruptured sites (A) and ruptured sites (B). Data are presented as mean ± SD (n=4 donor samples with 3 replicates for each test group). CA-chorioamnion, C-chorion alone and A-amnion alone.

3.4.3 The elasticity of the membranes:

The elasticity of the FM was studied initially by observing their capability of restoring their original position after removed the ball (indentation) from deformed membranes. The membranes were placed between the rings and tightened in the holder as same as ball indentation preparation (Figure 3.2 C). The membranes were loaded with the balls and at certain times the ball was taken away and the behaviour of the membranes were recorded via camera (Figure 3.2 D). Chorioamnion membranes were found to recover their deformation soon. After 3-4 hours of loading when the ball was removed from the membrane, it was deformed but it recovered very soon. (Figure 3.10)

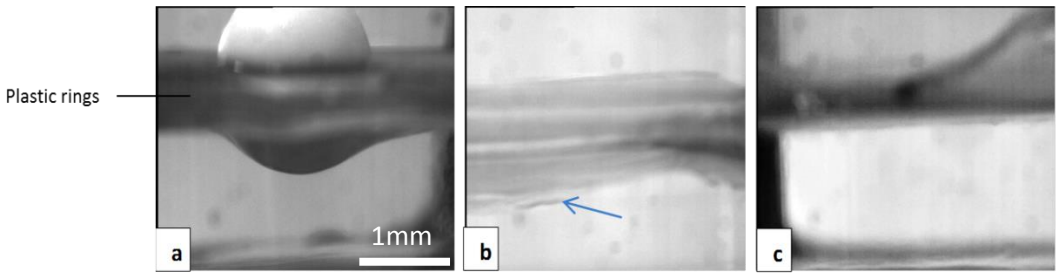

Figure 3.10: Representative Viscoelastic behaviour of fetal membrane. Chorioamnion membrane (non-ruptured) after a few hours of ball indentation (a), apparent deformation (b) recovered after 3-4 mins (c). Scale bar = 1 mm.

Amnion and chorion membranes showed a considerable difference in viscoelasticity during the ball indentation test in that the amnion membrane showed higher elasticity. There was no permanent deformation noticed in amnion membrane after the ball loading for 3-4 hours; a small deformation was detected after 6-8 hours of loading but that recovered immediately after the ball was removed, whereas the chorions maintained the deformation for a longer time and in some cases it showed permanent deformation. (Figure 3.11)

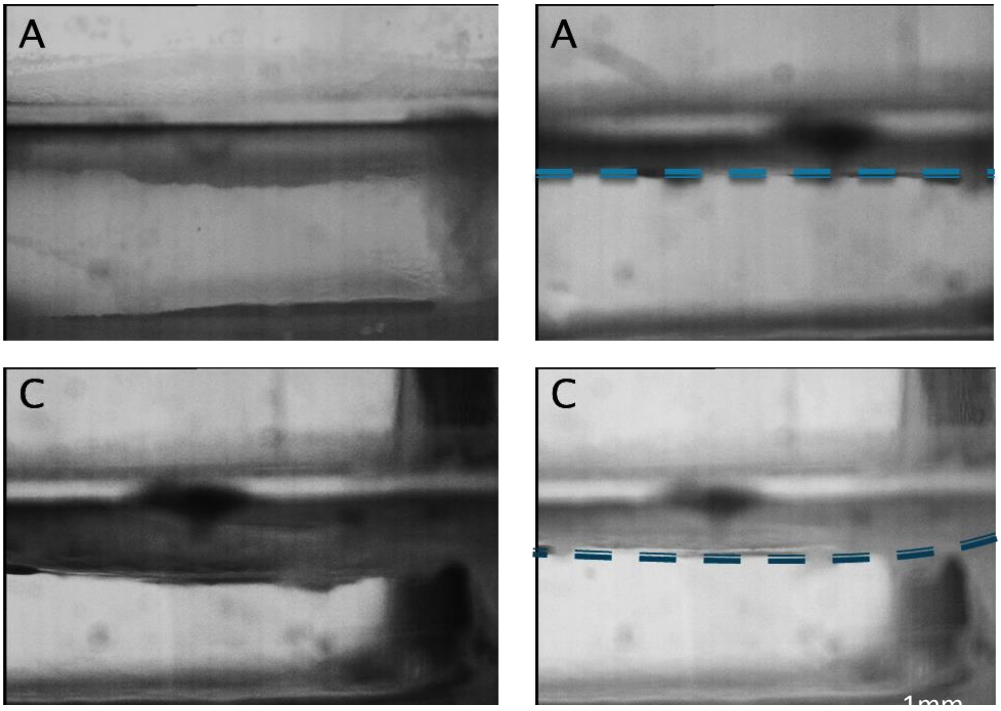

Figure 3.11: Comparison of deformation recovery capacity. Amnion (A) small deformation just after removing the ball (top left). Amnion showed immediate reformation (top right). Deformation of chorion (C) (bottom left) was permanent even after 8-10 hours (bottom left). Scale bar = 1 mm.

3.4.4 Modulus measured by OCE:

OCE images showed the mechanical property difference between ruptured and non-ruptured sites of the FM samples as well as the samples between full term and preterm membranes. The measurements were assessed throughout the pieces of the membranes in air and embedded on the agar platforms as references. Figure 3.12 shows the representative elasticity mapping images of full term and preterm membranes in ruptured and non-ruptured sites from OCE measurement. The heterogeneity of membrane stiffness and modulus were clearly visible between membranes. The stiffness of the non-ruptured site was higher than the ruptured site in general. However, the non-ruptured site of the preterm membrane showed a big difference in comparison with the

ruptured site. There was less difference in modulus between the two membrane sites in full term samples.

The preterm PROM samples had a large difference of modulus between ruptured and non-ruptured sites. Based on the colour bar the modulus of ruptured sites of PPROM samples were around 300-500 kPa while in non-ruptured sites it was around 600-1000 kPa. In case of full term samples both the sites have shown similar or very less differences of modulus, which were mostly within 200-400 kPa. The heterogeneous distributions of the stiffness were confirming the creep results from ball indentation tests. Within agar, the stiffness difference of the samples was more obvious.

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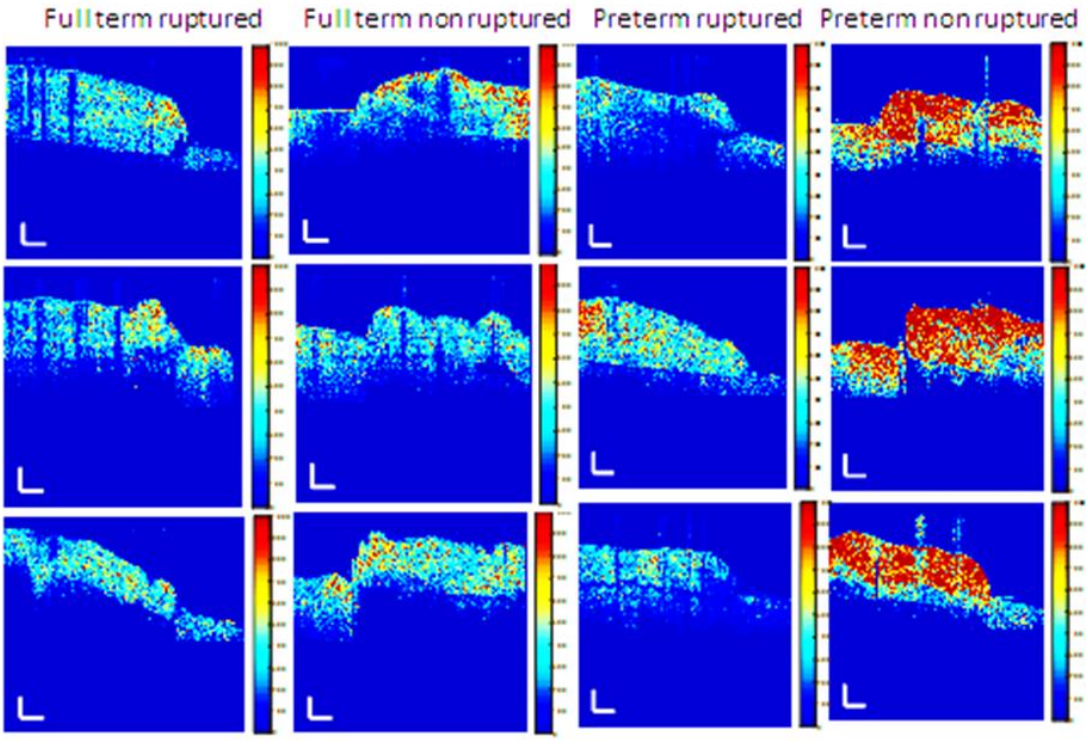

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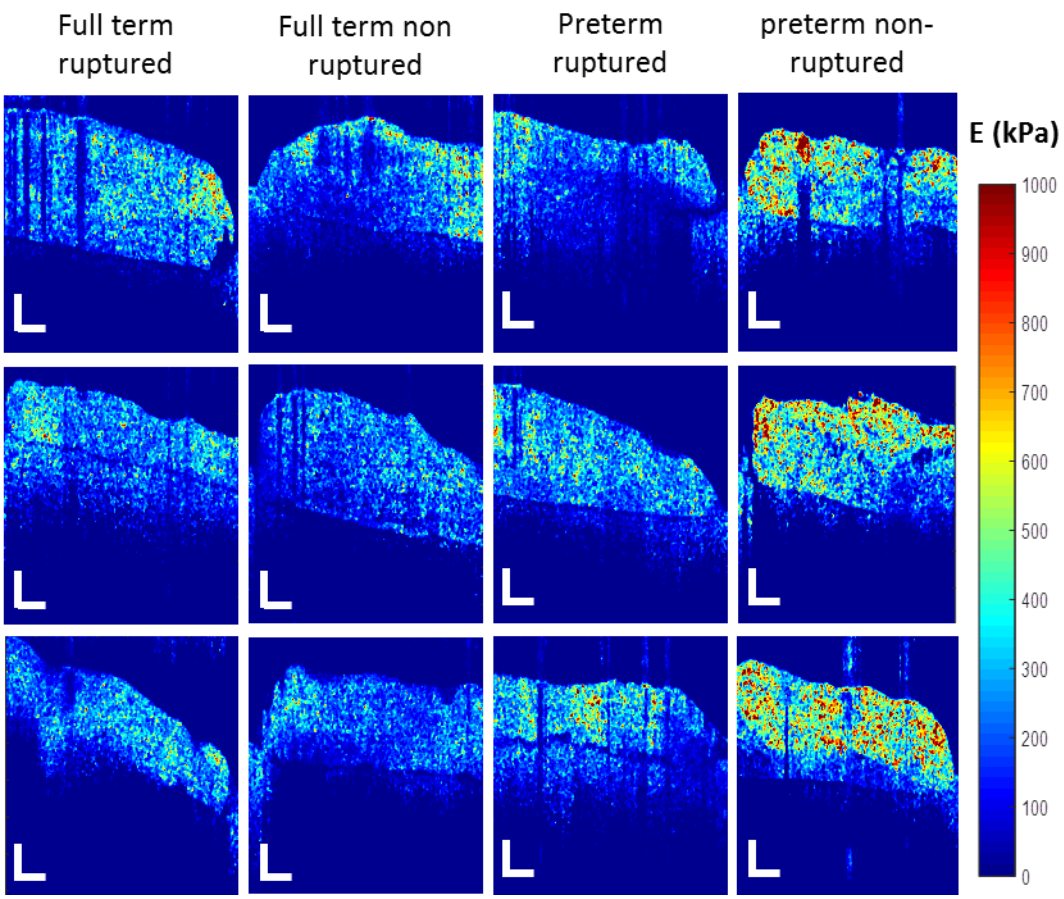


Figure 3.12: OCE images showing the modulus map of different sites of full term and preterm membranes. Cross-sectional views placed along the y-axis. Different modulus between preterm and full term samples in non-ruptured and ruptured sites were revealed (n=3 donor samples with 3 replicates). The colour scale bar indicates the relative modulus of the membranes in kPa. Scale bar = 300 µm. (A) Data before normalisation**.** (B) OCE data after normalisation. Colour bar indicates modulus.

3.4.5 Thickness of the membrane:

Two dimensional images of the FM samples were analysed using OCT. The images were taken from selected 3 areas of each sample. Thicknesses were obtained from images. Thickness of different groups of membranes measured by OCT showed that the membranes from non-

ruptured sites were thicker than the membranes from the rupture area. However, full term membranes were found thinner than preterm membranes, especially near ruptured site (Figure 3.13).

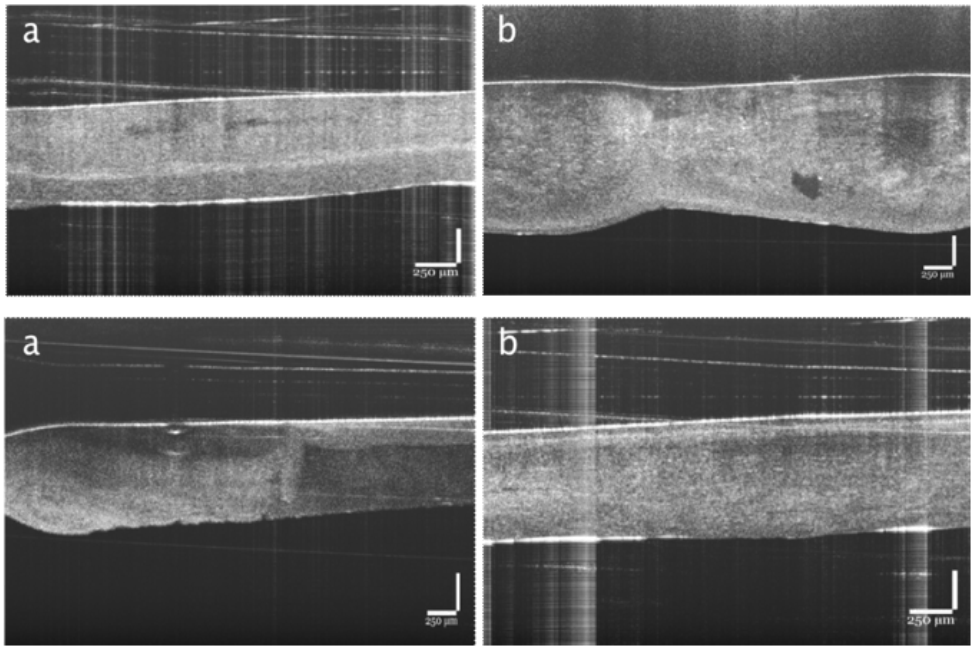


Figure 3.13 Thickness of membrane measured by OCT. OCT images showing the variation of thickness between full term and preterm samples in non-ruptured and ruptured sites. Scale bar = 250 µm. a-full term and b-preterm. Top row non-ruptured site and bottom row ruptured site. (n=4 individual membranes with 3 replicates)

The difference in thickness between two sites of full term membranes was less (0.1 mm). However in case of Preterm membranes, a greater thickness in non-ruptured sites was noticed when compared to the ruptured sites (thickness difference 0.38 mm). A difference between sites of the same membranes and preterm (0.766 ±0.094 mm) with full term (0.389 ±0.094 mm) membrane samples was also revealed (Figure 3.14).

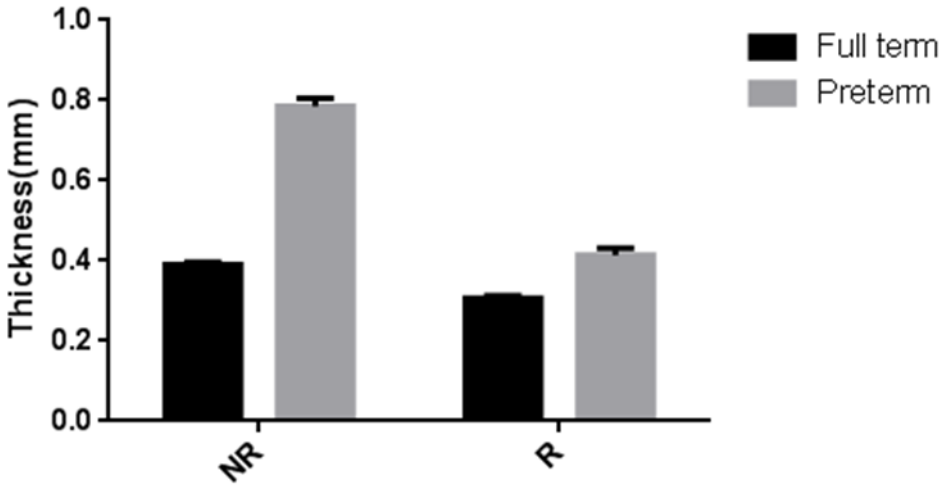


Figure 3.14: Quantitative data of FM thickness. Data showing the quantitative value of thickness of preterm and full term membranes (R-ruptured and NR-non-ruptured site). Data represents mean ± SD. (n=4 donor membranes with 3 replicates)

3.5 Discussion:

In this study, two new yet complementary mechanical test techniques to reveal the different heterogeneity patterns of mechanical properties in full term and preterm membranes were applied. The ball indentation system and the OCE technique both test the viscoelasticity and modulus of the membranes in a complementary fashion. The ball indentation technique is simple, flexible and it is possible to measure multiple samples at a time (e.g. different layers and location). Importantly, the test conditions mimic the in vivo environment as all creep tests were conducted in an incubator at body temperature and the force exerting to the membrane is radial. Membranes from ruptured areas showed larger deformation compared to the membranes far from the ruptured areas. Although we did not identify the 'Para-cervical weak zone' in the membranes as described by El Khwad et al., 2005 we postulate that the rupture site would cover the weak zone. From the ball indentation test and OCE results, it is clear that there was a large difference in the mechanical behaviour between membranes of preterm and full term deliveries,

and between locations within the membranes themselves. For the first time, we are able to provide evidence that the heterogenic difference of mechanical properties in preterm membranes between rupture and non-ruptured sites was far larger than those in full term membranes.

3.5.1 Chorioamniotic separation may be linked with membrane rupture

FM is composed of two different layers-amnion and chorion. Amnion is transparent and thinner than chorion. We have observed that these two layers were easily separable in chorioamnion sample obtained from normal vaginal delivery. In the preterm membrane, these were fused strongly, especially in the placental sites. Closer to delivery time, some degree of splitting up of amnion and chorion is natural. At the beginning of the gestation amnion and chorion exists separately and they fuse during 14-16 weeks. After second trimester, with increased gestational length, the adherence between these two layers is decreased. The separation most commonly and widely occurs after 37 weeks (Strohl et al., 2010). Also, uterine contractions during labour help further separation (El Khwad et al., 2005). Helmig et al has suggested that this happens due to the biochemical changes such as proteoglycan decorin and hyaluronan (Helmig et al., 2005). Therefore, the variation in chorioamniotic separation across all types is possibly linked with membrane rupture alongside other well-known factors such as gestational age, labour, biochemical changes and mechanical weakness.

3.5.2 The new mechanical technique (ball indentation test) determined the mechanical properties of different membranes:

To test the mechanical strength of FM, we have successfully established a new technique (ball indentation) which may overcome the flaws of previously used techniques. Ball indentation technique is simple, flexible, and possible to measure multiple samples at a time (e.g. different layers, locations) and loading condition can mimic the in vivo environment.

The mechanical test results presented a different behaviour in chorions. Amnions showed lower deformation and higher mechanical strength than chorions. The mechanical integrity of biological tissue is dependent on its collagen quantity and distribution. In FM, collagen type I, II, IV, V and VI are present and they are aligned in a particular pattern. Collagen type IV filaments help in binding the entire network to the basement membrane (Malak et al., 1994). As the collagens are mainly distributed on amnion layer and amount is nearly twice in comparison to that of collagen on chorion, the variation in mechanical strength between these layers is expected.

Another interesting phenomenon was noted in the fact that amnion and chorion had different viscoelasticity. When the ball was taken away after certain time, amnion was found to reform immediately whilst chorion took few more minutes to reform. After long hours of indentation (10-12 hours), chorion never reformed again while amnion showed very little permanent deformation even after 20-24 hours. Typically, elastin is responsible for providing elasticity of the FM. The presence of higher levels of elastin in amniotic membranes compared to chorion (George et al., 2014) can explain the immediate reforming capability of the amnion. Elastin also helps in transporting water and other soluble material (Fukuda et al., 1999). The short term recoverable deformation may be due to water overflow from collagenous filaments and the long-time loading causing permanent deformation may be due to change of collagen fibre alignments (Mauri et al., 2015). Significant variation in membrane weakness was noted between the two areas in preterm membranes.

3.5.3 A big variation of membrane strength between non-ruptured and ruptured areas in preterm membrane:

The ball indentation study confirmed heterogeneous mechanical property distribution in the same FM whether it is between the sublayers (amnion and chorion) or difference in isolation area (ruptured area or tear zones and non-ruptured areas). Membrane from ruptured area or tear zone showed larger deformation compared to the membranes far from the ruptured area or non-

ruptured areas thus supporting the fact that the area which is rendered weak, ruptures. Malak et al., have reported that an excessive and constant morphological alteration of FM takes place towards the end of gestation. It could happen in different ways such as basement membrane degradation of the amnion, trophoblast layer swelling and shrinking, or membrane thinning all together. This alteration is known as 'zone alter morphology' (ZAM) and it is supposed to be controlled by several biochemical factors. Also, it has predicted that this weak zone in full term FM could be the reason of membrane failure during labour (Malak et al., 1994, Perrrini et al., 2016). The mechanical behaviours of chorion and amnion individually were remarkably different, so the generated physical stresses in the bi-layered FM potentially could cause membrane rupture. In case of PPROM membranes, a mathematical model study has reported that the membrane physical stress was increased due to the chorion thickness and reduced lubrication between amnion-chorion interphase (Verbruggen et al., 2017). Although that study did not mention any weak zone in preterm membrane, they believed that the mechanical and structural change of amnion and chorion disrupt the integrity of FM and that could lead to membrane rupture in either normal or preterm rupture (Verbruggen et al., 2017). Our data were consistent with those studies. The big difference in creeping property and modulus in PPROM membranes could be the potential reason for preterm rupture. Therefore an obvious dissimilarity of mechanical creeping property was found between both the areas of membranes either from full term or preterm.

Full term				Preterm		
	NR	R	Difference	NR	R	Difference
CA (Creep) Pixels	80	94	15%	71	110	36%
CA (modulus) Kpa	300	200	33%	900	400	55%
CA (Thickness) mm	0.39	0.3	23%	0.77	0.4	48%

Table 3.2: Approximate percentage differences of mechanical behaviour (Creep, modulus and thickness) between ruptured and non-ruptured sites of samples from full term and preterm deliveries. In preterm membranes the differences were higher than the full term membranes. CA-chorioamnion. % = $(CA_{NR}-CA_R)/CA_{NR}$ x100. NR= Non-ruptured and R=Ruptured.

An interesting outcome was observed when the two areas of full term membranes were compared with the two areas of the preterm membranes. The preterm membranes deformed less than the full term membranes. As membrane gets weaker with an advancing gestational age, this result was expected. But what is interesting from the ball indentation test was a big difference in the mechanical behaviour between membranes of preterm and full term deliveries, and between locations within the membranes themselves. For the first time, as Table 3.2 has displayed, this study is able to provide evidence that the heterogenic difference of creeping and modulus in preterm membranes (36% and 55%) between rupture and non-ruptured sites was far larger than those in full term membranes (15% and 33%). The heterogeneity in stiffness in sites of membranes was confirmed with OCE. OCE findings were shown to correlate with outcomes from ball indentation test. It showed overall higher moduli of preterm membranes compared to the full-term ones. Notable heterogeneity in full thickness of the membranes of full term (23%) and preterm (48%) was observed via OCT. This is further supported by the results of mechanical behaviours of the FM samples which were carried out in our investigation of preterm rupture of

the membranes. The overall observations were interesting with noteworthy implications in PPROM aetiology. In addition, this heterogeneity is more likely the cause of the inferior mechanical properties of the ruptured sites and their untimely rupture.

3.6. Conclusion:

The successfully established ball indentation test technique used in this study has overcome some of the drawbacks associated with the previous techniques. Our study has clearly shown a large difference in the mechanical properties between ruptured and non-ruptured sites of full term and preterm membranes, as demonstrated creep, modulus and thickness analysis (Table 3.2). The new mechanical test techniques, ball indentation and OCE, independently and complementarily indicated that the non-ruptured sites in preterm membrane had a much higher strength than ruptured sites, whilst the strength difference between two different sites on full term membrane was small. This may therefore be a major factor leading to the weakening of the placental membranes during early gestation. Although we have extensively delved in mechanical properties of the membranes, further exploration of other factors which could influence the mechanical properties is required including the assessment of biochemical changes. This will be investigated in next chapter.

Chapter 4

Expression of key biochemical markers between fetal membrane from full term and preterm labour

4.1. Introduction:

It is prudent to establish a conceivable and reliable mechanical test in order to explore the mechanical behaviour of fetal membrane (FM). Also, there is need for a better understanding of the rupture sequence of the FM components (amnion &chorion). The triggering mechanism and factors involved are still questionable. Biochemical changes in the membrane are normally one of the main factors in FM rupture. However, its role in PPROM is unclear. It is known that mechanical failure of FM is an essential part of labour, including sequential events such as cervical softening and dilation, weakening of fetal membrane, contractions of myometrium and eventually FM rupture. (Soydinc et al., 2013)

ECM (extracellular matrix) is composed of glycosaminoglycan (GAGs), fibrous proteins and proteoglycans. Together, these components play a role in maximizing and preserving the integrity of human fetal membranes until normal labour at the end of gestation. Proteoglycans are a combination of GAGs and proteins (Chandar et al., 2012). Collagen provides resistance to shear forces, while elastin adds a resilience capability to tissue. In contrast to fibrous proteins, proteoglycans provide a strong resistance to compressional forces, due to the presence of GAG side chains. Two main proteoglycans found to be abundant in fetal membranes are decorin and biglycan (Meinert et al., 2001).Towards the end of pregnancy and during labour, proteoglycans have been known to play a role in ripening the cervix (Winkler et al., 1999). It is assumed that one of the factors which cause PPROM is due to an imbalanced expression of GAG molecules in FM especially the relative concentration of decorin and biglycan.

Matrix metalloproteinases (MMPs) are a group of specific enzymes, which play a vital role throughout gestation, in membrane weakening and rupture during labour. These enzymes regulate the degradation of collagen and non-collagen components. MMP 2 and MMP 9 have been found to be secreted in high concentrations once the contractions started and during labour, MMP 9 supersedes the MMP 2 concentration (Soydinc et al., 2013). Greenwood et al.,

noticed significantly higher levels of MMP 9 than MMP 2 during active labour, while MMP 2 levels were higher during the initial phase of contractions (Greenwood et al., 1995). MMP 13 was also found to present in amniotic fluid and in FM during labour, although it's role is not very clear yet (Fortunato et al., 2003).

Programmed cell death (apoptosis) is another event believed to contribute to ECM remodelling and its integrity. Apoptosis is thought to play a role in activating the onset of labour and by ECM degradation during this period (Menon et al., 2004). PARP, an apoptosis marker, is found to be highly expressed in the section of FM near internal cervical areas. The higher expression levels are believed to contribute to the reduction of the membrane strength (McParland et al., 2003). Notably, Kumar et al., reported that apoptosis can occur without inflammation. According to their study, TNF-α and IL-1β stimulate collagen remodelling and towards the term labour, cause FM to lose strength and prepare for rupture, correlating with apoptosis (Kumar et al., 2006).

Before pregnancy and during early gestation, progesterone is mainly produced by the corpus luteum of the ovary. Towards the end of pregnancy, it is produced in large amounts by the placenta and plays a vital role in labour. If progesterone levels decrease, this can pose a risk for premature rupture. PR-A and PR-B are two proteins expressed by progesterone receptors (Conneely et al., 2002). There is established knowledge appreciated progesterone use as prevention for preterm birth (Mackenzie et al., 2006) but very few studies have investigated the PR role in preterm labour pathology. Recently, FDA has approved progesterone supplementation for women at risk of premature birth. Although some studies have shown a positive result, others have not been convincing in demonstrating a successful outcome in reducing premature birth with progesterone supplementation (Briery et al., 2011).

This gives a clear indication that along with mechanical stress biochemical changes also play a major role in PPROM. Biochemical factors which are abnormally regulated or untimely activated can cause membrane weakness rendering membrane to rupture before term. It is essential to

compare the protein and hormone levels between term and preterm labours. It would also be interesting to find a correlation between physical and the mechanical changes and biochemical alteration leading to membrane rupture.

4.2 Objective:

The main aim of this chapter was to study the expression of key biochemical markers between ruptured and non-ruptured sites of membranes from full term and preterm labours. Distribution of the different types of collagen, proteoglycans and the structural components of ECM within the FM were assessed as well in order to investigate the impact of these expression patterns on PPROM. This chapter also aimed to define the correlation between physical properties and biochemical alteration leading to immature membrane rupture.

4.3 Methods:

4.3.1 Sample preparation:

FM samples from full term normal vaginal deliveries and preterm membranes from spontaneous PPROM deliveries were collected. Intact membranes from both ruptured and non-ruptured sites were studied. The sample collection method was described in chapter 2, section 2.1.

4.3.2 Sample sectioning:

Intact samples were sectioned through two different methods, paraffin embedding and cryo-sectioning following the protocol described previously in chapter 2, section 2.3.1.1 and 2.3.1.2 respectively.

4.3.3 Histochemical staining:

Different types of histochemical staining were performed on the paraffin sectioned samples. H&E staining was followed as described before in chapter 2, Section 2.3.2. Sirius red staining and

toluidine blue staining, was performed as per protocol as described previously in chapter 2 in sections 2.3.3 and 2.3.4.

4.3.4 Biochemical assays:

Hydroxyproline assay and DMMB assays were performed to quantify the total collagen and total GAG of the samples as described in chapter 2, section 2.5.2 and 2.5.1 respectively.

4.3.5 Cell viability:

To assess the viable cells in the FM samples before performing other biochemical tests, a live/dead staining kit was utilised. The protocol from chapter 2, section 2.5.3 was followed.

4.3.6 Immunostaining:

Immunostainings were done using cryosections of the samples to detect the biomarkers of ECM. These were for collagen I and III, proteoglycans-biglycan and decorin, MMP 9 and 13, IL-1β, fibronectin, and progesterone receptors. Membrane sections were stained using the protocol as described before in chapter 2, section 2.4 and imaged through confocal microscopy. Semi-quantification of fluoresce expression was conducted via ImageJ Software.

4.3.7 Western blotting:

Western blotting was performed for further quantification of the above mentioned bio markers using the same protocol described in chapter 2, section 2.6. ImageJ software was utilized to semi quantify of the protein expressions.

4.4 Results:

4.4.1 H&E staining result:

Intact membrane sections were stained. H&E stained samples were viewed and imaged under bright field microscopy. The distinct two layers were clear in all the FM cross-sections (Figure 4.1).

The amnion layer was transparent (avascular) and contained five separate sublayers. The epithelium layer cells were compactly arranged as indicated in Figure 4.1.

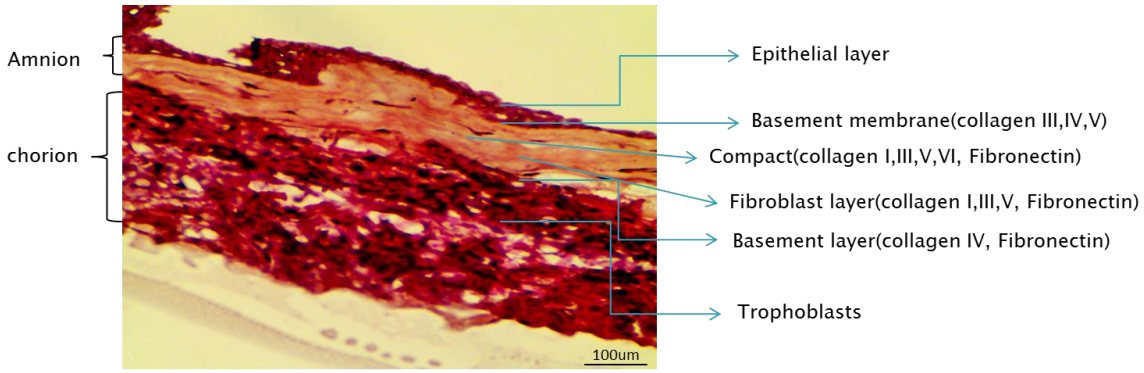


Figure 4.1: H &E staining of a cross section of the human fetal membrane showing cellular sub-layers of amnion and chorion and their compositions. Scale bar = 100 µm.

4.4.2 Cell viability result:

A Live/dead test was performed to check the cell survival of the membranes after collection from the hospital and also with the membranes which was stored in -80°C. Live/dead assay test results revealed that a good proportion of cells were viable even after 12 hours of delivery plus 3 hours of preparation time. Membranes were stored in -80°C immediately after collection. Confocal microscopic images demonstrated good cell viability (Figure 4.2) in which the viable cells fluoresced as green and non-viable cells red.

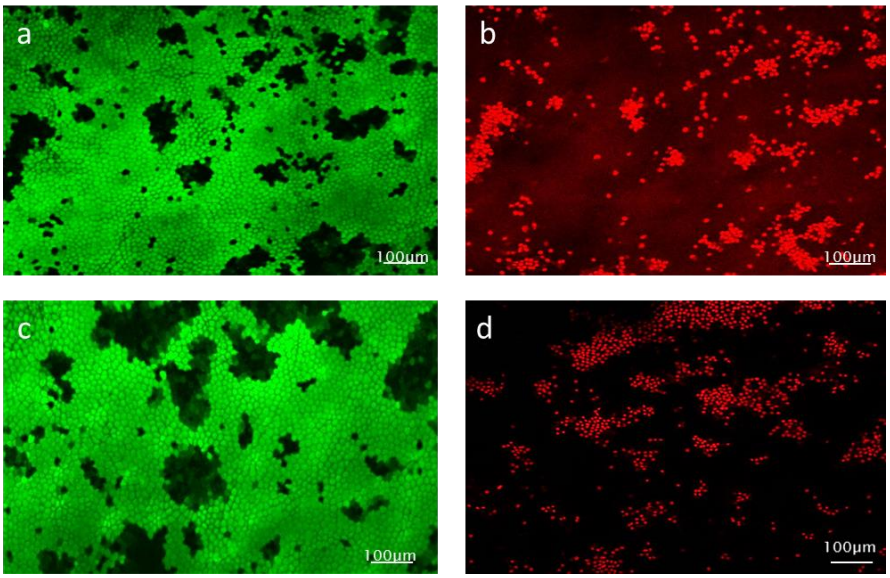


Figure 4.2: Live and dead staining of fetal membranes. Top row images representing the collected membranes after 12 hours of delivery. Bottom row images are representing the frozen membranes. (a), (c) the live cells of the membranes; and (b), (d) the dead cells. The Live cells stained green and dead cells red, scale bar = 100 µm. n=3 donors.

4.4.3 Collagen content and morphology of the membranes:

To understand the tissue collagen behaviour in full term and PPROM membranes, Pico Sirius red staining was performed on the non-ruptured sites of samples. The staining was viewed by the bright field light and polarized microscopy. Images from the bright field microscopy showed denser collagen fibers than the full term membranes while dark coloured areas denoted that the fibers were aligned more compactly in the preterm non-ruptured sites than the full term counterparts (Figure 4.3 A). Polarized microscope images showed more stained areas in preterm tissue than full term (Figure 4.3 B). Dark red stain represents the collagen fibres which are densely aligned.

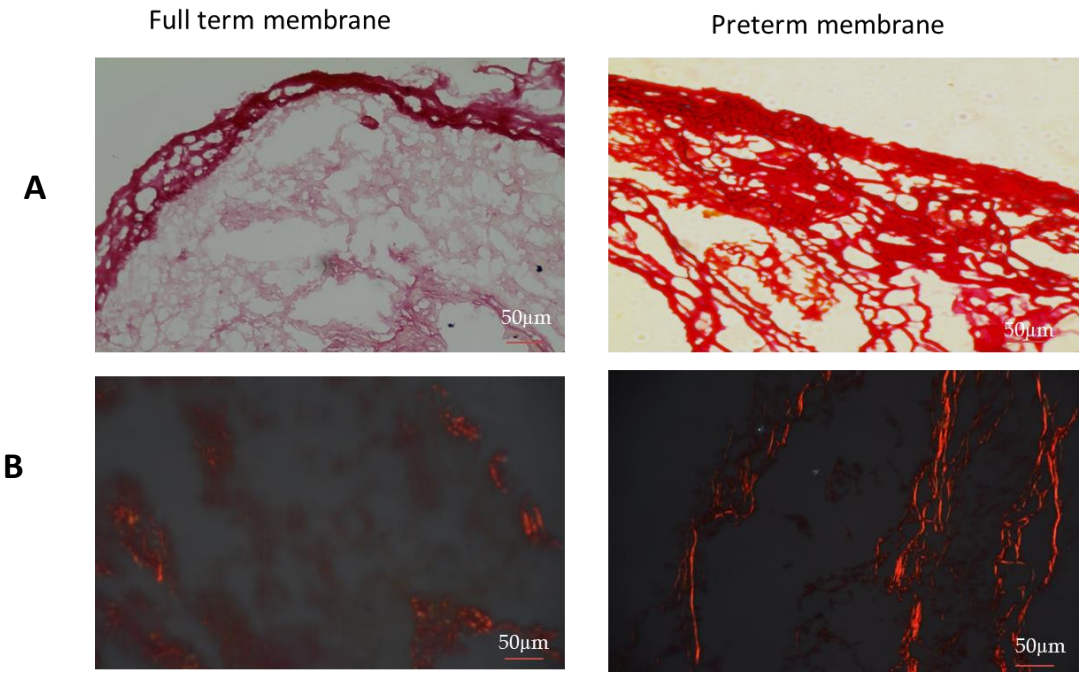


Figure 4.3: Representative Picro Sirius red staining images of membranes from full term and PPROM. (A) Picture was taken by brightfield microscope. (B) Images taken under polarized light. Both samples were from non-ruptured sites of normal delivery samples. Scale bar = 50 µm. (n=4 donors).

For further validation, total collagen quantification was performed using hydroxyl proline assay. This time, the test was carried out with both ruptured and non-ruptured sites of the membranes from full term and preterm (PPROM). The quantification of collagen using colorimetric hydroxyl proline assay results showed (Figure 4.4) differences in total collagen concentration between both the groups. In general, ruptured sites of the membrane showed lesser total collagen quantity than the non-ruptured sites. In ruptured sites of the full term membranes, the average amount of collagen was higher than the preterm ruptured sites. Whereas, the average collagen quantities of preterm non-ruptured site samples were higher than their full term counterparts. That means the total collagen content between ruptured and non-ruptured sites in full term membrane samples were smaller (22.7%) in comparison to the counterparts of the PPROM membrane samples, which showed lager difference (49.5%) (Table 4.1).

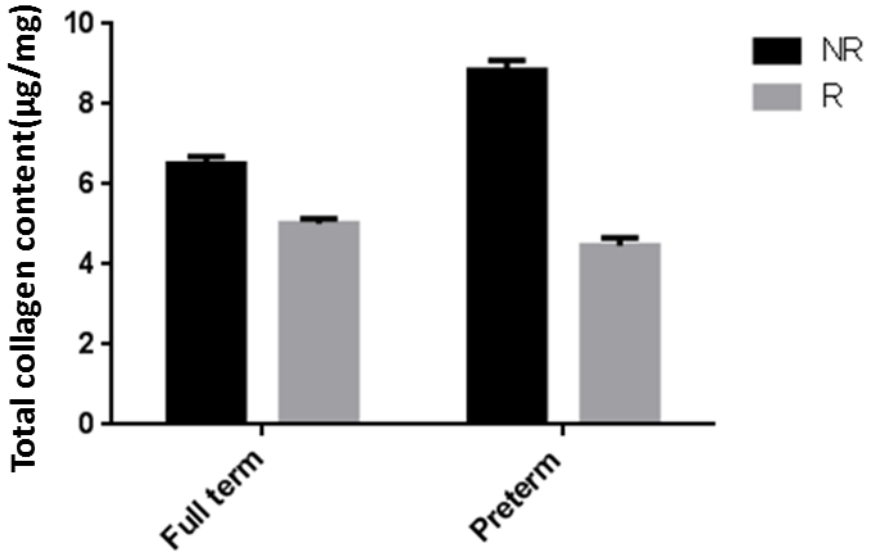
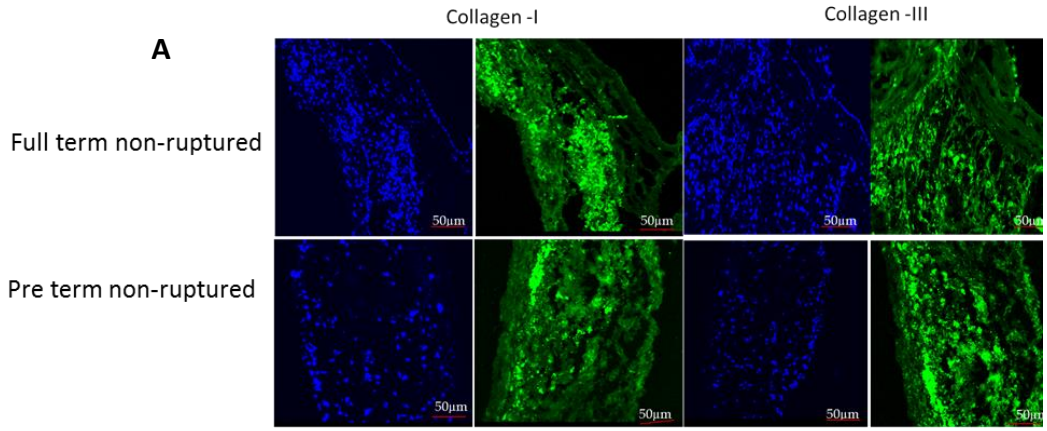


Figure 4.4: Total collagen content of fetal membranes. (Collagen assessment of the samples from full term and preterm membranes from ruptured and non-ruptured sites. (NR=non-ruptured site, R= Ruptured site). Data are presented as mean ± SD (n=3 donor membranes with 3 replicates).

	Full term			Preterm		
	NR	R	Difference (%) $(CA_{NR}-CA_R/CA_{NR})$ %	NR	R	Difference (%)
CA (Collagen quantity) µg/mg	6.500	5.022	22.7	8.835	4.464	49.5

Table 4.1: Differences of total collagen between non-ruptured and ruptured sites of full term and preterm membranes. NR=non-ruptured, R=ruptured.

The two types of predominant collagen in fetal membranes are collagen I and collagen III. The immunostaining result showed different expressions of collagen I and III between full term and preterm non-ruptured sites of membranes (Figure 4.5 A). Higher expression in preterm samples compared to the full term membranes corresponded with other results of total collagen observation. Further semi-quantification by ImageJ software of the fluorescence intensity was carried out to confirm the outcome (Figure 4.5 B).



B

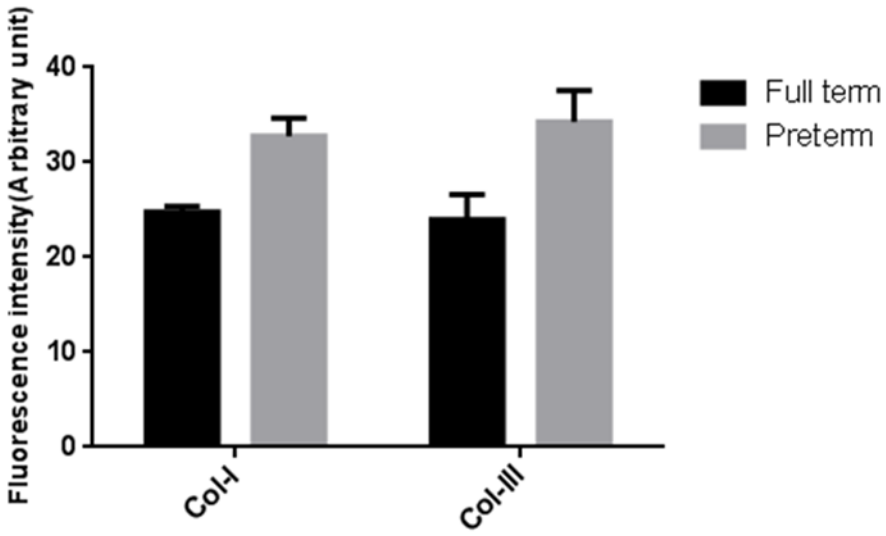
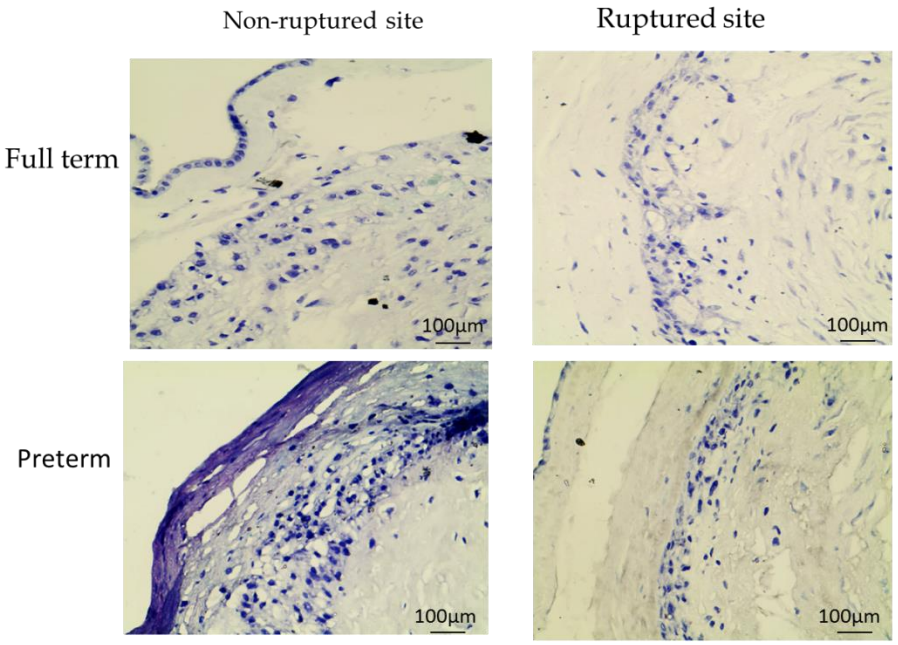


Figure 4.5: Immunostaining expression for collagen I and III. (A) Representative images showing collagen I and III expression (green) in non-ruptured sites of both full term and PPROM membrane. Nuclei were counter stained with DAPI (blue). (B) Semi quantification data of the fluorescence intensity showed higher intensity of collagen-I and collagen III in preterm samples than the full term samples. Data are presented as mean ± SD (n=3 donors and 3 replicates). Scale bar = 50 µm.

4.4.4 Sulphated GAG (sGAG) expression:

Total sGAG level of the membranes was estimated by toluidine blue staining. Toluidine blue staining revealed that ruptured sites of the membranes showed higher rate of expression than the non-ruptured sites in both full-term and preterm samples. Preterm ruptured site samples were stained darker than full term ruptured site of the membranes. Non-ruptured sites of both types of membranes were equally stained. Spongy layer, which is the interface between amnion and chorion, showed most prominent staining (Figure 4.6).

A



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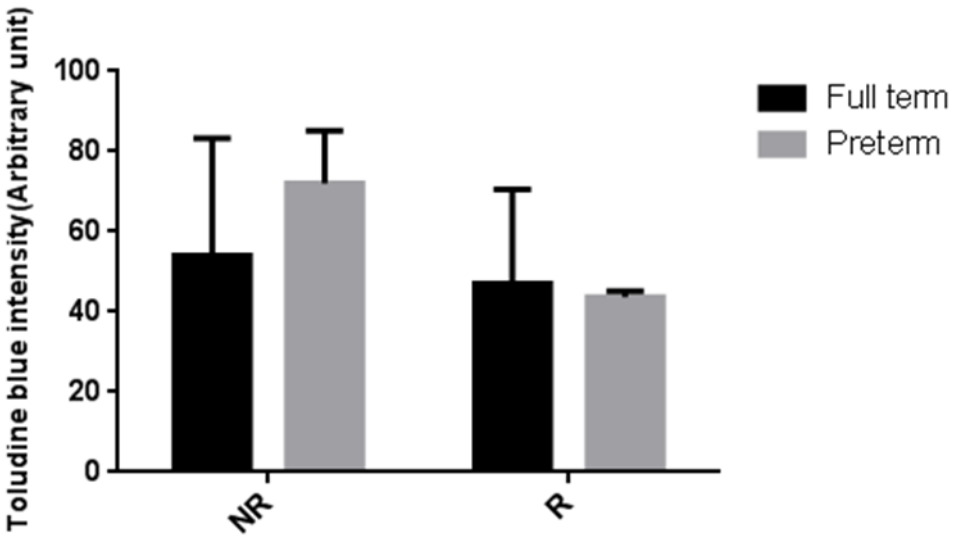


Figure 4.6: Toluidine blue staining for sGAG detection in fetal membranes. (A) Representative images showing the expression of total sGAG via toluidine blue staining in the membranes from full term and PPROM. Both Ruptured sites and non-ruptured sites were stained. Scale bar=100 µm. (B) semi-quantification of toluidine blue staining intensity. Data are presented as mean ± SD (n=3 donors with 3 replicates).

Further investigation of the GAG contents of FM samples were assessed by DMMB assay. The assay result concurred with toluidine blue staining outcome. Both preterm and full term of the membranes showed differences between ruptured and non-ruptured sites of the samples (Figure 4.7). A greater variation of GAG content on preterm membranes (46%) was found compared to the full term membranes (20%). Percentage was calculated by equation of $(CA_{NR}-CA_R)/CA_{NR}$ x 100 (Table 4.2).

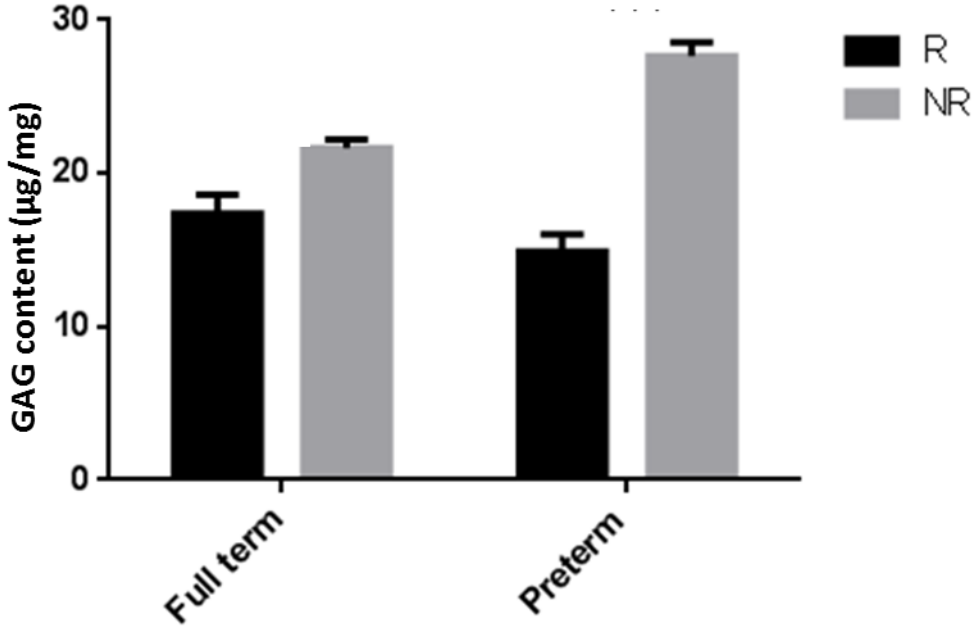


Figure 4.7: sGAG content from full term and preterm samples. sGAG result of DMMB assay of ruptured sites (R) and non-ruptured sites (NR). Data are presented as mean ± SD (n=3 donors and 3 replicates).

Full term				Preterm		
	NR	R	Difference (%)	NR	R	Difference (%)
CA (GAGs content) µg/mg	21.65	17.37	20	27.67	14.89	46

Table 4.2: Differences of total GAGs content between non-ruptured and ruptured sites of full term and preterm membranes. NR=non-ruptured, R=ruptured.

4.4.5 Decorin and bi-glycan expression:

Immunostaining was conducted to observe the expression of the two proteoglycans in fetal membrane, decorin and biglycan. Frozen sections of both the types of membranes and from both the different locations were included. Figure 4.9 shows that in the membranes from non-ruptured sites decorin expressed more than the ruptured sites of the membranes. In ruptured sites, both full term and preterm membranes appeared to have similar decorin expression. However, the non-ruptured sites of preterm membranes showed greater expression than the full term non-ruptured sites.

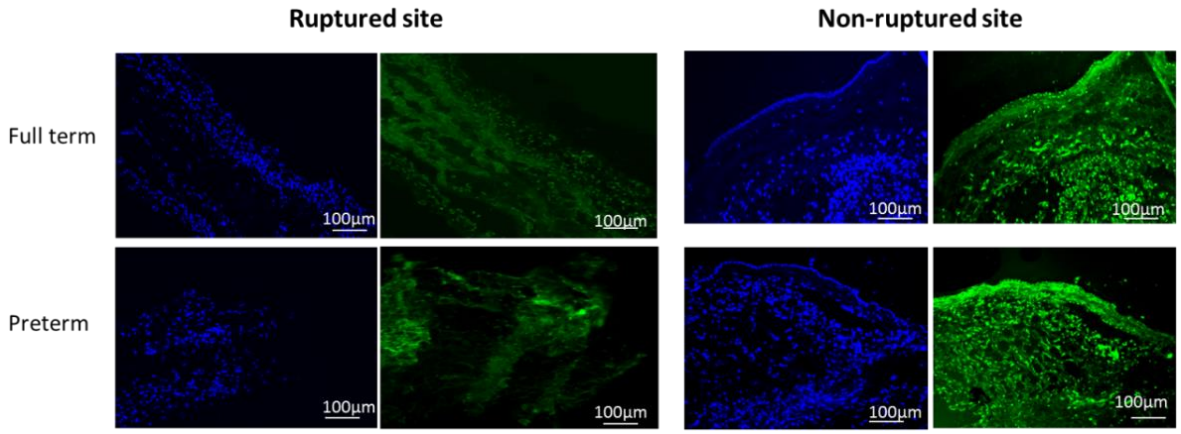


Figure 4.8: Immunofluorescence expression of decorin. Representative Immunostaining images showing decorin expression (green) in full term and preterm membranes and in both ruptured sites and non-ruptured sites. Sections were counterstained by DAPI (blue) (n=3). Scale bar= 100 µm

The immunostaining outcome of biglycan expression was found to have a similar pattern to the decorin expressed. However, the expressions in the non-ruptured sites of both membranes were not as high as decorin expression. (Figure 4.9)

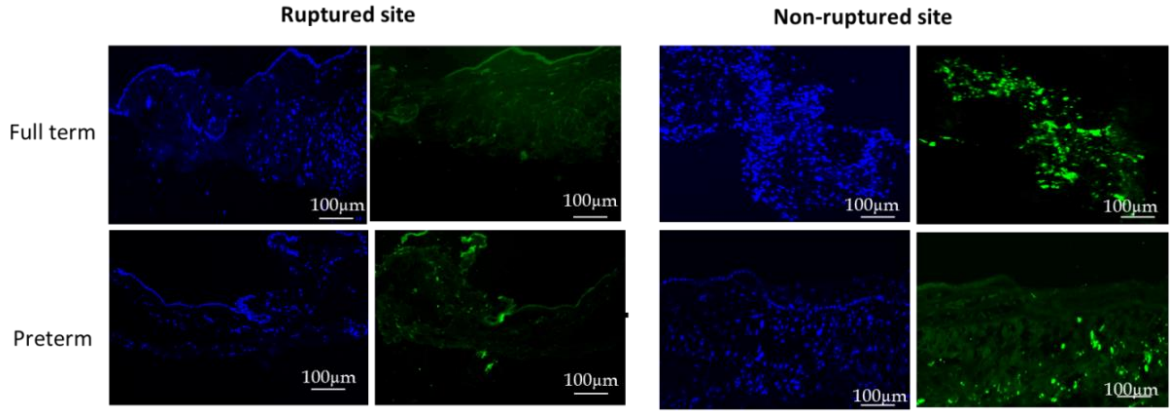
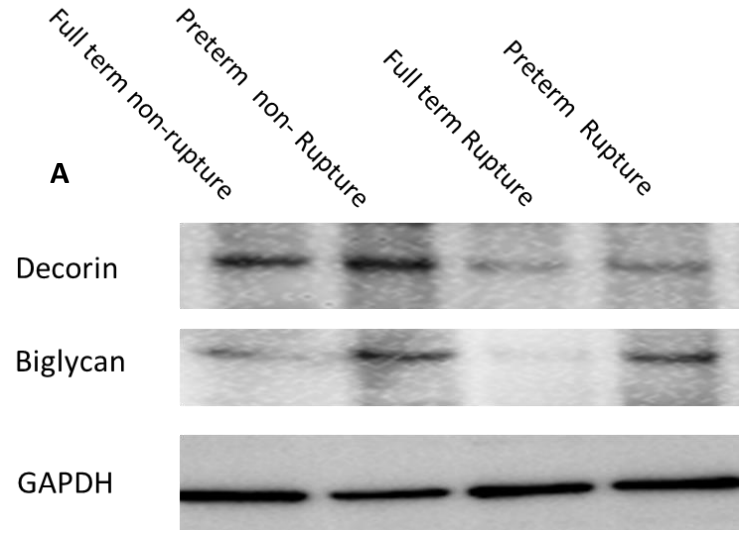


Figure 4.9: Immunofluorescence expression of biglycan. Representative Immunostaining images showing biglycan expression (green) in full term and preterm membranes and in both ruptured sites and non-ruptured sites of the membranes. Sections were counterstained by DAPI (blue). (n=3 donors and 3 replicates). Scale bar=100 µm.

For cross validation of the staining outcome, quantitative analysis of the presence decorin and biglycan in samples was carried out by Western blotting. In Figure 4.10 A, Western blot results indicate a higher expression of decorin and biglycan in non-ruptured sites of the membranes of both full term and preterm types than their ruptured sites. This data was consistent with the

immune-histological staining. The intensity of the protein expression of both decorin (Figure 4.10 B) and biglycan (Figure 4.10 C) corresponded with the Western blot outcomes.



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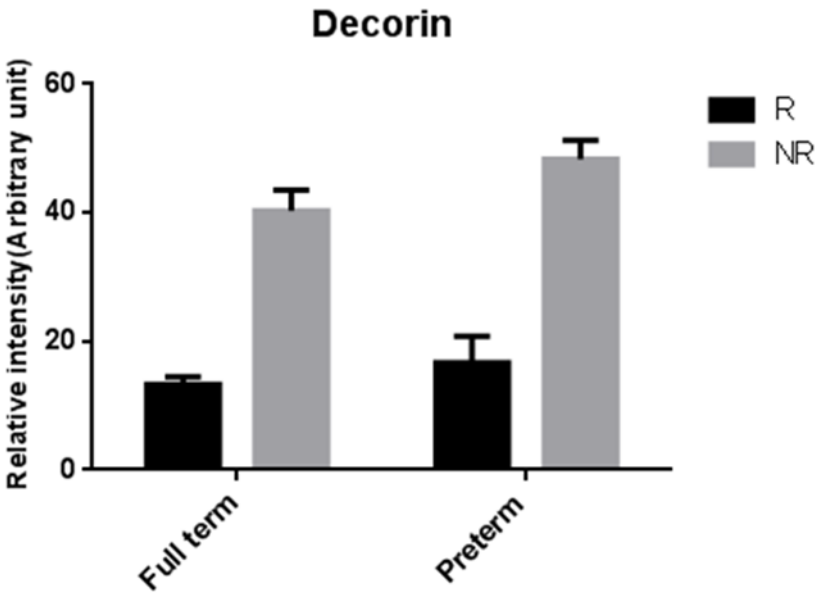

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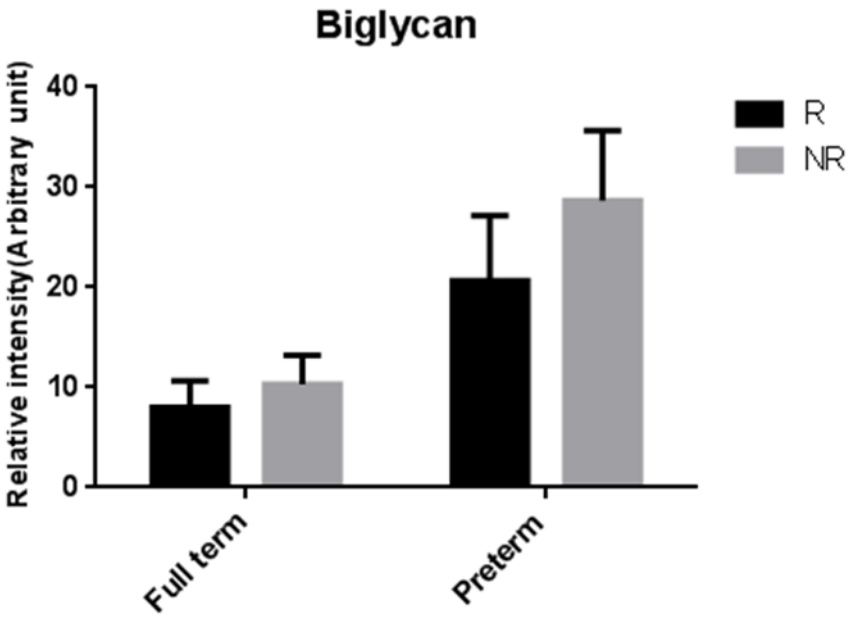
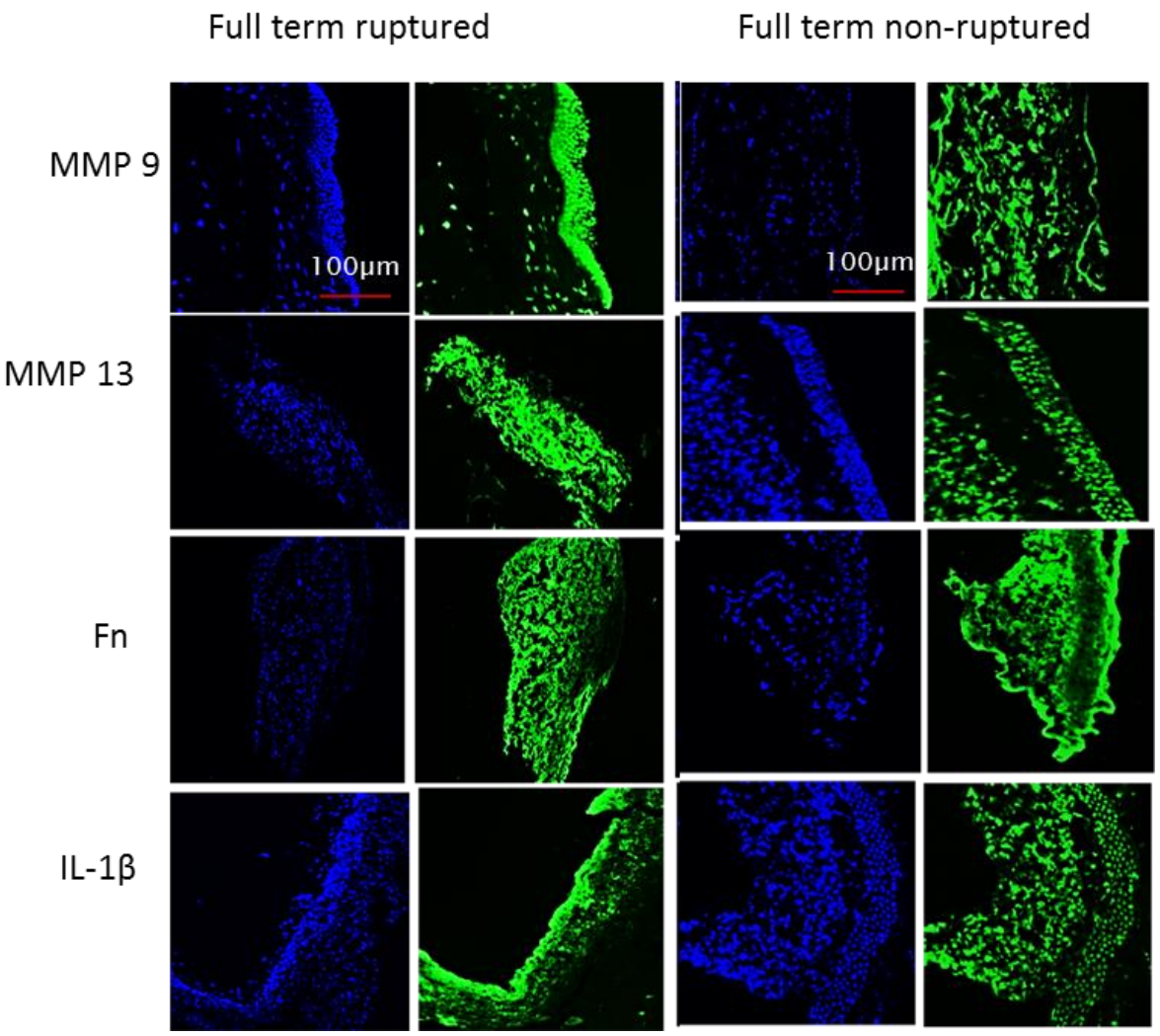


Figure 4.10: Western blotting result of biglycan and decorin. (A) Protein expression of decorin and biglycan in full term and preterm membranes and in non- ruptured and ruptured sites. Results are relative to the loading control GAPDH. (B) Semi-quantification of western blot data for decorin and (C) biglycan. Data are presented as mean ± SD (n=3 donors and 3 replicates).

4.4.5 Expression and detection of biochemical markers of full term FM:

Expressions of different biomarkers such as MMP 9, MMP 13, IL-1β and fibronectin (Fn) in FM samples were determined by immunostaining assays. Membrane sections (4-6 µm) from both the groups from ruptured sites were assessed. MMP 9, MMP 13, Fn and IL-1β expressions were higher in ruptured sites compared to the non-ruptured sites (Figure 4.11). Further semi-quantification of the fluorescence intensity was carried out to confirm the outcome (Figure 4.11 B.

A


Full term ruptured
Full term non-ruptured
MMP 9
MMP 13
Fn
IL-1β
100μm
100μm

B

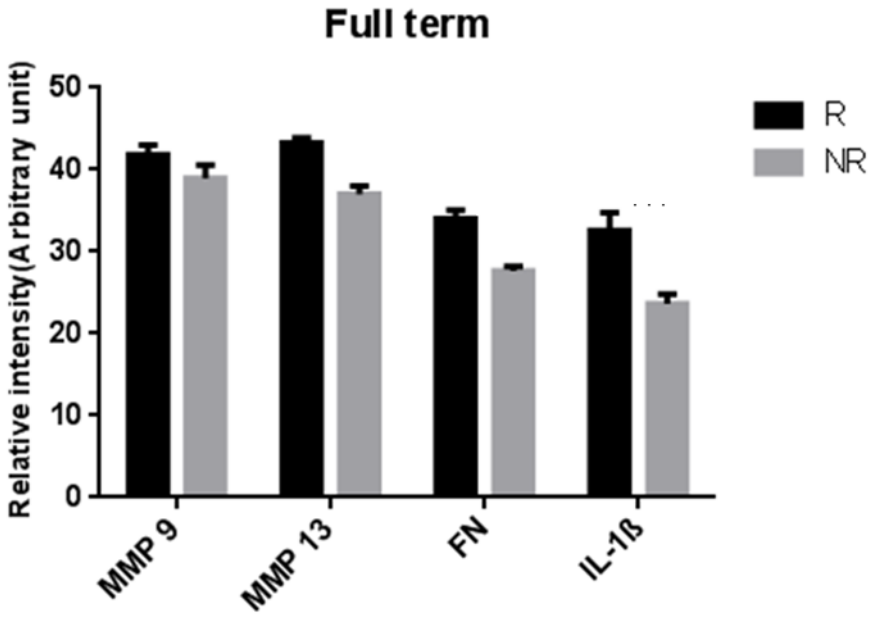
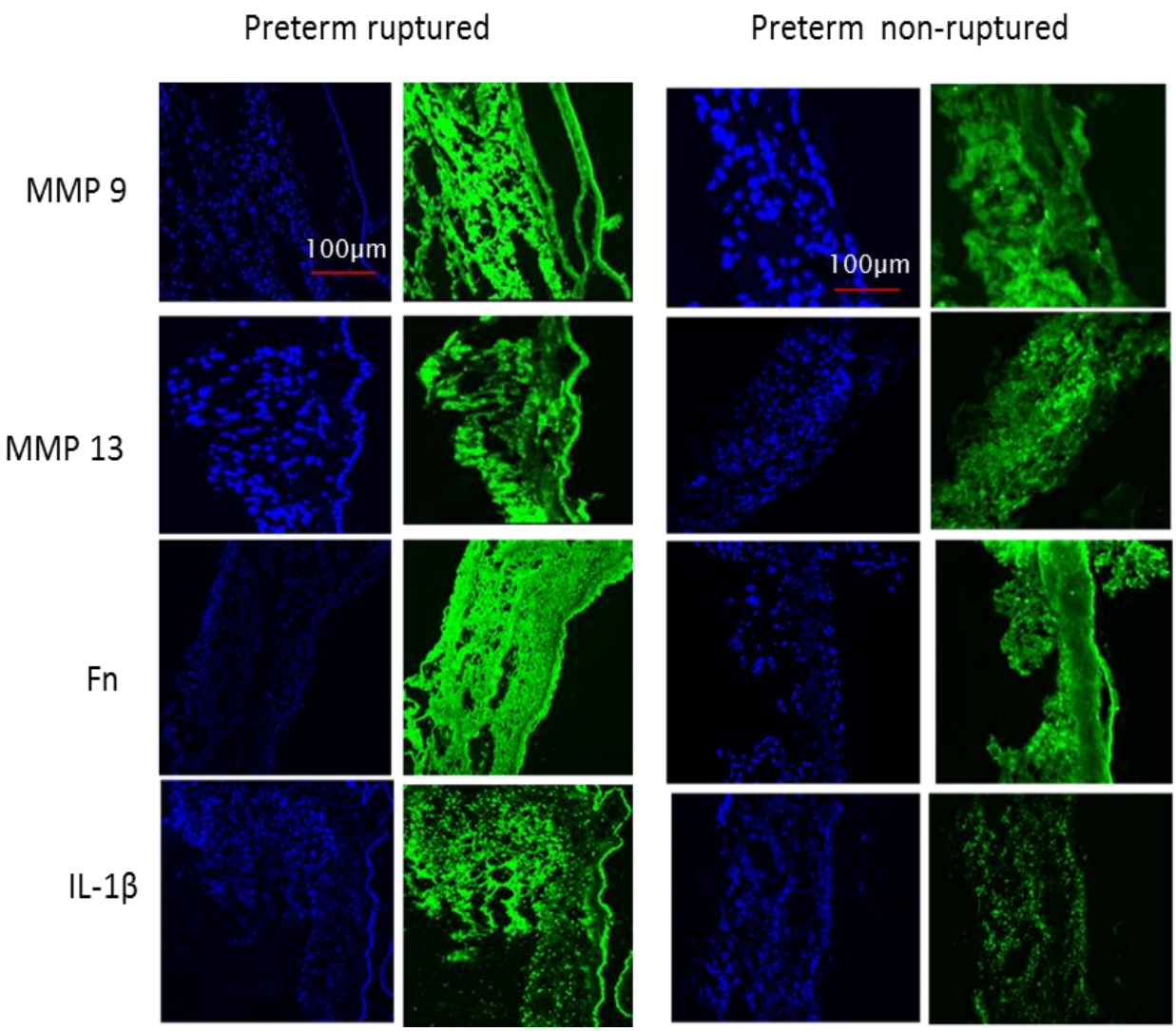


Figure 4.11: Expression of different biochemical markers at full term membranes. (A) Representative Immunostaining images showing different biomarkers (green) expression in full term at ruptured and sites with counter staining by DAPI (blue). Scale bars = 100 µm. (B) Semi-quantification of immunofluorescence data. Data are presented as mean ± SD. R-ruptured site and NR-non-ruptured site. (n=3 donors and 3 replicates).

4.4.6 Expression and detection of biochemical markers of preterm FM:

Similarly, the preterm samples were immunostained with MMP 9, MMP 13, IL-1β and fibronectin antibodies. In general, the expressions of biomarkers were lower in non-ruptured sites compared to the ruptured ends. The ruptured sites of preterm samples exhibited almost similar expression as full term ruptured sites (Figure 4.12). But the non-ruptured sites of preterm membranes showed lower expression of the biomarkers than the full term non-ruptured counterparts (Figure 4.12). Further semi-quantification of the fluorescence intensity was carried out to confirm the outcome (Figure 4.12 B) which indicated a big difference of immunostaining intensity between non-ruptured sites and ruptured sites of the preterm membrane samples. This data is consistent with the immunostaining expression.

A


Preterm ruptured
Preterm non-ruptured
MMP 9
MMP 13
Fn
IL-1β
100μm
100μm

B

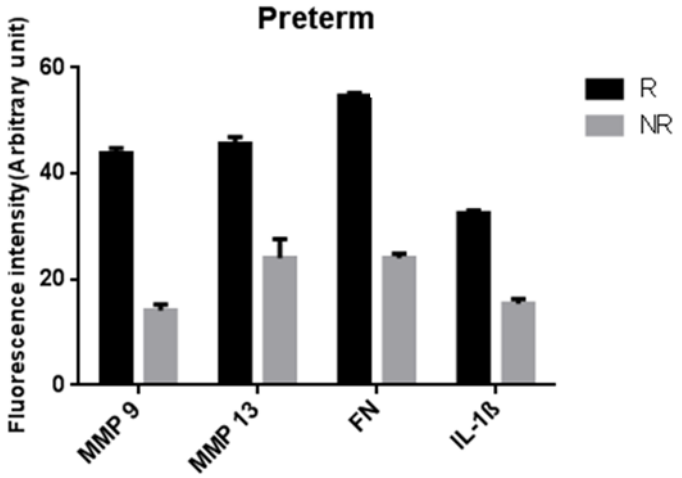
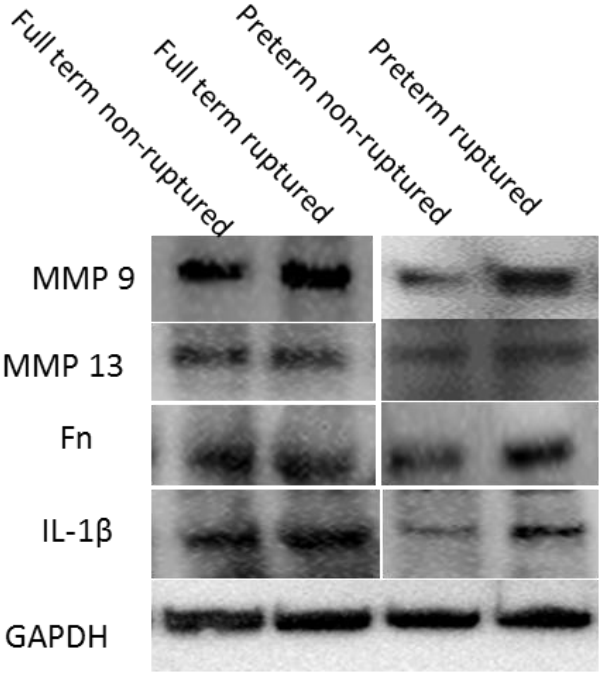

Preterm
Fluorescence intensity(Arbitrary unit)
60
40
20
0
R
NR
MMP 9
MMP 13
FN
IL-1ß

Figure 4.12: Expression of different biochemical markers at preterm membranes. (A) Representative immunostaining images showing different biomarkers (green) expression in preterm membranes at ruptured and non-ruptured sites with counter staining by DAPI (blue). Scale bars = 100 μm. (B) Semi-quantification of immunofluorescence data. Data are presented as mean ± SD. R-ruptured site and NR-non-ruptured site. (n=3 donors and 3 replicates).

4.4.7 Western blotting result:

Further detection of MMP 9, MMP 13, IL-1β and Fn in the samples was performed by Western blotting Figure 4.13 A shows, higher protein expression in ruptured sites of preterm and full term than in the non-ruptured sites in general. Both sites in full term showed less difference of expressions (Figure 4.13 C). But, in preterm membranes, ruptured sites of protein expression were much higher than the non-ruptured sites. This corresponded to the immunostaining results and showed similar expression patterns of MMP 9, 13, fibronectin, IL-1β in both sites. The immunostaining expression was found to be consistent with the western blotting result. The intensity of the protein expressions were corresponded with the Western blot outcomes (Figure 4.13 B). A big difference in bio marker expression was noticed in both sites of preterm membranes (Table 4.3).

A


Full term non-ruptured
Full term ruptured
Preterm non-ruptured
Preterm ruptured
MMP 9
MMP 13
Fn
IL-1β
GAPDH

B

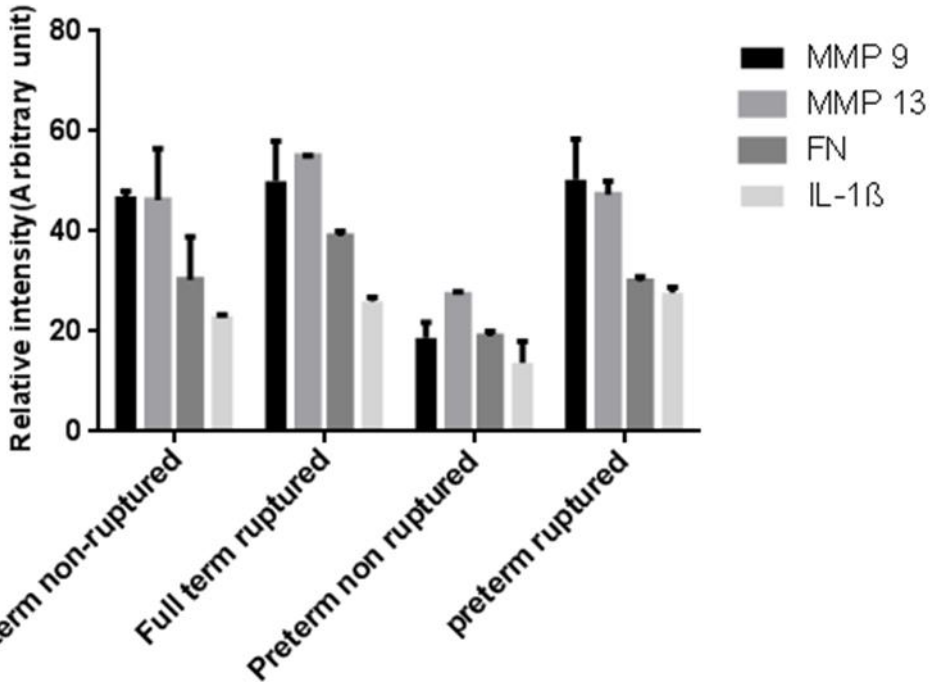

Relative intensity(Arbitrary unit)
80
60
40
20
0
MMP 9
MMP 13
FN
IL-1ß
Full term non-ruptured
Full term ruptured
Preterm non ruptured
preterm ruptured

Figure 4.13: Western blotting result of biochemical markers. (A) Protein expression of MMP9, MMP13, IL-1β and Fn in full term and preterm membranes and in both non- ruptured and ruptured sites. Results relative to the loading control GAPDH. (B) Semi-quantification of Western blot data. Data are presented as mean ± SD. (n=3 donors and 3 replicates).

	Full term			Preterm		
	NR	R	Difference (%)	NR	R	Difference (%)
CA (MMP 9)	47	58	23	18.33	50.83	57
CA (MMP 13)	46.14	55.12	19	26.9	47.34	74
CA (Fn)	30.11	39.03	29	19.03	29.2	53
CA (IL-1β)	23.25	26.5	14	13.76	27.18	97

Table 4.3: Estimated differences of different protein expression between non-ruptured and ruptured membranes of full term and preterm samples based. NR=non-ruptured, R=ruptured.

4.3.8 Progesterone receptors expression:

The expression PR isoforms were assessed by western blot analysis of full term and preterm membranes from both ruptured and non-ruptured sites. Immunostaining for PR (PRA & PRB) were performed with all the samples. Figure 4.14 shows preterm sample had uniform staining across the membranes, whilst the term sample expression was concentrated on amnion location. A clear expression of progesterone was found in full term membranes. Although both amnion and chorion of preterm samples displayed progesterone receptors expression, this was less compared to the full term membranes.

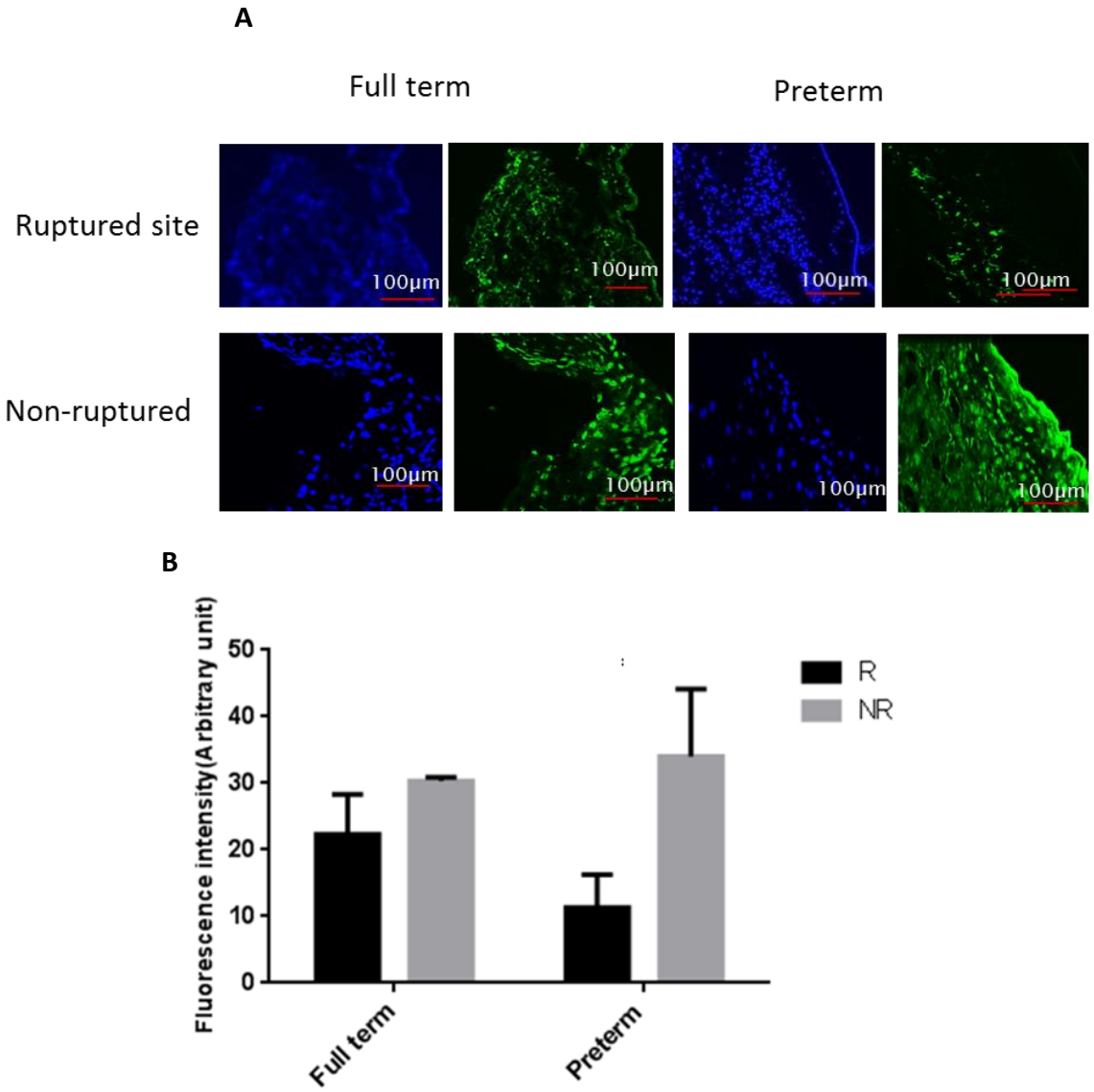


Figure 4.14: Expression of progesterone receptor on fetal membranes. (A) Representative images showing PR receptor expression (green) in full term and preterm groups of membranes at their ruptured and non-ruptured sites. Nuclei were counterstained by DAPI (blue). Scale bar=100 µm. (n=3). (B) Semi-quantification of immunofluorescence data. Data are presented as mean ± SD. R-ruptured site and NR-non-ruptured site. (n=3 donors and 3 replicates).

4.5 Discussion:

Rupture of membranes is a normal planned mechanism of pregnancy which is supposed to happen at the end of gestation. The problem with PPROM is that this planning is disrupted early; causing prematurity complications and the reason behind that remains as a question mark. Previous chapter revealed the different heterogeneity pattern of mechanical properties in full term and preterm membranes. In this chapter, multiple signal molecules including key MMPs have been assessed to provide knowledge in relation to preterm membranes. The considerable different expression of these molecules in rupture and non-rupture sites between full term and preterm membrane were demonstrated.

4.5.1 Change of collagen content relating to membrane weakness:

H&E stained images confirmed that chorion was densely arranged in FM but the chorion itself had a loosely arranged network. That considered as one of the reason of its mechanical weakness. The heterogenic distribution of the collagen content (Table 4.1) was found to correlate with the mechanical behavioural differences of preterm membrane in this chapter. The Picro Sirius Red staining result showed the collagen fibre of preterm membranes were thicker and aligned more compactly, hence they were darker in colour than the termed samples which stained lighter as collagen were thinner and loosely bound. Skinner et al., noticed that the volume of decreased collagen content during the end of term pregnancy in full term was higher compared to the preterm collagen content (Skinner et al., 1981). The mechanical integrity of biological tissue is dependent on its collagen quantity and distribution. Collagen turnover is a continuous process during pregnancy to combat the rising amniotic fluid volume and mechanical tension to maintain the gestation (Al-Zaid et al., 1988). In fetal membrane, the widely banded collagen type I (col-I) and III (col-III) are crosslinked by type V and VI collagen fibres (Malak et al., 1994). As the collagens are mainly distributed in the amnion layer and the amount is nearly twice in comparison to that of collagen in chorion, variation in mechanical behaviour between these layers is expected. Similarly, discordant collagen distribution and quantity was found in different sites

within the same preterm membranes. According to previous studies, decrease in collagen I and III are associated with low mechanical strength (Rangaswamy et al., 2012, Nallasamy et al., 2017). However, the short term recoverable deformation of the fetal membranes possibly due to water overflow from collagenous filaments and the permanent deformation mostly due to the change of collagen fibres' alignments (Skinner et al., 1981).

4.5.2 Alternation of GAGs proportion disrupting the FM strength:

GAG distribution analysis in the FM showed significant differences between ruptured sites ($p=0.001$) and non-ruptured sites ($p=0.016$) in full term and Preterm membranes. Collagen provides the tissue with a resistant capability to shearing forces, while elastin adds a resilience capability for the tissue. In contrast to fibrous proteins, proteoglycans provide great resistance to compressional forces, and this property is due to the presence of GAGs side chains (Meinert et al., 2009). So far, there are very few studies investigated GAG related with PPROM. We hypothesised that the cause of PPROM is may be due to an imbalance between the expression of GAG molecules in the fetal membranes; leading essentially to rupture of membranes. More specifically, decorin in the amnion is experiencing a less expression; causing a general weakness of the pressure-bearing membrane. In comparison, biglycan is being expressed in high amounts in the chorion. Wu et al, noticed that decreased level of biglycan and decorin in mice fetal membranes could cause morphological abnormalities (Wu et al., 2014). Our data supported this finding. In early stage of gestation decorin plays a major role in tissue remodelling, so that FM grow quicker to accommodate the fetus. Biglycan plays a role in signalling pathway. Decorin and biglycan both regulate the expression of MMP 9 (Wu et al., 2014).

4.5.3 Increased expression of MMP 9 and MMP 13 near ruptured sites of FM:

The immunostaining result of MMP 9 and MMP 13 showed higher expression in preterm membranes than termed membranes. The degradation of collagen in FM is regulated by MMPs.

MMP 9 or gelatinase B and MMP 13 or collagenase III is generally found in FM and amniotic fluid. Goldmen et al have reported that increased level of MMP 9 secretion is found in chorioamnion after the contraction starts (Weiss et al., 2007). Otega et al, mentioned MMP 9 is not expressed enough before labour, the concentration changed dramatically near the delivery (Vadillo-Ortega et al., 2005). Greenwood et al also noticed that only during active labour MMP9 showed higher level of expression (Greenwood et al., 1995). According to previous studies, MMP 9 increases significantly in the chorioamnion during the labour time at term.

The present study showed higher expression of MMP 9 in human FM also at preterm. Hence, increased level of MMP 9 production in FM could be a key step for both preterm and term rupture of the membrane. Athayde et al. found high concentration of MMP 9 in amniotic fluid of women with PPROM (Athayde et al., 1998). Later Xu et al proposed that high expression of MMP 9 in the FM is possibly accountable for increased level of MMP 9 production in amniotic fluid (Xu et al., 2002). Goldmen et al and Yonemoto et al reported that increased MMP 9 activity was found only in amnion, as it produced predominantly by amnion epithelial cells (Yonemoto et al., 2006, Goldmen et al., 2007). Similar result was found in this study too although MMP 9 was found to expressed by preterm chorion as well. Xu et al. observed MMP 9 production by chorion tropoblast in their in vitro study. It was felt that this could be dependent on matrix distribution (Xu et al., 2002).

So far studies of MMP 13 in relation to preterm birth are very limited. Fortunato et al. found MMP 13 expression in amniotic fluid and a much lower expression in chorioamnion but denied any significant role of it in membrane rupture (Fortunato et al., 2003). However, Soydinc et al. reported high expression of MMP 13 in vaginal fluid washings of women with PPROM (Soydinc et al., 2013). Our result showed similar pattern of MMP 13 expression as MMP 9, higher in preterm membrane than full term, which suggested elevated concentration of both the MMPs are

associated with rupturing of membrane and because of its higher levels membrane weaken and rupture earlier in PPROM than term.

4.5.4 Involvement of fetal fibronectin in membrane rupture:

Fibronectin, another key component of the FM, is widely distributed from amnion to the decidua. This multidomain protein binds to collagen, ECM receptors and helps in adhesion with the uterine lining. It also delivers the structural support for the fetal membrane (Lookwwod et al., 1991). Fibronectin has been used as a clinical marker of spontaneous preterm labour. Usually, chorion separates from decidua in the lower uterine segment during the term. This separation mostly occurs as fibronectin binding splits up from ECM and is released through the cervix vagina. These exudations of fetal fibronectins help in detecting the likelihood of preterm labour (Lookwwod et al., 1991, Greenwood et al., 1995). However, it has not proved very helpful to predict preterm birth (Sanchez-Ramos et al., 2009).

In this study, we found a significantly higher expression of fetal fibronectin in preterm membranes than full term, indicating that this likely has some specific connection with preterm labour, more than just matrix binding. Okamura et al. reported that a particular fibronectin domain can activate MMP 9 in response to inflammation (Okamura et al., 2001). Mogami et al. also reported that fetal fibronectin participate actively in up regulating the MMP 1 and MMP 9 mRNA (Mogami et al., 2013). Our finding supports this, in preterm membrane both Fn and MMPs showed increased expression. Thereby, there could be a link between these biochemical markers and they may well play an important role in the mechanism of premature membrane rupture.

4.5.5 Association of IL-1β to early FM rupture:

Cytokines, produced by FM are known to play a key role in the mechanism of normal parturition. In this study, preterm membranes also exhibited higher rate of IL-1β expression compared to the

full term samples. It could be due to the cell death or locally generated infection and necrosis during delivery and handling the sample. However, high or low amount of cytokine activation is common in preterm labour with or without the presence of infection. It has been evident from previous studies that IL-1β is responsible for MMP 9 elevation and apoptosis induction (Roh et al., 2000, Xu et al., 2002). Epithelial cells in amnion undergo apoptotic cell death towards the beginning of labour and lead to ECM degradation (Perry et al., 1998). Fortunato et al., suggested that increased concentration of IL-1β in amniotic fluid may provoke higher rate of cell death of FM which may lead to preterm labour. They also reported lower efficacy of interleukin markers on apoptosis activation but this can alter in the presence of intra amniotic infection (Fortunato et al., 2003).

4.5.6 Role of lower level of progesterone receptor in membrane rupture:

Very few previous studies have investigated progesterone involvement with preterm birth. Progesterone hormone plays an important role in maintaining a healthy pregnancy. It is found to suppress the MMP activities (Sato et al., 1991) and higher levels of progesterone are associated with decreased collagenase production (Rajabi et al., 1991). In this study, progesterone showed greater expression in term membrane than preterm, which supports that progesterone may reduce the MMP concentration which is associated with membrane rupture. In present times, progesterone is used as a supplement believed to prolong the delivery for women with PPROM. Progesterone is able to block induced apoptosis within the fetal membrane, inhibit the inflammation, and reduce the gap junction which may be the strategies to prevent preterm labour. Despite this, the prevention rate is very low. (Perry et al., 1998, Sfakianaki et al., 2006,Luo et al.,2010). So, from this study, it could be concluded that lower levels of progesterone may increase the MMP concentration or cell death thus increasing the likelihood of membrane rupture.

4.6 Conclusion:

In-depth assessment of multiple structural and biochemical properties in fetal membranes by quantitative and qualitative approaches clearly demonstrated that there was bigger heterogeneous expression pattern between rupture and non-rupture sites in preterm membranes than in full term and preterm. This was mirrored well with the biomechanical property in full term and preterm membranes measured in chapter 3. The findings from this study suggested that a specifically weakened area of preterm membranes was of insufficient strength to support the fetus and the amniotic fluid. This was due to their abnormal collagen distribution which was regulated by MMP activation and abnormal expression of the MMPs might lead to other biomarker activation or vice versa. These also correlated with decreased thickness and lower elasticity of fetal membranes.

The biochemical changes displayed indicate their involvement in membrane degradation and rupture. Increased levels and activity of MMP 9 and MMP 13, fibronectin and IL-1β, as well as decreased levels of progesterone detected in preterm membrane indicated that these play a major role in the weakening of fetal membrane. Different types of collagen distribution and estimation of GAGs gave a clear idea that these were linked with the microstructural alternation of fetal membranes. An imbalance in GAG distribution and decorin and biplycan expression seen in preterm membrane could explain the separation of amnion and chorion early which is a vital cause of membrane weakness. Altogether, all these factors might lead to PPROM.

Chapter 5

Induction of biochemical molecules expression change in membranes by external force and maternal risk factors

5.1. Introduction

An intact FM is the main load bearing tissue during gestation and plays a major role in maintaining amniotic fluid homeostasis throughout pregnancy. Despite of facing multiple challenges and going through varying stressful environments, FM continues to support the growing fetus and maintains the mechanical and biochemical balances needed during fetal growth. This fetus and membrane bonding is necessary to sustain until full term when the fetus matures (Menon et al., 2016). Pathophysiology of preterm rupture of membrane is unclear. Our previous investigation proved that both biomechanical and biochemical factors contribute to membrane rupture. It also showed that these factors are interrelated and possibly act simultaneously to initiate and regulate the preterm rupture of the membrane. Although association of these factors and their co-relation is probably a part of normal delivery, we believe there could be other external forces which trigger activation of these factors before their scheduled time of activation or cause an imbalance in their co-relationship leading to PPROM and preterm birth.

Bio-medically, mechanical forces act as a therapeutic purpose to improve the healing process which is brought on with a change to the cell and tissue mechanics and biochemical pathways involved in disease development as well (Ingber et al., 2003). In the case of FM, it has been observed that pregnancies of more than one fetus have higher risk of preterm birth than those of a single fetus. Neonatal complications were higher and stillbirth rate was 12-fold higher than singleton pregnancies (Keith et al., 2002). This may be linked to the in vivo fetal movement or other external force leading to preterm membrane rupture. We believe that some external mechanical forces *in vivo* might promote erratic behaviour in the biochemical activities of the FM extracellular matrix. These could be in any particular area of the membrane causing weakness and leading to rupture. Based on the observations detailed in previous chapters, it can be suggested that

when biomechanical and biochemical behaviour of FM are mistuned, it can cause preterm rupture. No other study has suggested this phenomenon before.

It is difficult to mimic the stress that intact FM undergoes throughout the gestation period. It is constantly subjected to the mechanical stress load of the growing fetus, the amniotic fluid volume and fetal movements which the FM successfully withstands. Also, there are various biochemical factors which constantly change as the gestation period progresses and this coupled with the general health of the host (i.e. the mother) which can all affect the mechanical factors of FM. To date, there have been no robust experimental setups that could mimic the mechanical and biochemical ambience that FM undergoes which could potentially lead to preterm premature rupture of membrane. In this chapter, we have investigated about the influence of in vivo external force on the biochemical changes of FM which can cause membrane weakness, for the first time, using *In vitro* model.

Cigarette smoking during pregnancy has long been known to be a risk factor for preterm birth. Maternal smoking is associated with obstetric morbidity and various neonatal complications (Peacock et al., 1995, Burguet et al., 2004, England et al., 2013). However, not all studies were successful in discovering any significant correlation between maternal smoking and preterm delivery. Most of these were epidemiological studies, so, further information is needed to investigate this association and the possible mechanisms involved. Maternal glycaemia or Diabetes is another risk factor associated with maternal and fetal morbidity. Hence, in most cases, induction of preterm delivery is warranted. However, the cause behind spontaneous preterm rupture of membrane due to diabetes is still unclear (Köck et al., 2010).

Dexamethasone is a corticosteroid widely used as treatment option for women with a risk of preterm delivery, to minimise premature birth and related complications. However,

there are disagreements between neonatologists and obstetricians regarding the benefits of antenatal corticosteroid. The role of dexamethasone in its preventing action towards the preterm rupture of FM also remains debatable. (Crowley et al., 1990, Harding et al., 2001)

To address these factors, this chapter has investigated and will discuss the effects of exposure to nicotine and glucose during pregnancy and their influence towards the early rupture of FM. The present study has also focused on the potential preventive effect of dexamethasone in membrane rupture before term. The possible mechanisms of preterm membrane rupture in the presence of nicotine and glucose and the potential preventive mechanism of dexamethasone were studied *in vitro*.

5.2 Objective:

The objective was to establish an experimental set up of an *in vitro* model with physiological relevance similar to FM *in vivo*. Once established, the in vitro model will be used to explore and determine the changes in biomarker activation when exposed to external mechanical forces mimicking fetal movement and amniotic fluid pressure. We hypothesised that mechanical force upregulates inflammatory cytokines, MMPs and fibronectin through transducing the force from mechanical to biochemical, resulting in membrane weakness; whilst preterm membranes are more sensitive to loading and chemical environment changes, which could lead to preterm rupture. Risk factors such as maternal smoking and maternal hyperglycaemia activate biomarkers which may lead to membrane weakness and rupture before the scheduled time (i.e. full term).

5.3. Material and methods:

5.3.1 Sample collection:

FM samples from elective caesarean section deliveries were used for this experiment. Both full term (37+week's gestation) and preterm (33 to 36 weeks gestation) samples were collected. Samples from non-ruptured sites or from close to placental border lines were collected and were stored immediately in -80℃ as explained at chapter 2, section 2.1.

Cell viability was confirmed before the start of the investigation. Cell viability by Live and dead assay was used to confirm the suitability of the membranes before the investigation started. The method was performed as described at chapter 2, section 2.5.3.

5.3.2 Experimental set up:

As previously described (chapter 3), ball indentation protocol is simple and test conditions which mimic the in vivo loading environment. Therefore, the new loading system was constructed based on the ball indentation device with the intention that the samples could experience the in vivo stretching condition as close as possible.

The new experimental model consisted of two parts. First part was arrangement of the membrane samples. The metal plates, plastic hoops, screws were taken as same as ball indentation test set up (described in chapter3, section 3.3.2). Metal balls were used here instead of plastic ball (Figure: 5.1). The membrane pieces with approximately 20 mm diameter from non-ruptured sites were placed between two plastic hoops which were placed between two metal plates. Three replicates of each types sample were executed at the same time. The plates were screwed tightly after placing the membranes and placed in a Petri dish with basal media.

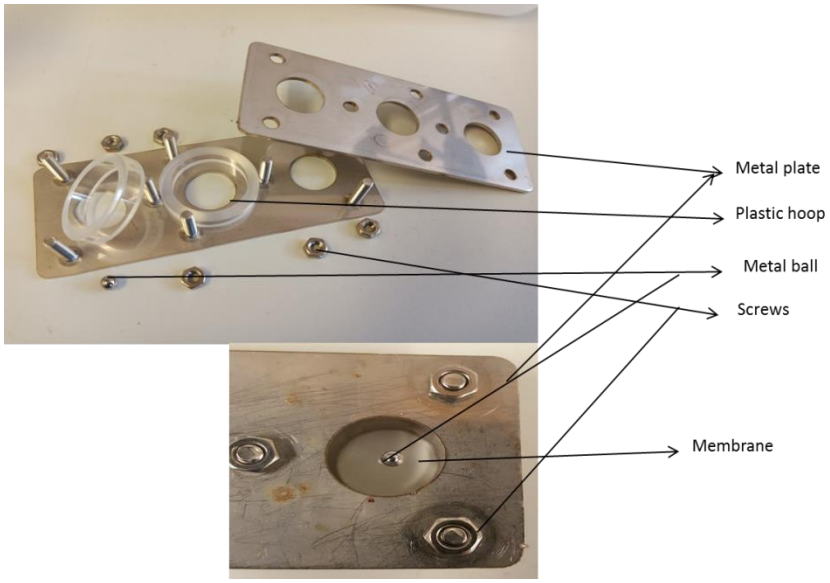


Figure 5.1: Schematic illustration of sample holder for mounting the fetal membranes. Sample pieces were mounted between the O-shaped plastic hoops within the plates and secured by screws. A metal ball was placed onto the membrane.

Second part was a mechanical system which could mimic the external force on sample membranes. A see-saw motion rocker (Cole-Palmer, UK) was programmed to facilitate the cyclic loading at a particular speed on the membrane through dropping and extracting of a metal ball on to the membrane. To achieve this, a metal handle was attached firmly to one side of the rocker platform. A magnet was positioned at another side of the metal hand. The strong magnetic attraction and the metal ball on the membrane cohesively exerted an impact force (weight of the ball) on the membrane. With the help of a tape, this metal and magnet assembly were secured properly.

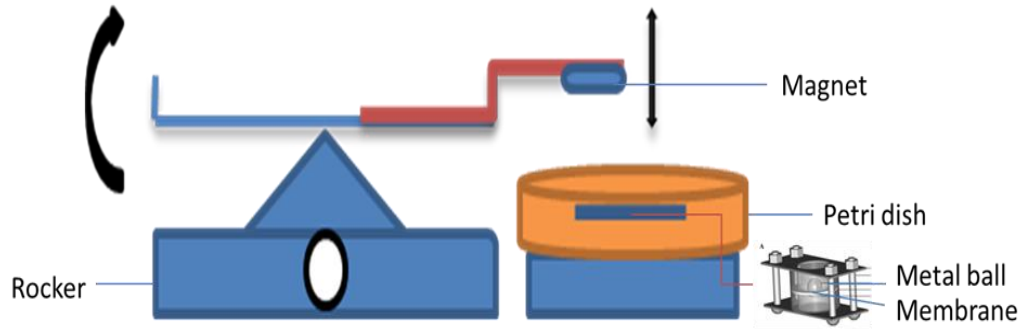


Figure 5.2: Diagram of the whole experimental set up.

Once the Membrane samples were assembled, a metal ball (0.3 gm stainless steel ball) was placed onto each piece of membrane inside the hoops and the Petri dish was closed and secured with tape on each sides. This was then put in an incubator. The rocker with metal magnetic arm was placed inside the incubator and petri dish holding the membrane assembly was paced under the magnetic arm (Figure 5.2). The speed of the rocker was set up at 30 rmp. After ensuring that the incubator door was closed properly, the rocker was switched on. The metal balls which deformed the membranes were going up and down in cyclic manner by the magnetic arm in the incubator. Though there were 3 hoops present in the ball indentation sample holder, only 2 magnetic arms could perform accurately at the same time. Hence, only two pieces of FM samples were mounted instead of 3 and the middle hoop was left empty. Incubator was maintained at 37°C and 5% CO_2. The rocker's rocking speed of 30 rmp triggered the metal ball dropping on the membrane at 0.5 Hz frequency. The strain applied to the membrane was almost instant and could not be controlled as it was the external magnetic force that dropped the metal ball on the membrane instantly. Membranes were loaded and exposed to the cyclic mechanical loading for 3 hours in the incubator. After that, the membranes were post incubated statically in the media for 4 hours in 37 °C incubator. Then the tissues were processed for further investigation.

5.3.3 Sample preparation:

Two groups of samples including full term and preterm were prepared for the investigation and minimum sample sizes of 3 from each group were investigated. Frozen membrane pieces were mounted immediately after thawing and they were placed into the incubator. The membranes incubated without loading were considered as control samples. After defined incubation period, part of those sample tissues were lysed by the RIPA buffer for protein extractions and the rest of them were fixed by 4% PFA for immunostaining. Other samples after loading (3 hours) and incubation (4 hours) were similarly prepared for immunostaining and protein extraction. Extracted proteins were stored at -80°C.

Nifedipine, a calcium-channel blocker (table 2.1) solution in DMSO was prepared. Samples were loaded with 10 µM and 30 µM nifedipne solutions in media. Two different concentrations: 10 µg/ml and 100 µg/ml of nicotine (table 2.1) were prepared in media. Full term and preterm samples (n=3, three different donors) were exposed to the 10 µg/ml and 100 µg/ml of nicotine solutions separately in 6 well plates and incubated for 3 hours at 37°C. Then samples were washed by PBS and half of them followed same the loading steps described above and half of them were incubated without loading within the basal media. Similar steps were performed after exposing the samples in glucose solution (10 mM in basal media) and dexamethasone solution (3 µg/ml).

5.3.4 Immunochemistry:

This procedure followed the same protocol as described previously (chapter 2, section 2.4). The only difference is that intact FM samples were used instead of cry sections. Sample pieces from 3-4 donors of each type and 3 areas of each sample were tested. After loading, the membrane areas located inside the rings exposed to the ball were used as experimental group and membranes without loading were used as control group. 3D scanning from bottom to top of the samples after

staining was performed by confocal microscopy to obtain z-stack images with 25 µm intervals. All the images were obtained by using Imaris 8.1 software.

5.3.5 Western blotting:

Western blotting was performed on all samples with and without loading. Proteins of loaded samples were extracted from the inner ring surfaces. The previously described protocol in chapter2, section 2.6 was followed.

5.4 Result:

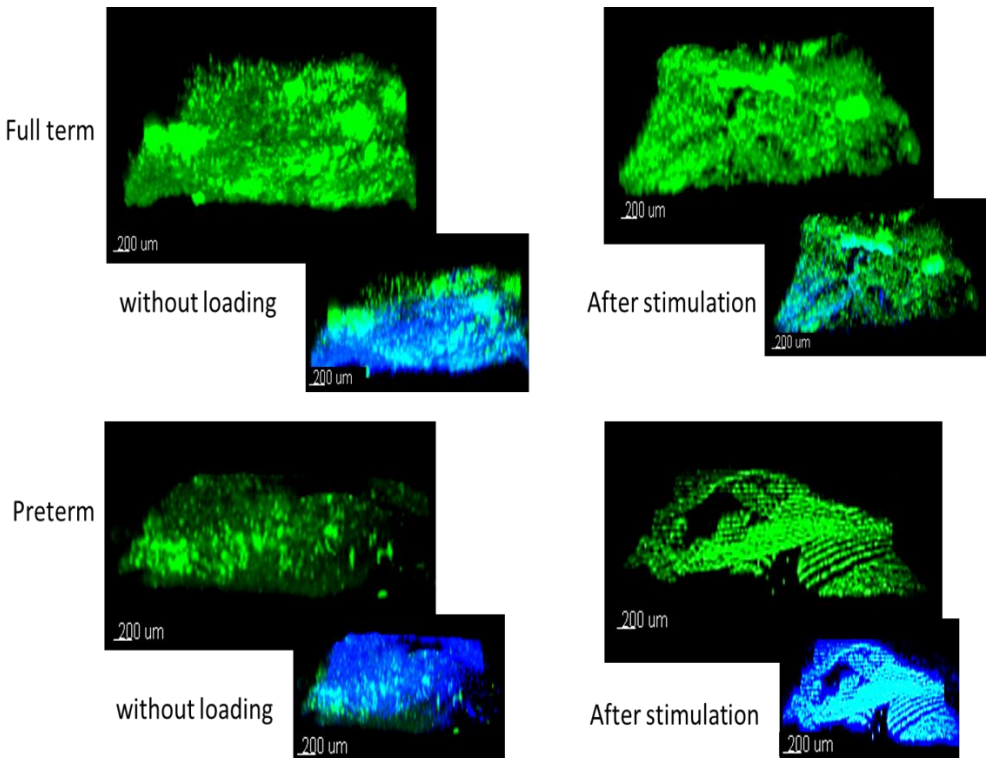
The experimental model set up was very simple, easy to handle, can easily be positioned in the incubator and not time consuming. It was observed that the ball deformed the membrane transiently. The main advantage of this model was the ability to control the speed and the loading weight accurately. The loading weight acted as a mimic similar to the pressure exerted by the kicks of the fetus during pregnancy.

5.4.1 Effect of external force-expression of biomarkers:

5.4.1.1 MMP 9 expression:

FM samples from full term and preterm were imaged for immunofluorescent-stained marker, MMP 9. The membranes after continuous loading for 3 hours and incubation for another 4 hours were imaged to see their MMP 9 expression change. The full term membranes before stimulation showed higher expression than preterm counterparts. The MMP 9 expression after stimulation showed higher expression in both full term and preterm membranes than before stimulation. In preterm membranes the expressions after stimulation were much higher than before stimulation while in full term the expression did not differ that much between before and after stimulation. Semi quantification of fluorescence intensity approved the outcome further (Figure 5.3).

A


Full term
without loading
After stimulation
Preterm
without loading
After stimulation
200 um

B

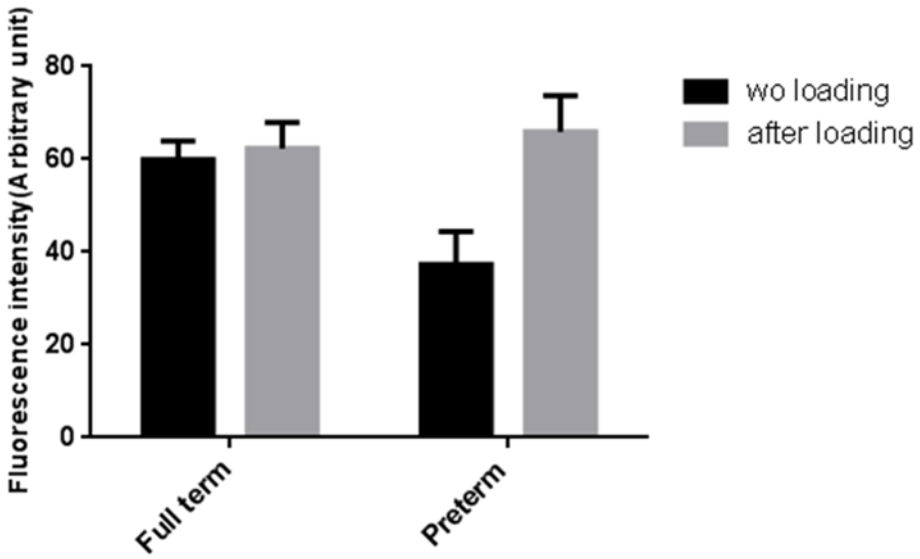
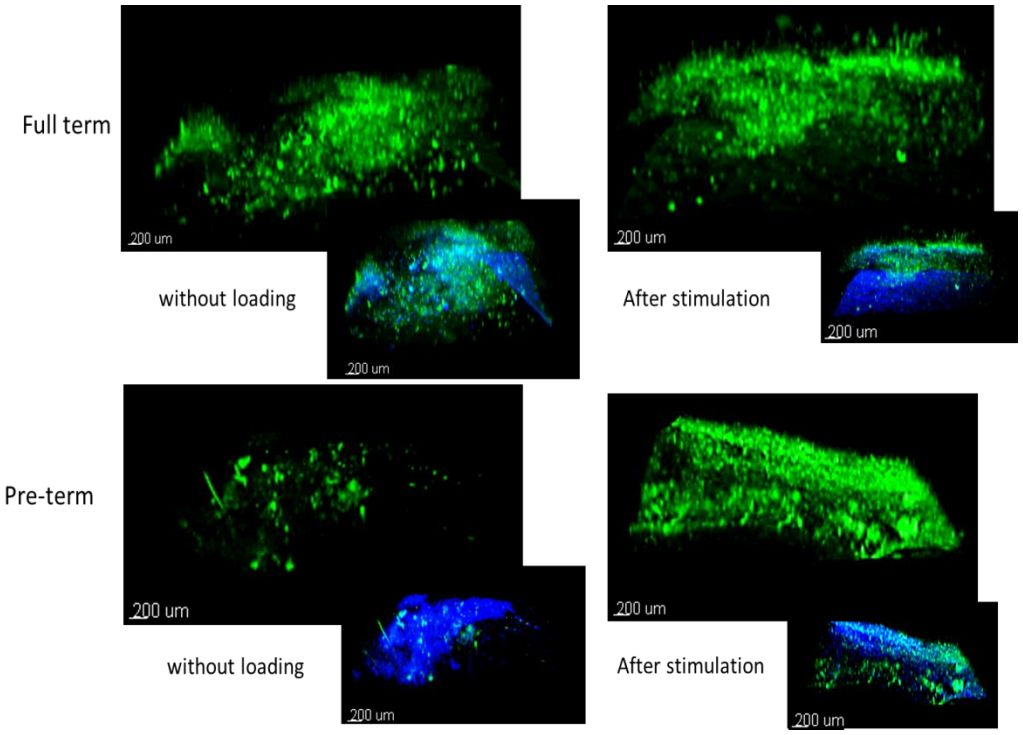

Fluorescence intensity(Arbitrary unit)
80
60
40
20
0
wo loading
after loading
Full term
Preterm

Figure 5.3: Effect of mechanical force on MMP9 expression alteration (immunostaining). (A) MMP 9 expression **(**green) in full term and preterm membranes before and after exposure to the loading/ external force. Blue -DAPI staining. (B) Semi quantified data of MMP 9 expression (wo=without). Data are presented as mean ± SD (n=3 donors). Scale bar= 200 µm.

5.4.1.2 MMP13 expression:

Immunostaining was also performed for MMP 13 on both types of membranes (full term and preterm) on test samples and their controls. Similar to MMP 9, MMP 13 too showed increased expression on samples exposed to external force than the controls. Preterm samples showed more sensitivity with the external forces than the full term membranes. Preterm control samples showed lesser expression than full term controls. Following stimulation both preterm and full term samples had similar expressions (Figure 5.4 A). Intensity of the staining in preterm samples was significant (Figure 5.4 B).

A


Full term
200 um
without loading
200 um
200 um
After stimulation
200 um
Pre-term
200 um
without loading
200 um
200 um
After stimulation
200 um

B

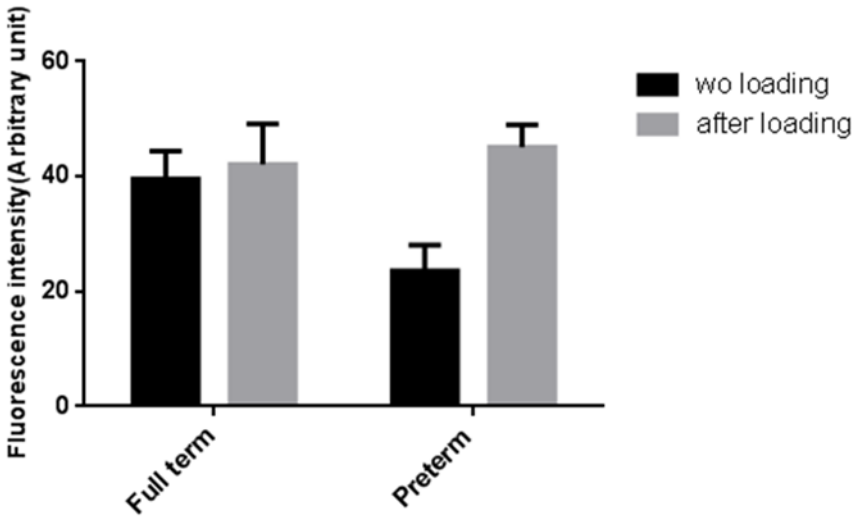
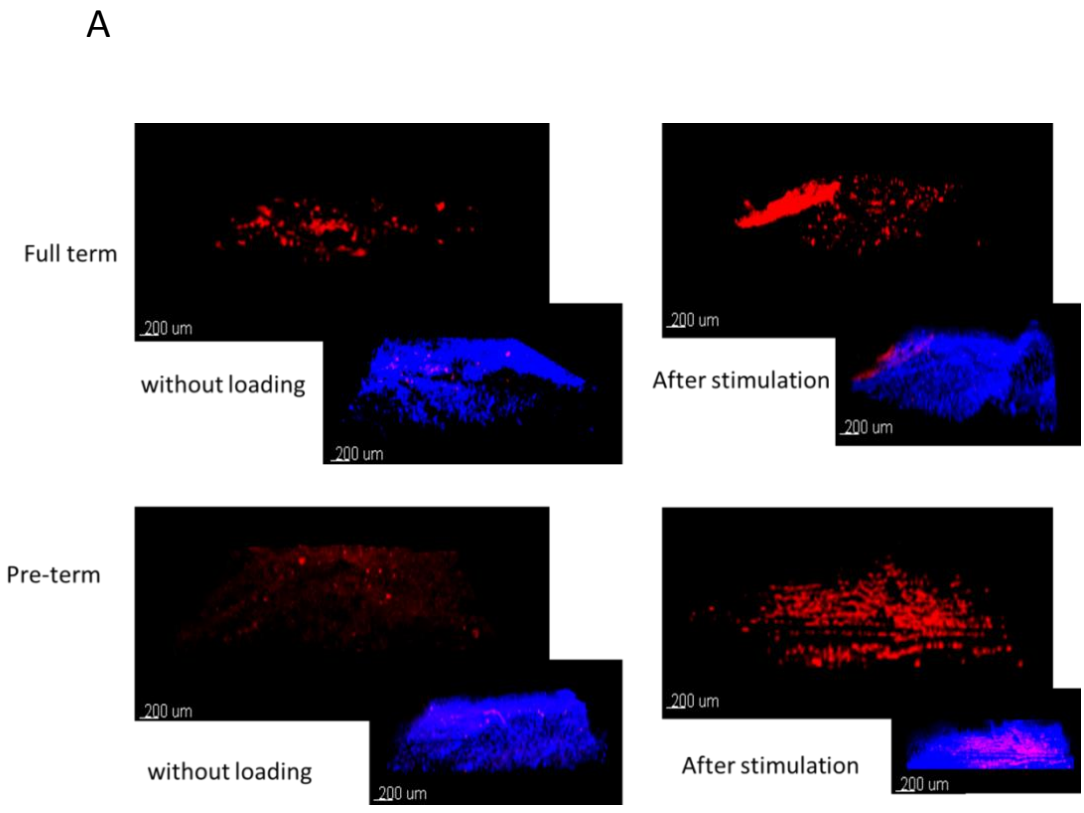

Fluorescence intensity(Arbitrary unit)
60
40
20
0
Full term
Preterm
wo loading
after loading

Figure 5.4: Effect of mechanical force on MMP13 expression alteration (immunostaining). (A) MMP13 expression (green) in full term and preterm membranes (non-ruptured sites) before and after exposure to the loading/ external force. Blue -DAPI staining. (B) Semi quantified data of MMP13 expression (wo=without). Data are presented as mean ± SD (n=3 donors). Scale bar= 200 µm.

5.4.1.3 IL-1β expression:

Very minimal expression of IL-1β was noticed in both FM samples before loading. After stimulation, both preterm and full term membrane samples expressed more IL-1β than the non-stimulated ones. Preterm samples were found to express more in response to external force stimulation than full term samples. (Figure 5.5)



B

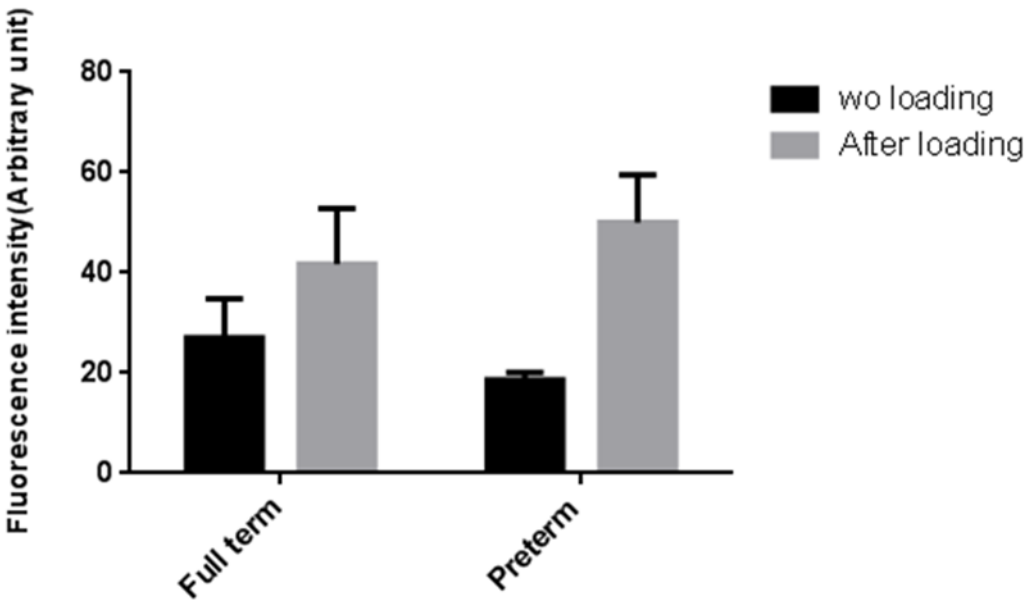
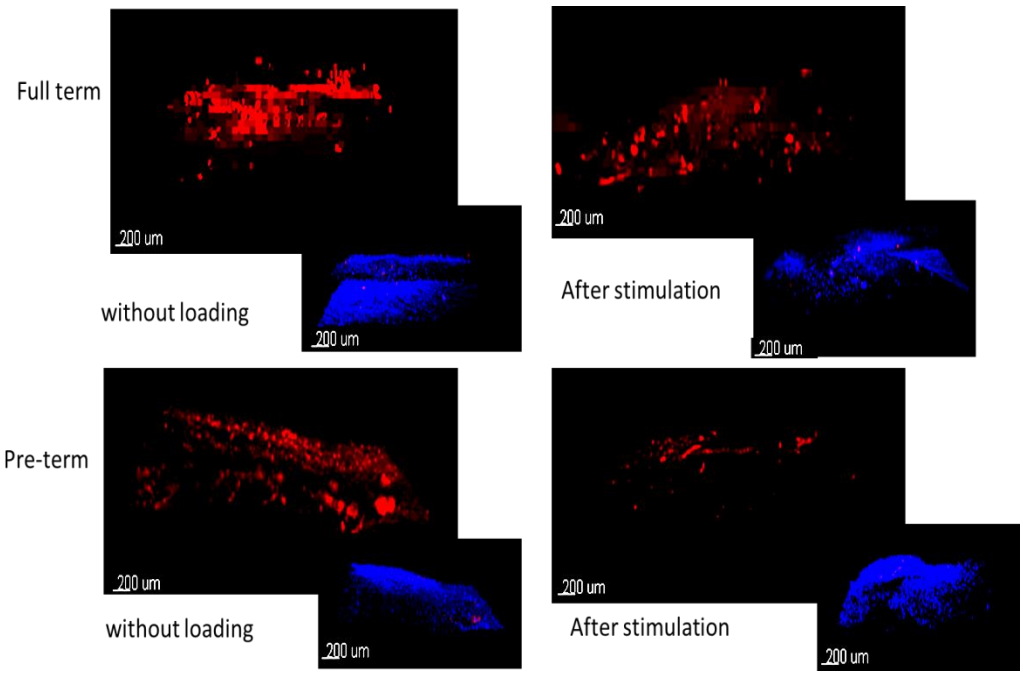


Figure 5.5: Effect of mechanical force on IL-1β expression alteration (immunostaining). (A) expression of IL-1β (red) in full term and preterm membranes (non-ruptured sites) before and after exposure to the loading/ external force. Blue- DAPI staining. (B) Semi quantified data of immunostaining expression (wo=without). Data are presented as mean ± SD. (n=3 donors). Scale bar= 200 µm.

5.4.1.4 Progesterone-receptor expression:

When full term and preterm membranes were immunostained for progesterone receptor antibodies, the progesterone receptors (PRA+PRB) expression was less after stimulation than the controls. Preterm membranes were found to be affected the most. The semi quantified result revealed that the difference of expressions were significant (Figure 5.6)

A


Full term
200 um
without loading
200 um
200 um
After stimulation
200 um
Pre-term
200 um
without loading
200 um
200 um
After stimulation
200 um

B

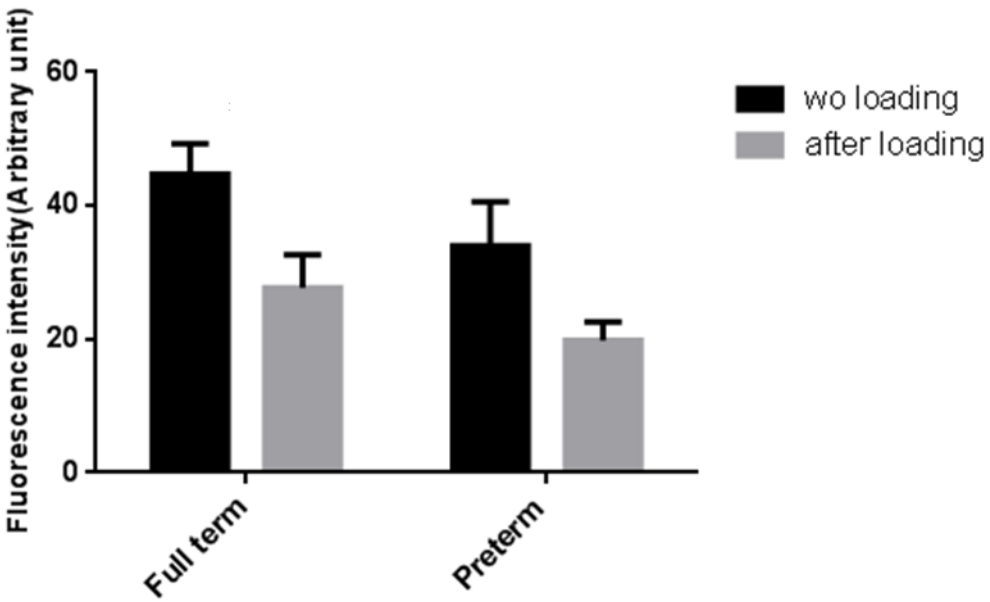
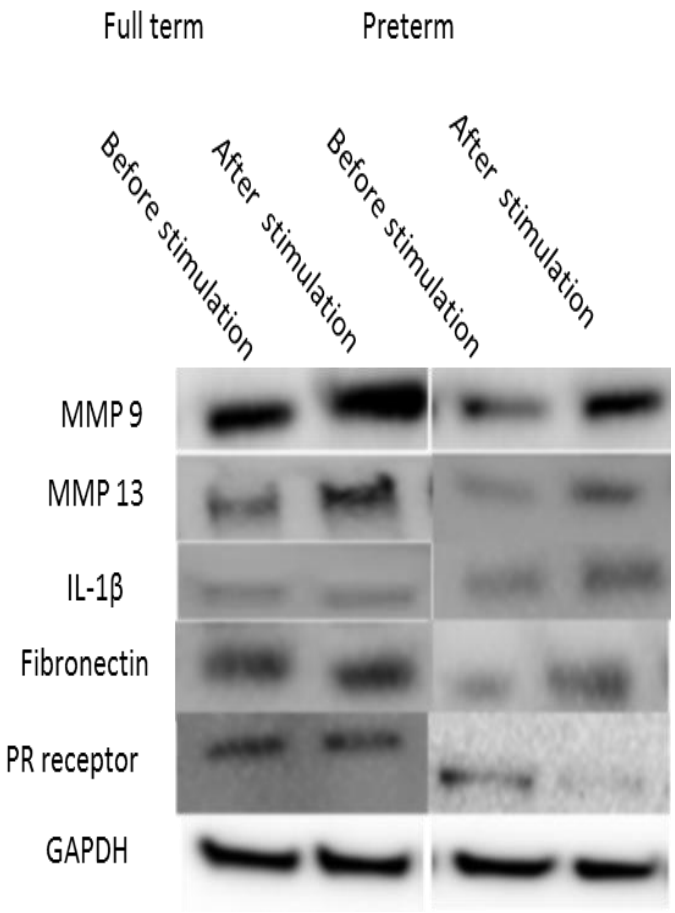

Fluorescence intensity(Arbitrary unit)
60
40
20
0
Full term
Preterm
wo loading
after loading

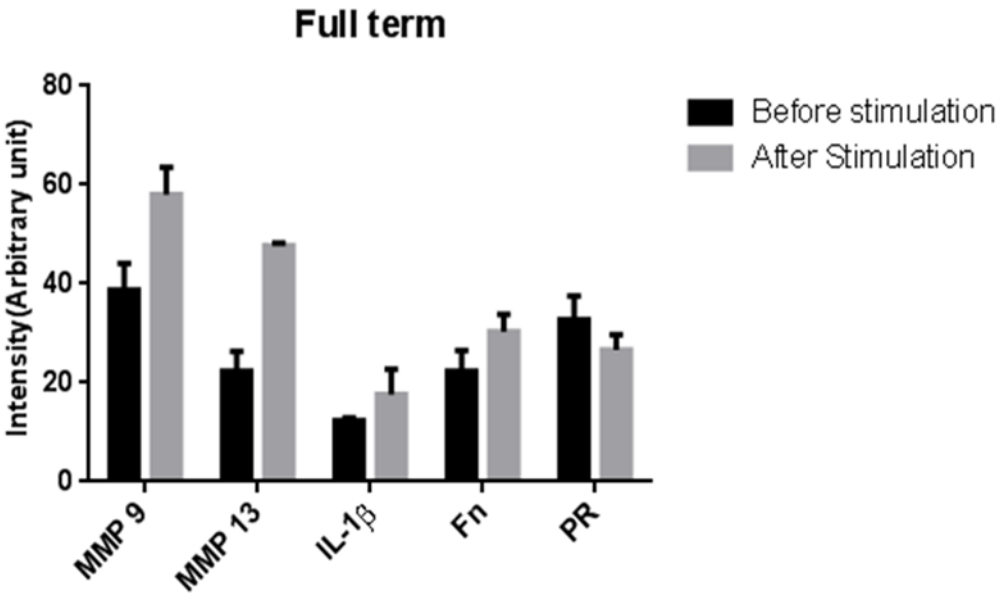
Figure 5.6: Effect of mechanical force on progesterone receptors expression alteration (immunostaining) (A) Expression of PR (red) in full term and preterm membranes (non-ruptured sites) before and after exposure to the loading/ external force.(B) Semi quantified data of immunostaining expression (wo=without).Data are presented as mean ± SD. (n=3 donors).

5.4.2 Western blotting expression of biomarkers:

Western blotting analysis was performed to re-evaluate and confirm the immunostaining data. Extracted proteins from both controls and samples of full term and preterm membranes were assessed. MMP 9, MMP 13, IL-1β and PR expression of the samples subjected to the loading were studied and compared with their controls. Western blot result showed higher expression of MMP 9, MMP 13 and IL-1β after stimulation in both the type of samples compared to the controls. These outcomes were consistent with the immunostaining data. Fibronectin showed almost similar expression in full term samples after and before stimulations. In case of preterm samples, there was increased expression of Fn after stimulation than before. The initial expression of fibronectin in full term was higher than pre term samples though. Progesterone receptor expressions were higher before loading compared to after stimulation in preterm samples. In full term, the difference of the expression was not much. The intensity of the protein expression corresponded with the Western blot result (Figure 5.6).

A


Full term
Preterm
Before stimulation
After stimulation
Before stimulation
After stimulation
MMP 9
MMP 13
IL-1β
Fibronectin
PR receptor
GAPDH


Full term
Intensity(Arbitrary unit)
80
60
40
20
0
Before stimulation
After Stimulation
MMP 9
MMP 13
IL-1β
Fn
PR

C

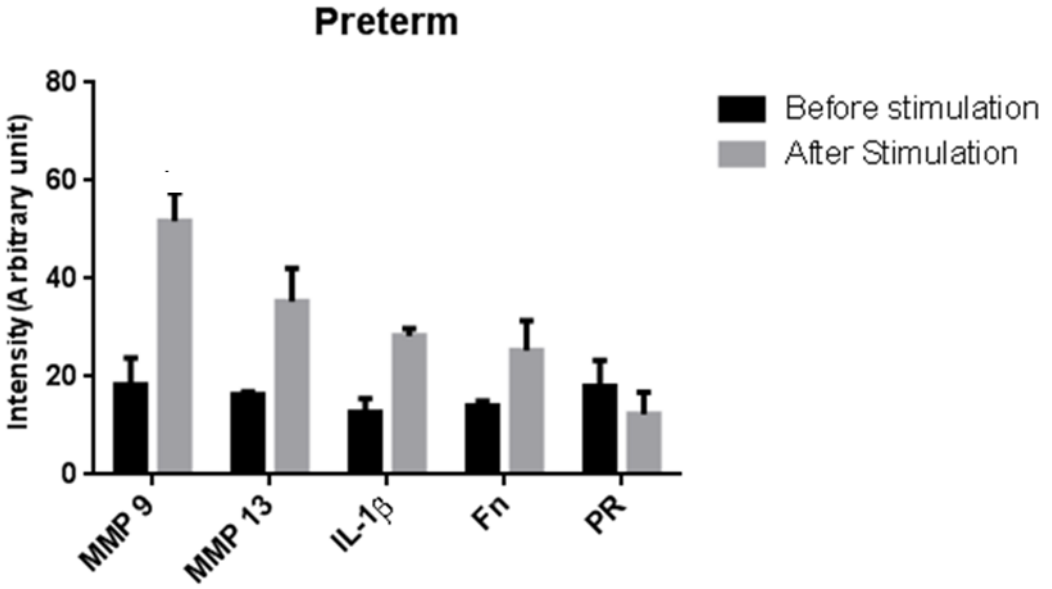


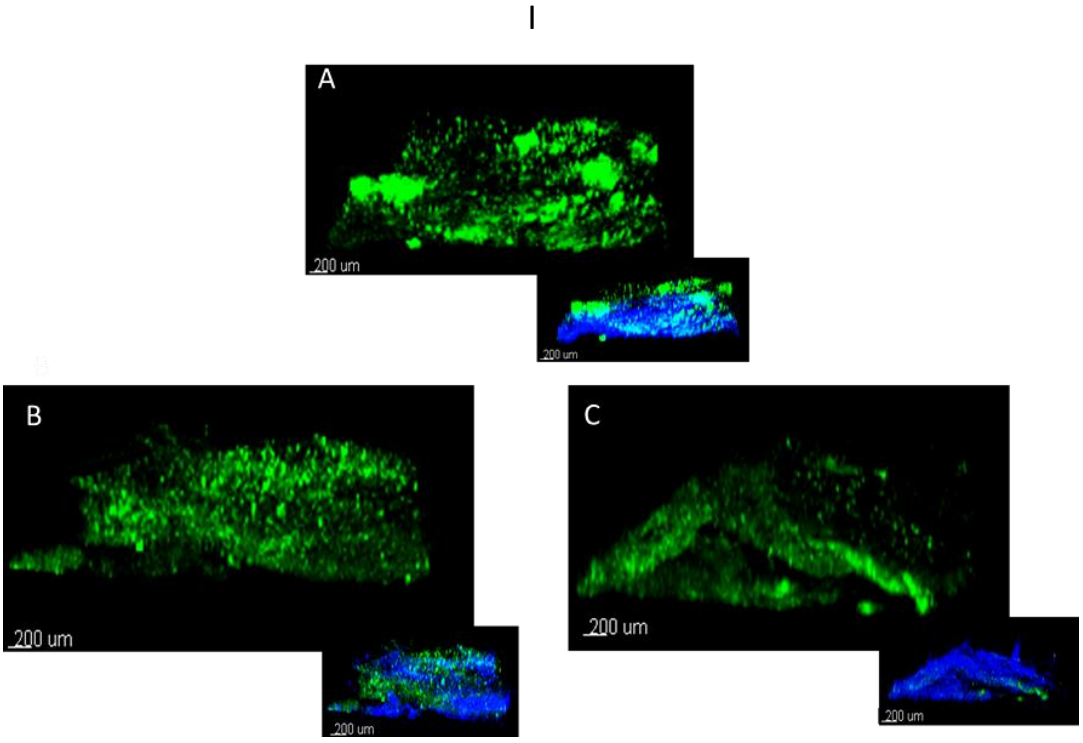
Figure 5.7: Western blotting result. (A) Protein expression of MMP 9, MMP 13, IL-1β, fibronectin (Fn) and progesterone (PR) receptor expression in the full term and preterm non-ruptured sites of the membrane samples, before and after loading stimulation. Results relative to the loading control GAPDH. Semi-quantification of Western blot data for full term (B) and (C) preterm samples. Data are presented as mean ± SD (n=3 donors).

5.4.3 The effect of calcium channel blocker (nifedipine):

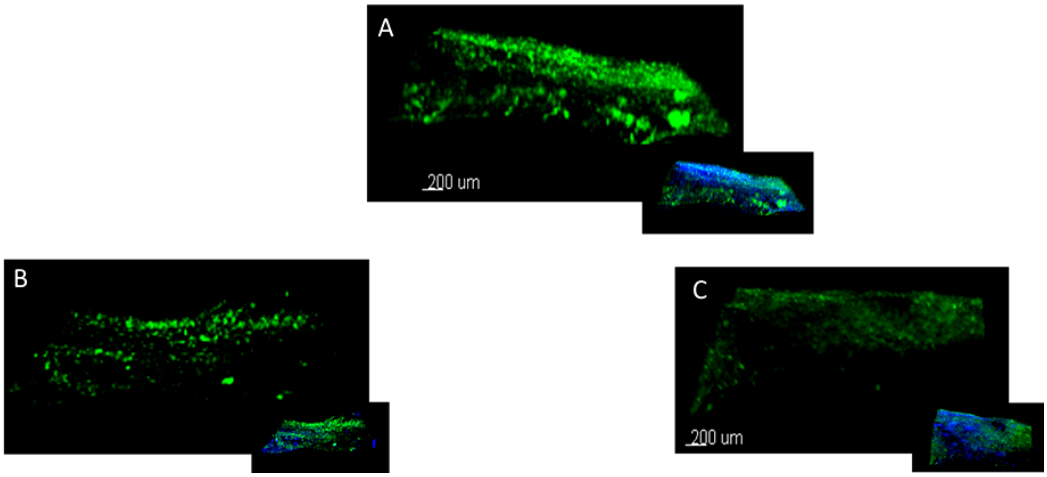
Samples from full term (n=3 donors) and preterm deliveries (n=3 donors) were incubated with different doses of nifedipine (10 µM and 30 µM) for 3 hours and subsequently incubated for 4 hours. Following that, biochemical markers like MMP 9, MMP 13 & IL-1β expressions were assessed via immunostaining method. Some samples from full term and preterm deliveries (n=3 donors) were loaded and incubated simultaneously but without nifedipine, were considered as controls.

5.4.3.1 Expression on full term samples:

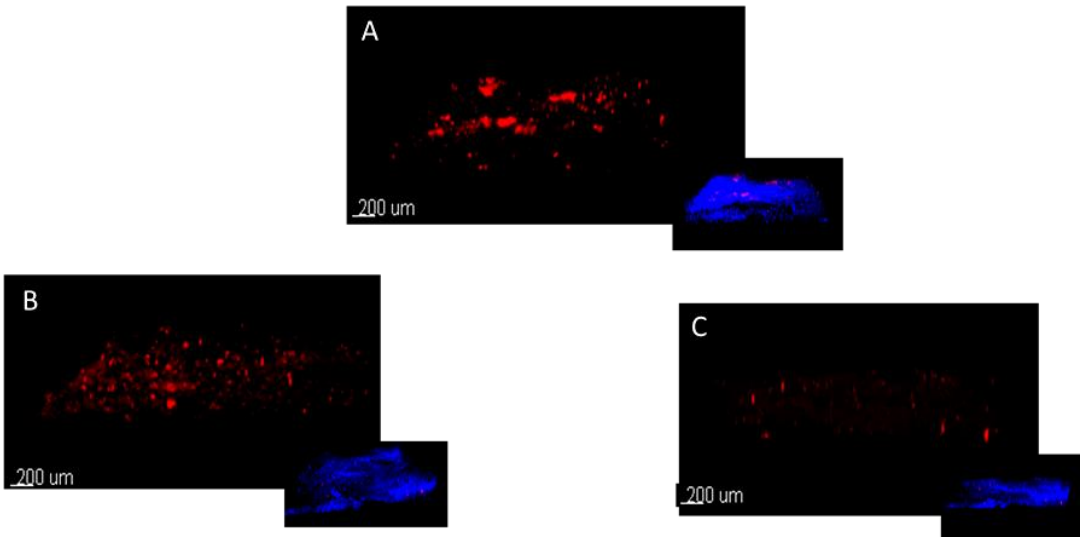
Full term samples after exposure to external force presented increased expression of MMP 9, MMP 13 and IL-1β. After loading and exposing to the nifedipine solution, the expressions of all the biomarkers were noticed to reduce. Higher concentrations (30 µM) of nifedipine showed better result in reducing biomarkers expression compared to the lower concentrations (10 µM) of nifedipine solution. Semi quantification of fluorescence intensity correlated well with the expression of MMP 9, MMP 13 and IL-1β after loading and after being exposed to two different concentrations of nifedipine solutions (Figure 5.8).



II



III



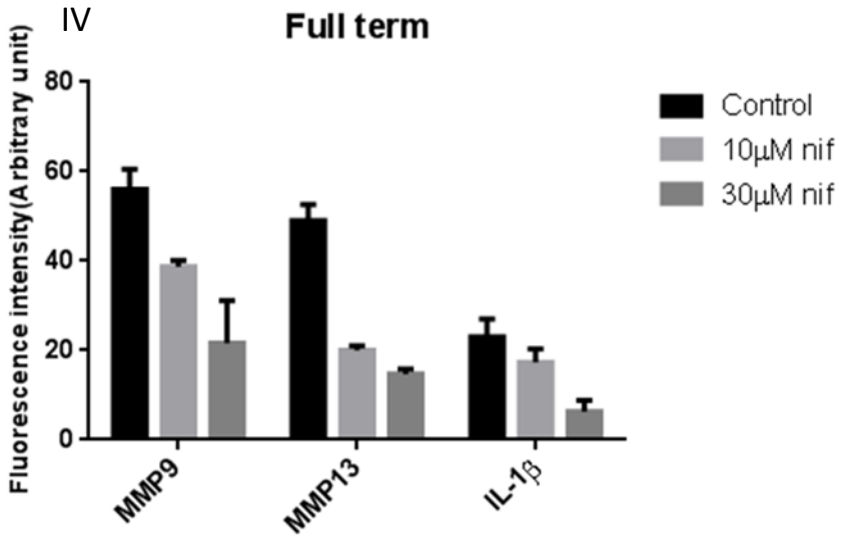


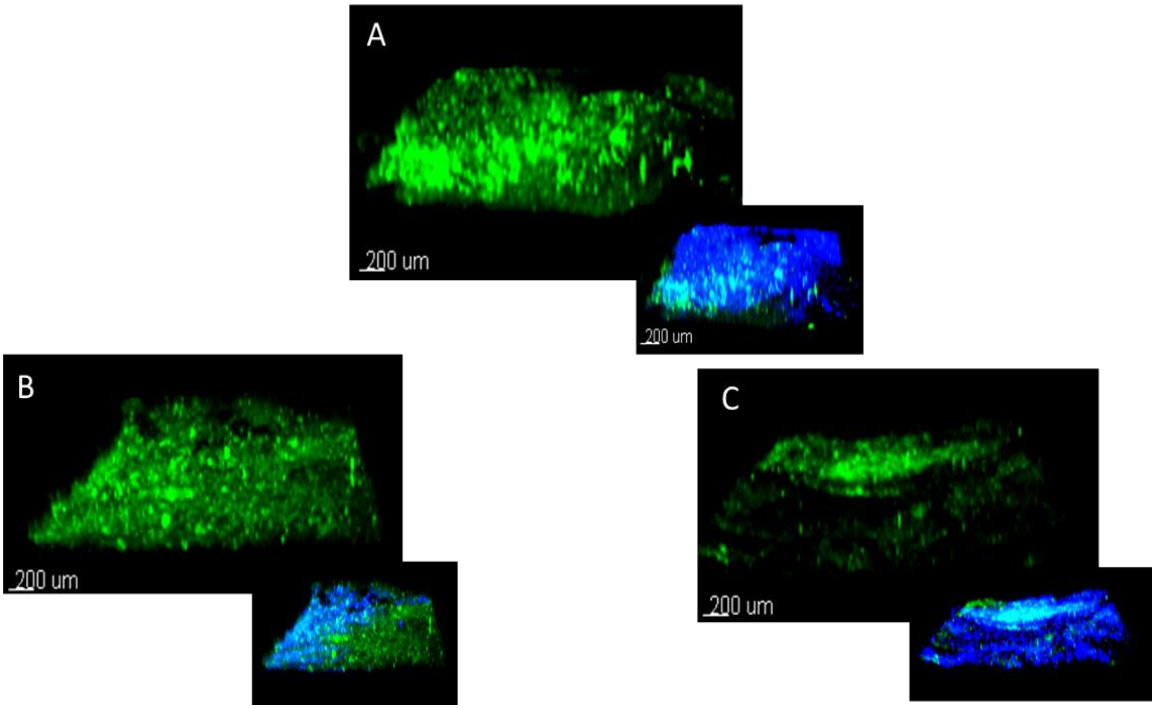
Figure 5.8: Immunofluorescence expression of biomarkers in full term membranes after exposure to nifedipine solution with loading. Immunostaining 3D images showing I) MMP 9 (green); II) MMP 13 (green); and III) IL-1β (red) expression in full term non-ruptured membranes. A- After exposure to the loading/ external force, B-after Loading and 10 μM nifedipinee solution, C-after loading and 30 μM nifedipine. Blue - DAPI staining. Small windows merged images. (n=3 donors). Scale bar=200 μm. IV) Semi-quantification of immunofluorescence intensity in full term samples. Data are presented as mean ± SD.

5.4.3.2 Expression on preterm samples:

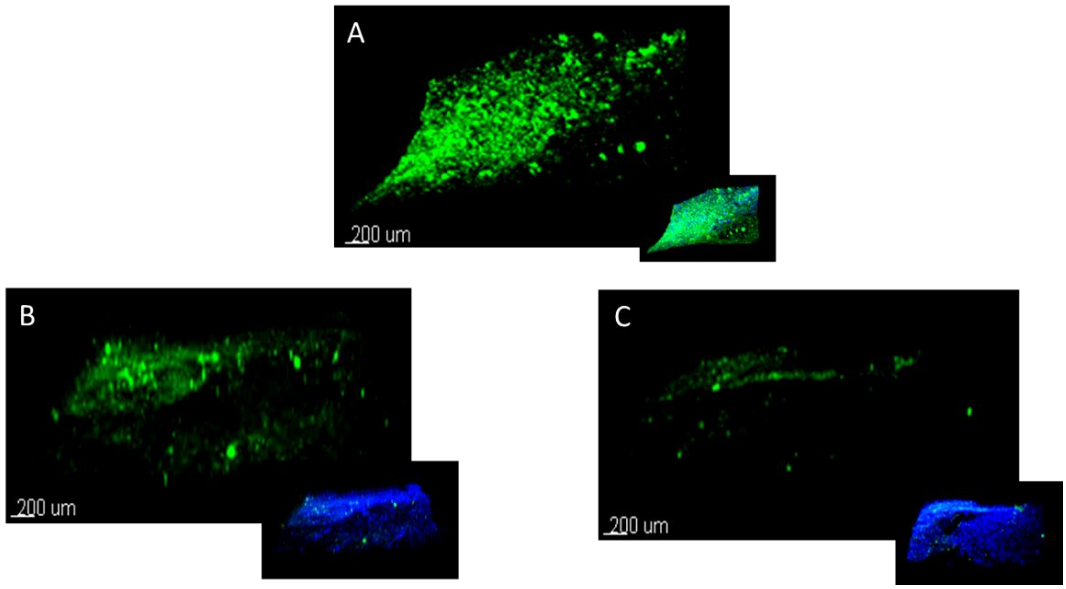
Figure: 5.9 presents the expressions of MMP 9, MMP 13 and IL-1β in immunostained FM samples. Stimulated samples in the presence of nifedipine solution showed reduced expression of biomarkers than the simulated samples. The expressions of MMP 9 were decreased more with the higher concentration of nifedipine (30 μM) than the lower concentration (10 μM). Figure 5.8-I shows MMP 9 expressions of stimulated samples after

exposing to nifedipine solutions. Expression of MMP 9 was found to reduce after exposing to the Nifedipine solution, but it was found to work better in 30 µM concentrations than 10 µM. However, 10 µM concentration of nifedipine also showed good effect on MMP 13 and IL-1β reduction (Figure 5.9 II and III).

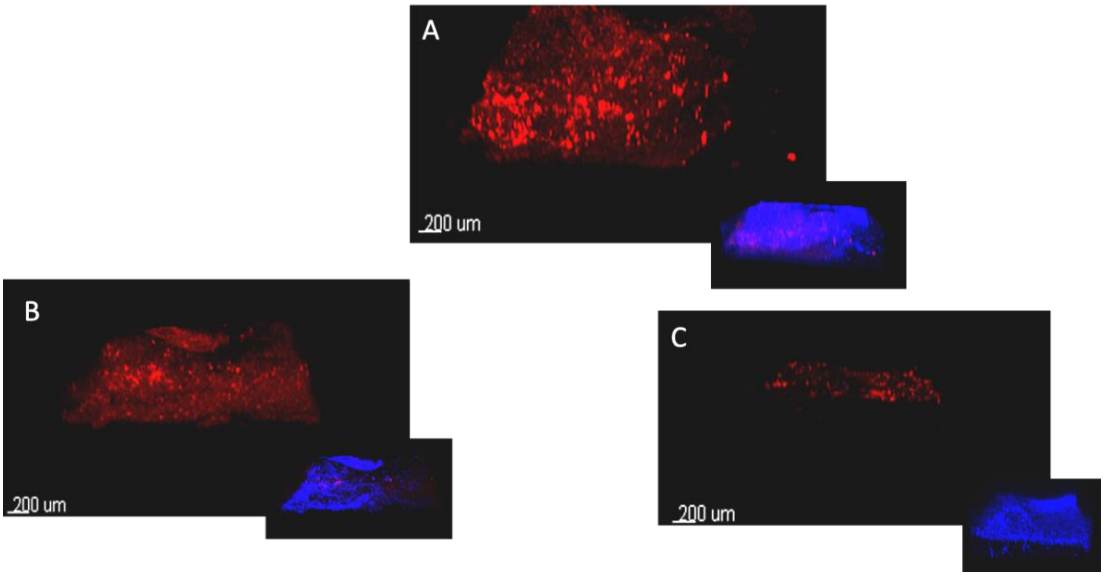
I



II



III



IV

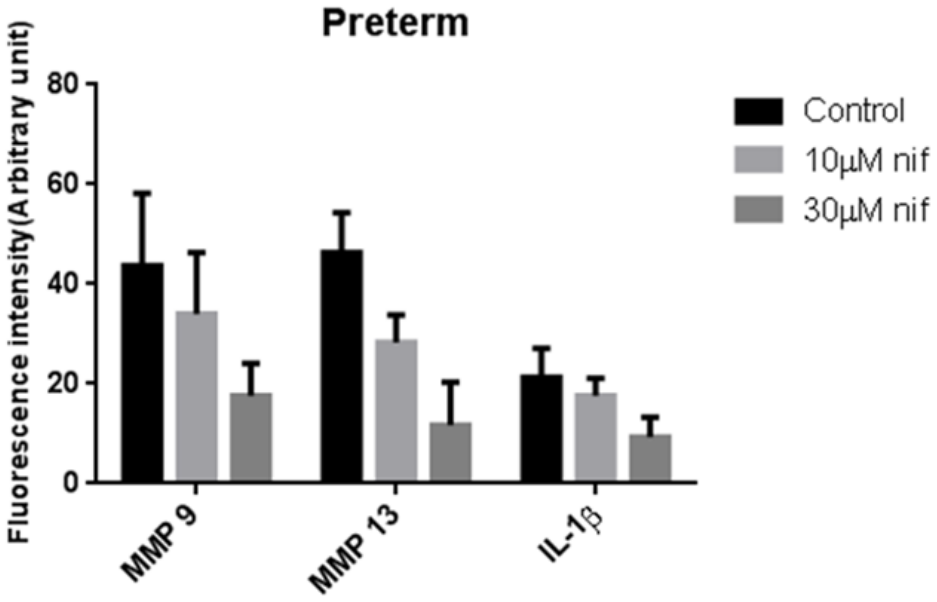


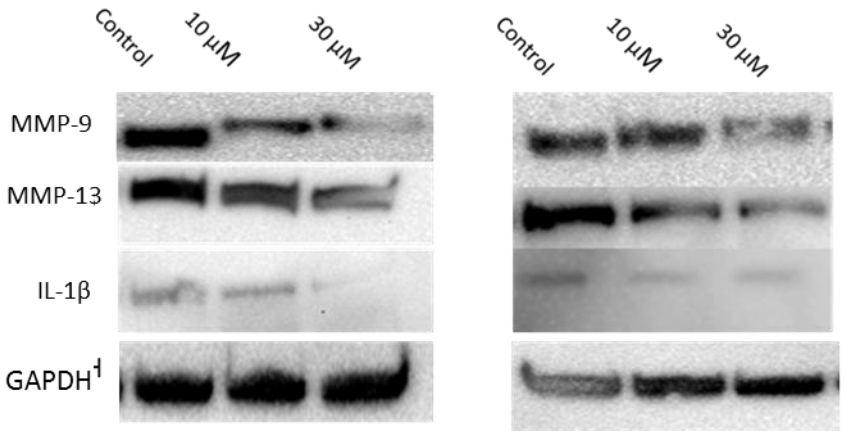
Figure 5.9: The expression changes of biomarkers in preterm membranes after exposure to nifedipine solution with loading. Immunostaining 3D images showing MMP 9 (I, green), MMP 13 (II, green) and IL-1β (III, red) expression in preterm non-ruptured membranes. (A) After exposing to the loading/ external force, (B)after Loading with 10 μM nifedipine in culture medium, (C) after loading and 30 μM nifedipine in culture medium. Blue is DAPI staining. IV, Semi-quantification of immunofluorescence intensity in preterm samples. Data are presented as mean ± SD (n=3 donors). Scale bar = 200 μm.

5.4.3.3 Western blots results:

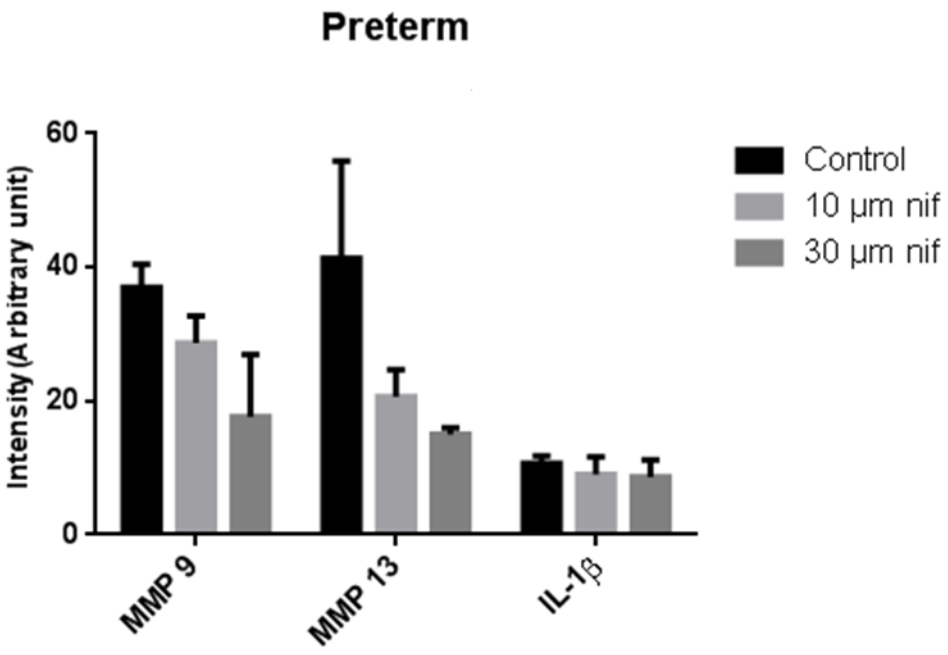
Similar to immunostaining, Western blot analysis was performed using extracted protein from three types of samples from both full term and preterm membranes. After exposed to external force only, the biomarker expressions on both the membrane samples were high. But when it was exposed to external force with nifedipine solutions, the biomarker expressions were decreased. Reduction of MMP 9, MMP 13 and IL-1β expressions were inversely proportional to the higher concentrations of the nifedipine solution (Figure 5.10

A). The western blot data were consistent with the immunostaining results. The intensity of the protein expressions further corresponded with the data for both full term (Figure 5.10 B) and preterm (Figure 5.10 C) samples.

A



B



C

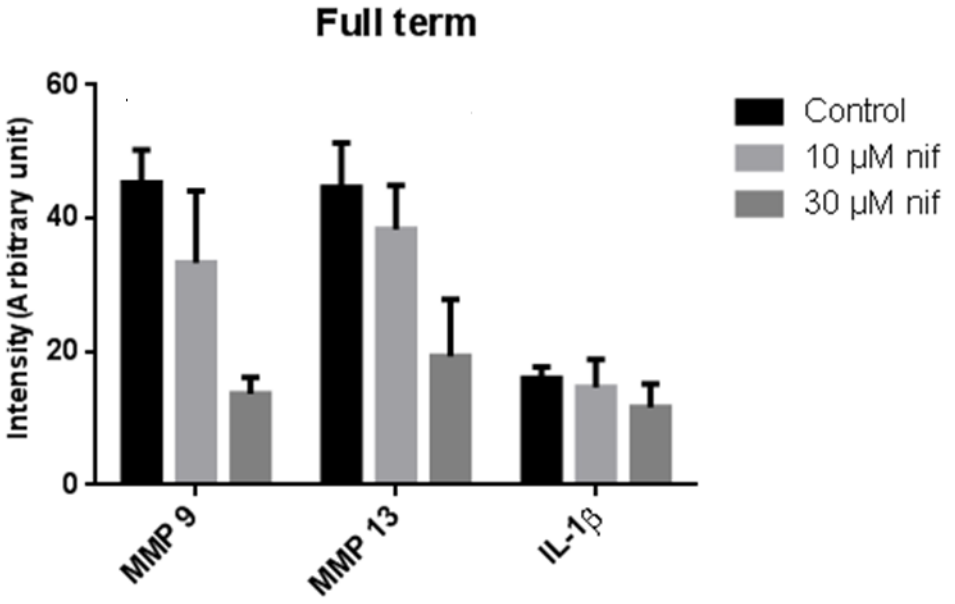


Figure 5.10 Western blot results after nifedipine exposure. (A) Expression of full term and preterm samples in three conditions, after loading (control), after loading with 10 µM and 30 µM of nifedipine solutions. Semi quantification of the Western blotting results for full term (B) and preterm (C) samples. Data are presented as mean ± SD (n=3 donors and 3 replicates).

5.4.4 Effect of maternal smoking/nicotine on FM:

The effect of maternal smoking was assessed on full term and preterm membranes by using nicotine solution. Two different concentrations of nicotine 10 µg/ml and 100 µg/ml were used. Initially, the expression of MMP 9 and IL-β1 were compared between the non-ruptured FM from preterm smoker mothers and non-smoker mothers (n=3). Non-smoking maternal samples showed lesser expressions than other samples in general (Figure 5.11).

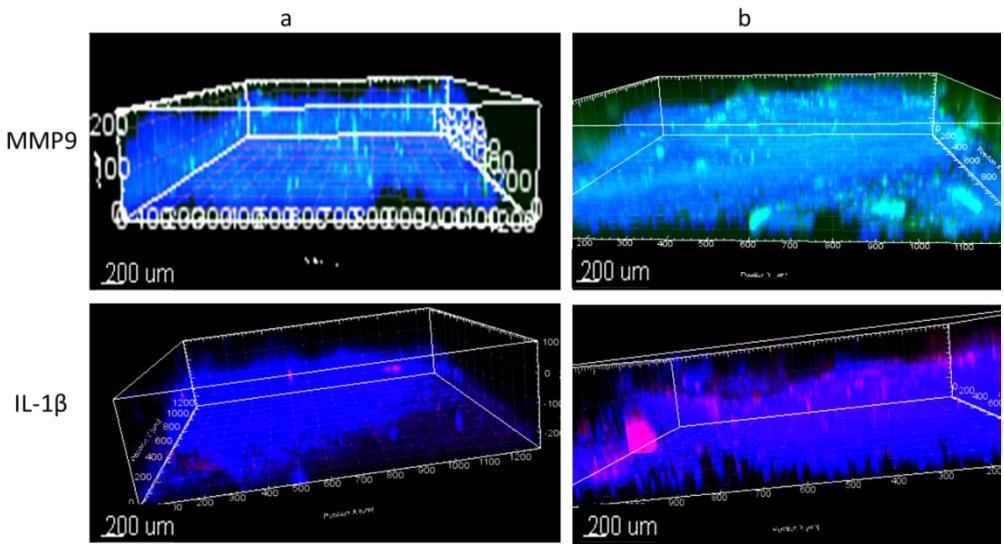


Figure 5.11: Immunostaining 3D images showing effect of maternal smoking. Representative images showing expression of MMP 9 (green) and IL-1β (red) of preterm membranes from non-smoker mothers (a) and smoker mothers (b). Blue is DAPI staining. (n=3 donors).

When FM from non-smoker mothers and non-ruptured sites were exposed to the nicotine solution, these expressed a higher rate of MMP 9 and IL-1β than membranes without exposure to nicotine. The expression rate was even higher with the higher concentration of nicotine concentration. Full term membranes also showed similar pattern of expressions. Although the expression difference between two concentrations in preterm samples were (in 10 µg/ml & 100 µg/ml) more than full term samples (Figure 5.12).

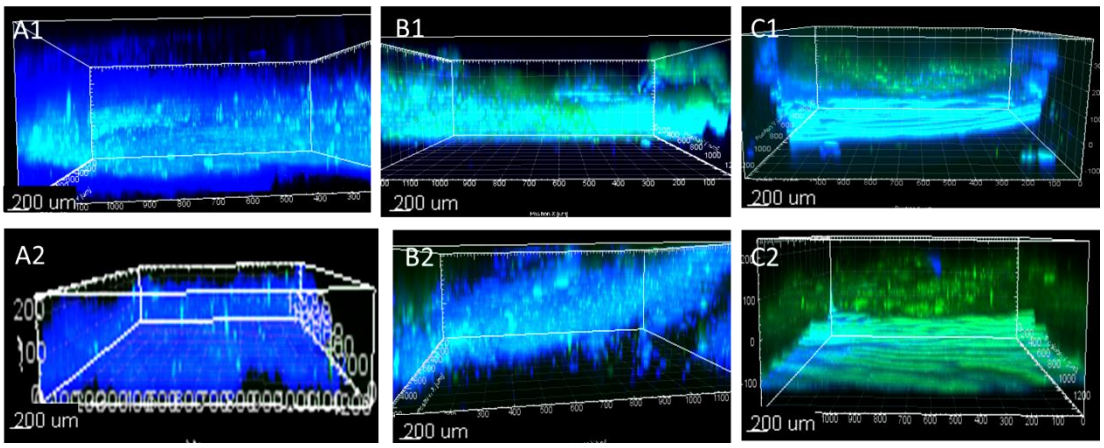
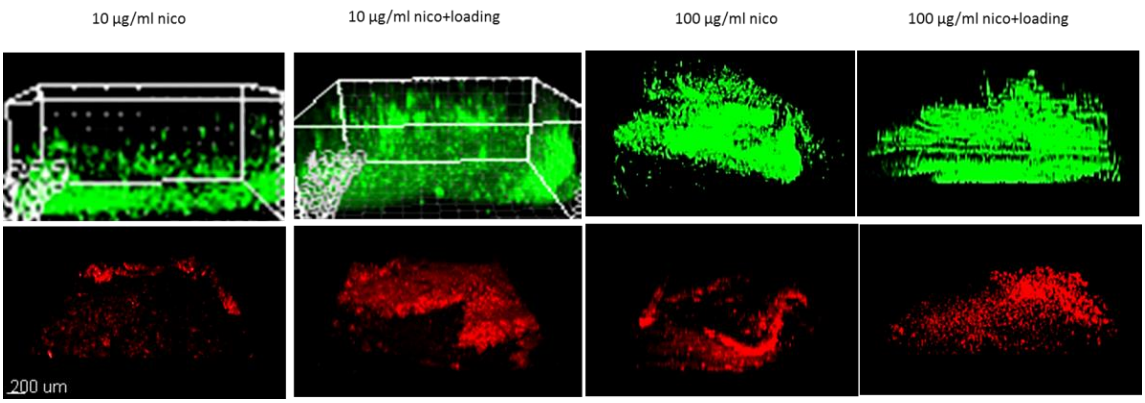


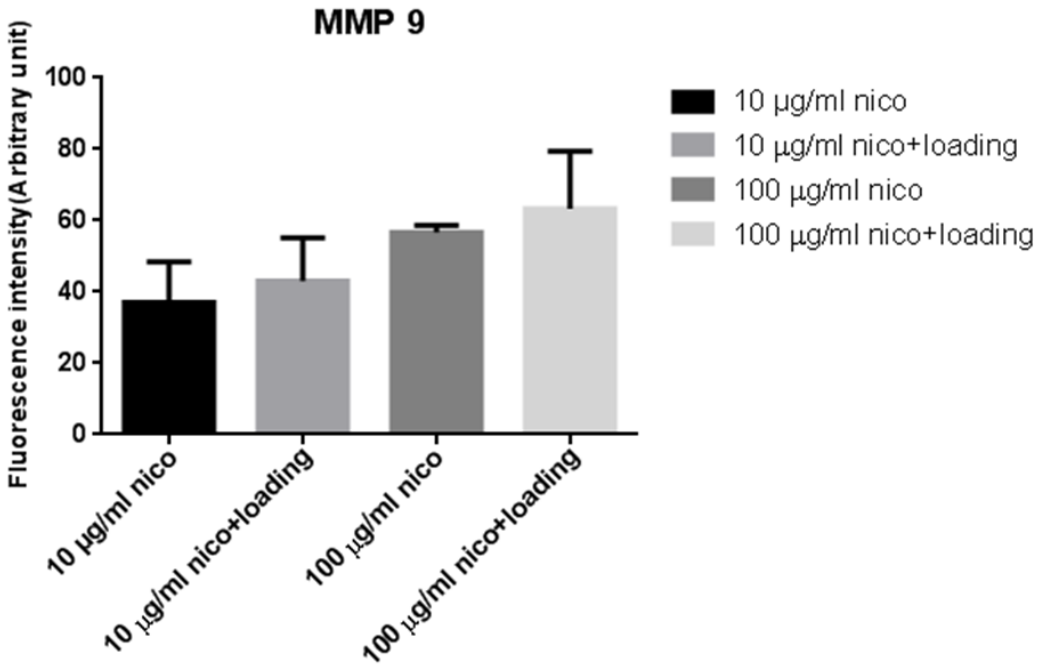
Figure 5.12: Effect of nicotine on full term and preterm samples. 3D confocal images of immunostaining showing MMP 9 expressions(green) in full term (A1) and preterm (B1) samples from non-ruptured sites, after incubation in 10 µg/ml (B1-full term & B2-preterm) and 100 µg/ml (C1-full term and C2-preterm) solution. Blue-DAPI staining. (n=3 donors). Scale bar=200 µm.

Preterm membranes exposed to nicotine solution also were loaded with external force. Figure 5.13 indicated that after adding the external force the expression of MMP 9 and IL-1β both increased than the membranes which had exposure to nicotine solution only. The pattern was same in case of either 10 µg/ml or 100 µg/ml of nicotine concentrations. Intensity of the biomarker expressions were correlated with the immunostaining data.

A


10 µg/ml nico
10 µg/ml nico+loading
100 µg/ml nico
100 µg/ml nico+loading
200 um

B


MMP 9
Fluorescence intensity(Arbitrary unit)
100
80
60
40
20
0
10 µg/ml nico
10 µg/ml nico+loading
100 µg/ml nico
100 µg/ml nico+loading
10 µg/ml nico
10 µg/ml nico+loading
100 µg/ml nico
100 µg/ml nico+loading

C

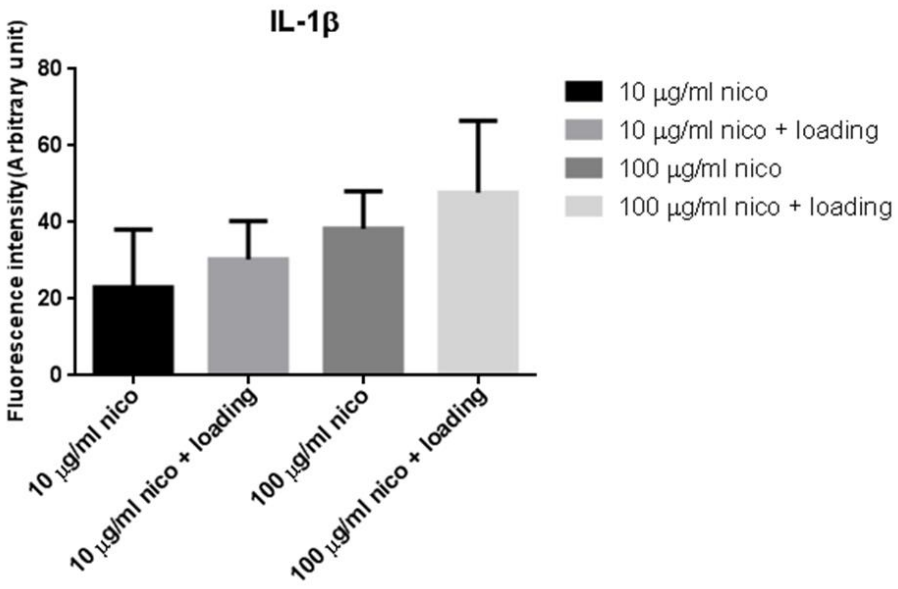


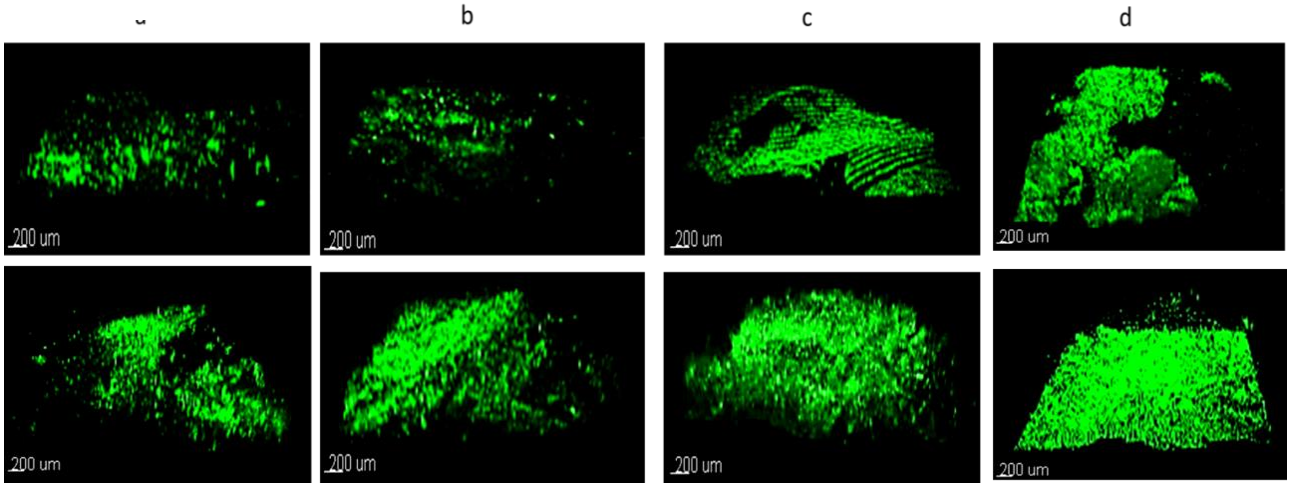
Figure 5.13: The effect of loading and nicotine exposure on biomarkers expression of preterm samples. (A) Representative Immunostaining results of preterm samples which were exposed to 10 μg/ml concentration of nicotine solution (10 μg/ml nico), 10 μg/ml nicotine solution and then to external force (10 μg/ml nico+loading), 100 μg/ml nicotine solution (100 μg/ml nico) and to external force (100 μg/ml nico+loading). MMP 9 (green) and IL-1β (red). (n=3 donors). Scale bar=200 μm. Semi-quantification of immunofluorescence intensity of MMP 9 (B) and IL-1β (C) in preterm samples. Data are presented as mean ± SD.

5.4.5 Effect of glucose on biomarker expression:

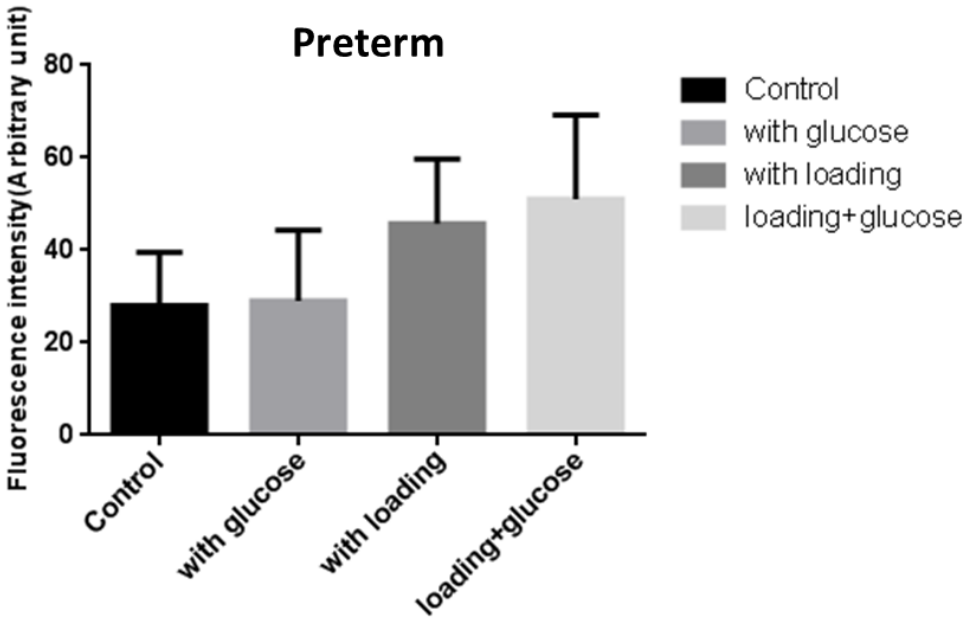
Preterm and full term samples from non-diabetic mothers were studied for glucose sensitivity. Samples were incubated with glucose solution (10 mM in basal media) for 3 hours and then exposed to external force for 3 hours and then incubated. Immunostaining was performed separately on samples immersed in glucose solution but not loaded and on samples loaded but not exposed to glucose solution from non-diabetic mothers. Samples from non-diabetic mother exposed to glucose solution were not found to have any changes of expression of MMP 9 when compared to the samples not exposed

to the glucose solution. However, when these samples were exposed to external force as well, they showed higher expression than before. But, the difference of expression was not much compared to the load only (without glucose) ones, especially in preterm samples (Figure 5.14)

A



B



C

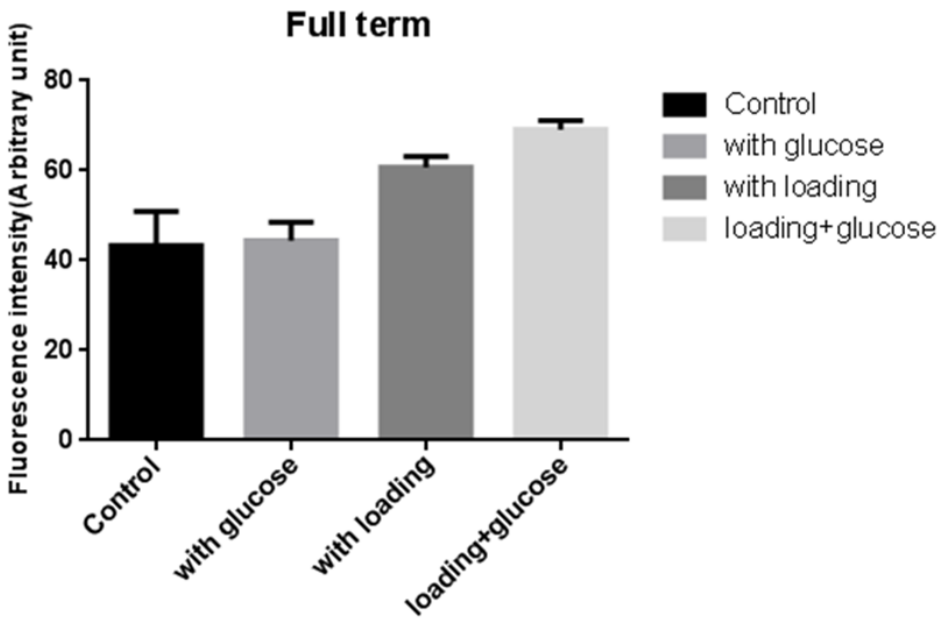
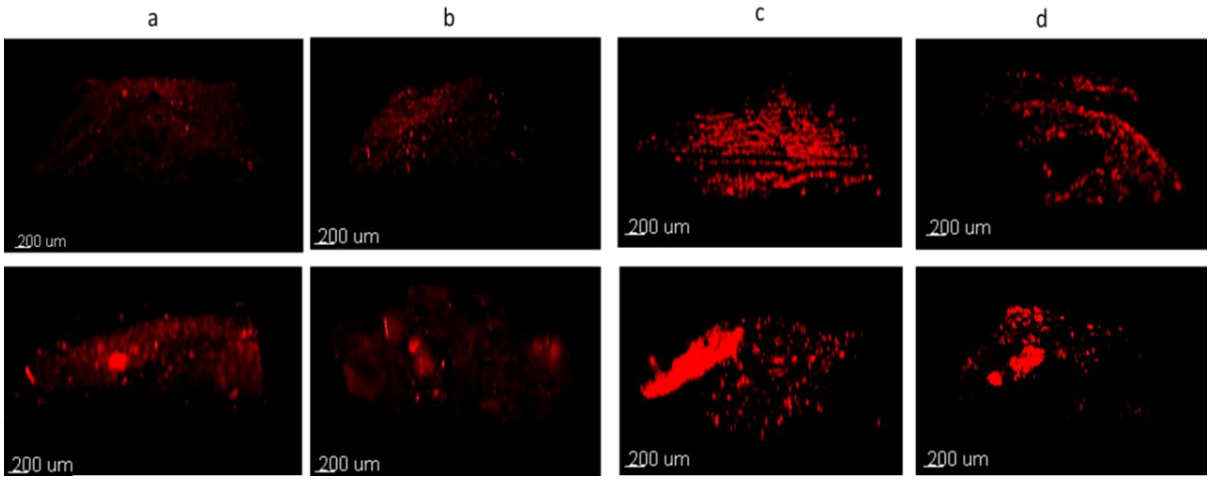


Figure 5.14 Immunofluorescence expression of MMP9 in FM after exposure to glucose solution and loading. (A) Expression of MMP 9 in preterm (top row) and full term (bottom row) samples. Samples are (a) non-ruptured sites, (b) after exposed to glucose solution, (c) after loading, (d) after exposed to glucose solution and loading. Scale bar= 200 µm. (n=3 donors). Semi quantification of immunostaining results for preterm (B) and full term (C) samples. Control was the sample without any treatment. Data are presented as mean ± SD.

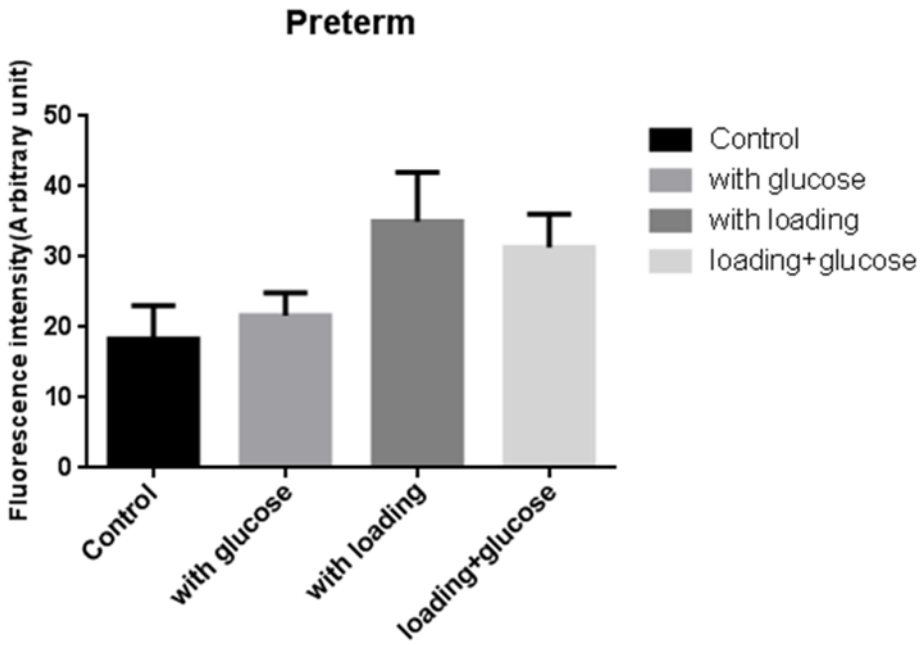
Similarly Figure 5.15 showed IL-1β expression on the 4 different types of samples from preterm and full term membranes. No difference of expression was found in the samples after exposing them to glucose solution. Samples after loading showed higher expression of IL-1β compared to the other two types, but, the expression was similar when compared to the samples which were exposed to glucose solution as well as loading. The pattern

was the same in full term membranes too. The intensity of the staining was similar to the immunostaining result. (Figure 5.15)

A



B



C

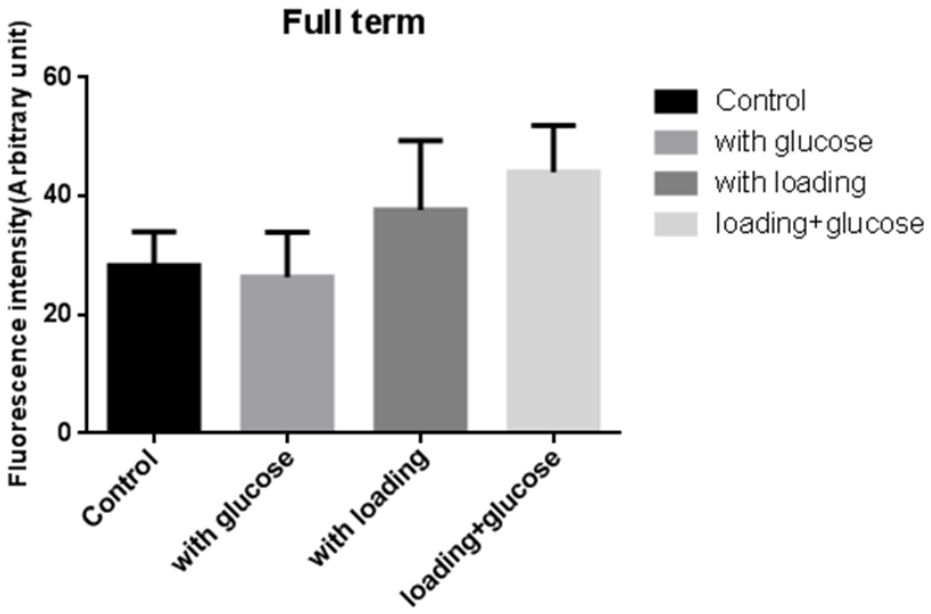
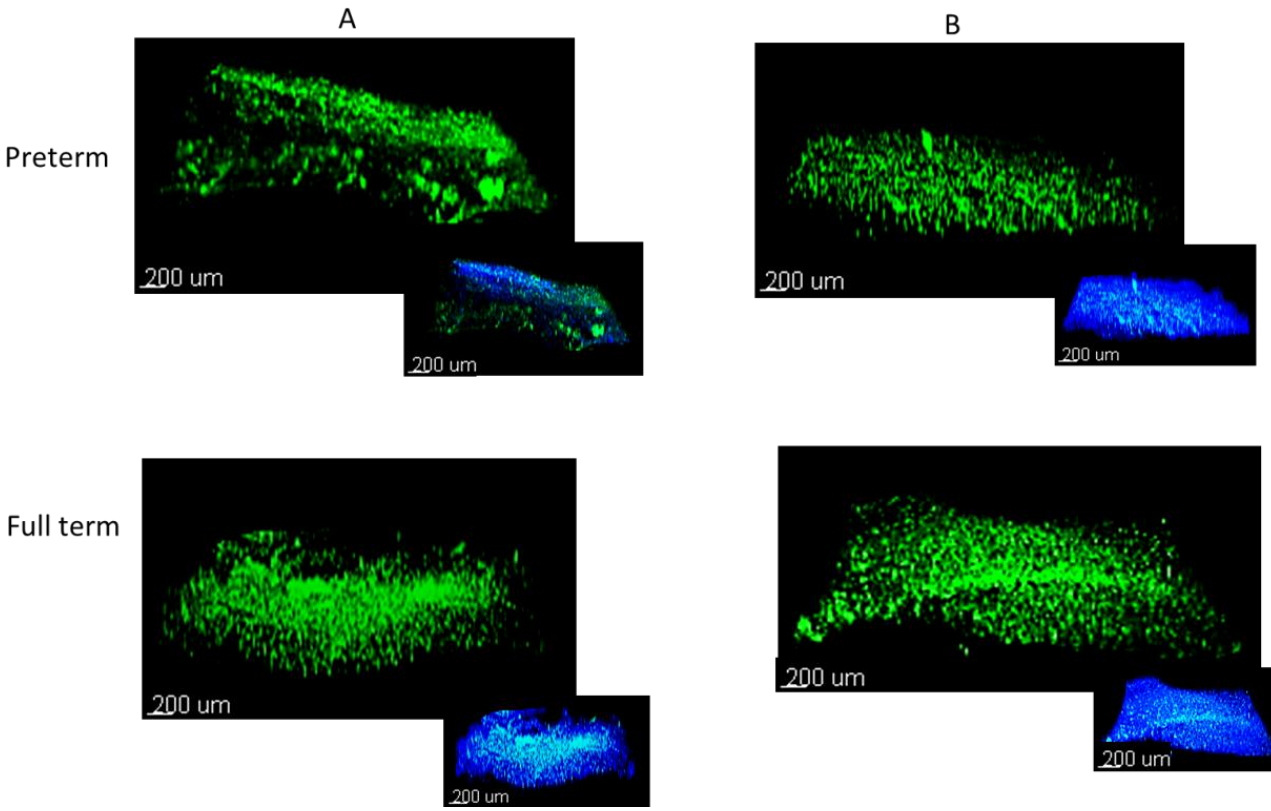


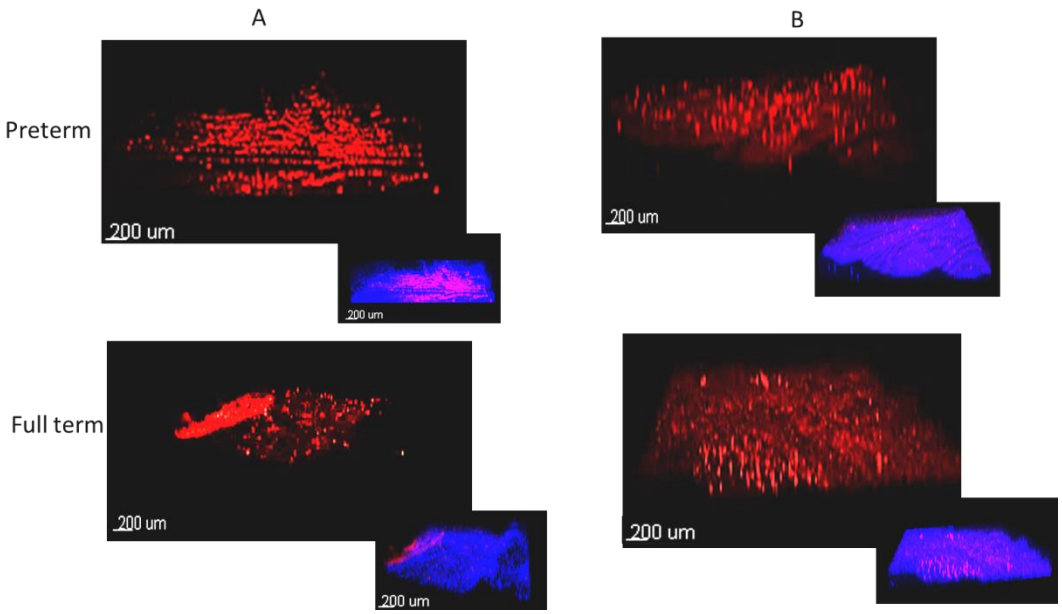
Figure 5.15 Immunofluorescence expression of IL-1β in FM samples after exposure to glucose solution and loading. (A) Expression of IL-1β (red) in preterm (top row) and full term (bottom row) samples. Samples are (a) non-ruptured sites, (b) after exposed to glucose solution, (c) after loading, (d) after exposed to glucose solution and loading. Scale bar= 200 µm. (n=3 donors). Semi quantification of immunostaining results for preterm (B) and full term (C) samples. Control was the sample without any treatment. Data are presented as mean ± SD.

5.4.6 Effect of dexamethasone:

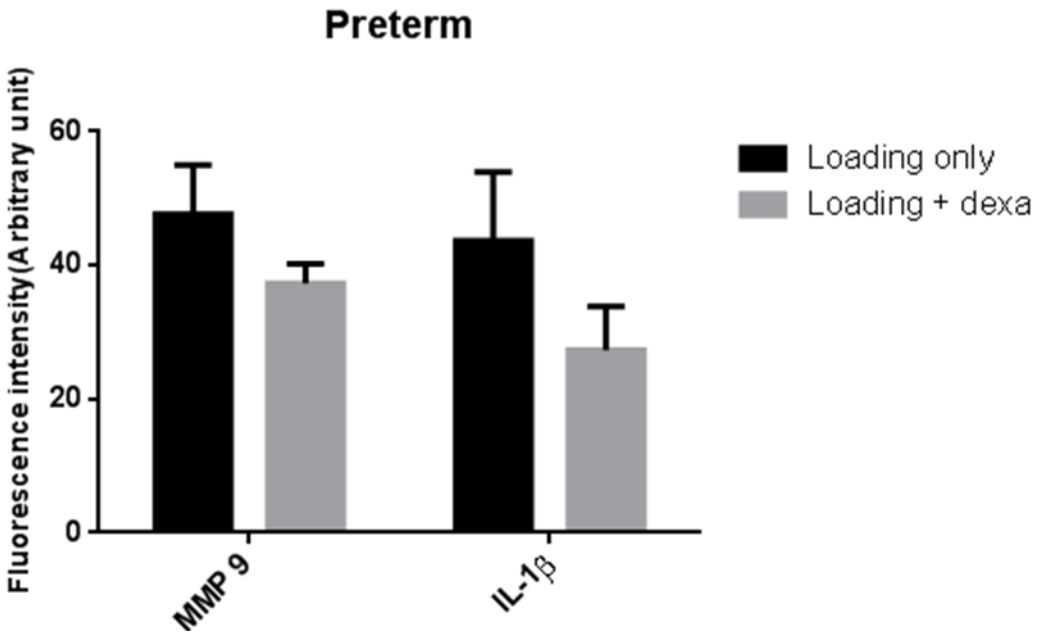
Samples from non-ruptured sites after loading with external force expressed high level of MMP 9 and IL-1β. But, in the presence of dexamethasone and external force, there was reduced expression of MMP 9 and IL-1β level.



II


A
B
Preterm
Full term
200 um
200 um
200 um
200 um
200 um
200 um

III


Preterm
Fluorescence intensity(Arbitrary unit)
60
40
20
0
MMP 9
IL-1β
Loading only
Loading + dexa

IV

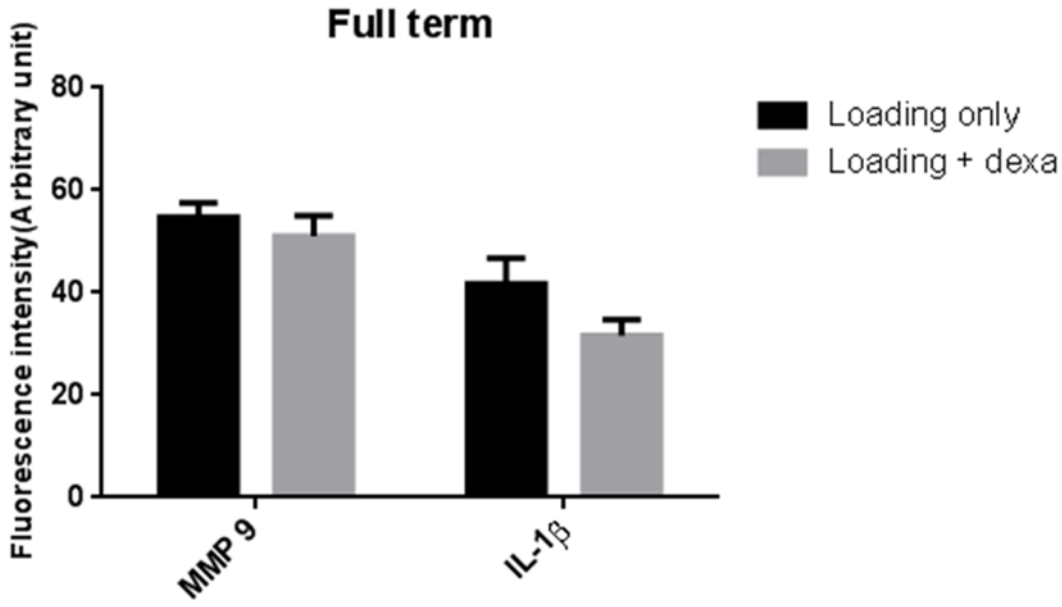


Figure 5.16: Immunofluorescence expression of MMP 9 and IL-1β in FM after exposure to dexamethasone and loading. Immunostaining images are showing I) MMP9 (green) and II) IL-1β (red) expression in preterm and full term non-ruptured sites of membrane after loading (A) and the membranes after exposed to the dexamethasone solution and loading (B). DAPI staining is blue. (n=3 donors). Scale bar = 200 µm. Semi quantification of immunostaining results for preterm (III) and full term (IV) samples. Data are presented as mean ± SD.

5.5 Discussion:

The aetiology of mechanical failure in FM is poorly understood resulting in a lack of detection and prevention of preterm rupture of membranes. There are very few studies which have explored mechanical force in relation to the membrane and its chemical environments. However, an ideal experimental model which could mimic the environment of a force from fetal movements acting on the FM in vivo has not been successful. This ex vivo model study showed the association of external force and

maternal risk factors acting together or acting on their own could activate the biomarkers. Preterm membranes are more vulnerable and susceptible to change in the chemical environment which weakens the memebrane. To perform dynamic loading on FM few requirements are necessary to take care including maintaining a toxic free hygienic environment. For multiple experiments, sample dimensions were needed to be small. Extra care was taken to maintain ideal temperature and carbon dioxide levels. Meticulous planning was done so that the whole set up could fit into the incubator. For application of repeated cyclic force in a stipulated time, proper clamping and sealing was assured. The model successfully fulfilled all the criteria.

5.5.1 External force upregulating the biochemical expression:

This study investigated the biochemical activation effects when mechanical stimulation was applied to human FM leading to membrane rupture particularly in preterm. The present study model showed the expression of biomarkers change with the time of exposure to certain amount of external force. To date, there has been no ideal model study exploring the relation or alternation of biomarkers expression in FM samples with application of pressure. Normally, during third trimester of the labour, membrane resistance gradually decreases and simultaneously various exerted pressure increases. Either one of these or combination of both leads to timely rupture of the membrane (Wright et al., 2007). Oyen et al. indicated in their study that chorioamnion membrane needs a force to rupture. Of note, this force stops increasing after 20 weeks of gestation and rather plateaus till term time (Oyen et al., 2006). Cyclic force alone is found to upregulate the interleukin-8 production and activation by alveolar epithelium cells. This can trigger lung inflammation (Yamamoto et al., 2001). Interestingly, we found that there was a higher biomarker expression in preterm non-ruptured sites after continuous pressure from external loading. This was similar to the ruptured sites in preterm

membranes. In full term membranes, similar trend was noticed. Apoptosis and over expression of fibronectin is known to cause disruption of collagen structural integrity. Therefore, increased levels of fibronectins and IL-1β were observed with a suspicion that they could possibly influence the mechanical stability of the membrane by interrupting and reducing the collagen and elastin levels. Also, IL-1β released under the influence of external force in our model may induce the activation of MMP 9 which is known to be associated with PPROM. The activity and presence of tissue inhibitors of metalloproteinases could be downregulated by force and induced MMP 9. A new cytokine, Pre-B-cell colony enhancing factor (PBEF) mainly exists in amnion was found to respond to the membrane stretching in FM. PBEF was found to be upregulated by IL-1β (Ognjanovic et al., 2001, Simona et al., 2002, Wright et al., 2007) Further investigation to validate this effect would be beneficial. Additional investigation of the mechanism of synthesis, release, and activation of biomarkers triggered by external force in vivo would benefit in better understanding of the same. We sought to investigate this aspect by exposing calcium channel blocker to the samples.

5.5.2 The potential pathway –calcium signalling pathway:

From the third trimester of pregnancy, downstream signalling via mechanotransduction in human FM prepares it to remodel and rupture (Claire et al., 2007). Using calcium channel blocker (nifedipine), expression of MMP 9 and IL-1β was significantly reduced in the membranes. In an animal model study, nifedipine (calcium channel blocker) inhibited tissue remodelling by inducing oxidative stress and MMP production in the vascular tissue. (Marcal et al., 2011) In rat, when vascular tissue was treated with calcium channel blockers, it was noted to suppress the expression of nitric oxide synthesis mRNA, ROS and TNF-α production. It facilitated upregulation of TIMP-1 and downregulation of MMP 9 through inhibition of Nf-kβ, MAPKs, JNK and Akt transduction signalling pathways (Yeh et

al., 2013). In another study, calcium channel blockers could reduce MMP 9 levels with no alternation of TIMP-1 or MMP 2 levels. (Martinez et al., 2006) Unlike any other tissue, remodelling and maintaining the tissue functions of human FM are regulated via different cell signalling triggered by mechanotransduction (Diver et al., 1995, Claire et al., 2007). Reduced expression of metalloproteinases in the nifedipine exposed samples indicates that it does play a vital role in activating integrin signal thus downregulating the inflammatory cytokines and MMPs. Hence, this protects collagen fibres from degradation and membranes from rupture. Therefore, it can be explained that the external force generated by fetus movement or the placental pressure could possibly be the source of induced biomarkers which then activates MMPs untimely through the calcium signalling pathway. Present study showed greater upregulation of biochemical markers including MMPs and IL-1β in preterm FM samples which were exposed to mechanical forces externally compared to the control samples. And, as shown in the previous chapter, it was observed that upregulated cytokines and enzymes altered the tissue morphology or collagen alignments. This process might have started with mechanotrasduction, where mechanical force was converted to biochemical signals which control the cellular activities. A stretch activated channel can directly activate the ion influx (Baumgarten et al., 2013). The force generated via this mechanical stimuli possibly led to FM stretching which opened up the protein gates, e.g. calcium ion Chanel and calcium ions flowed into the cells. These ions activated the pro-inflammatory cytokines and matrix degrading the proteins that triggered mechanotrsduction. Down regulation of these cytokines and MMPs due to calcium channel blocking has indicated that calcium channels probably played a fundamental role here in cellular responses of FMs to external stimuli including the mechanical force in vitro and membrane weakness. However, this observation and its mechanism need to be explored and studied more in detail further.

The main aim of this study is not to test the already approved two drugs, Dexamethasone, Nifedipine, and their dose range. The aim of this chapter is to investigate the role of external force in triggering different biochemical expression and the effectiveness of these drugs in activating these biochemical expressions on the membrane. To confirm the mechano-transduction mechanisms, calcium channel blocker has been used to block the downstream pathway. The aim of using these two drugs was not to test their optimized dose range. We only used clinically relevant dose in this study, but not study dose-response effects.

According to the NICE guideline the dose of dexamethasone for patients is 0.5-10 mg per day (NICE.org). We have taken simulated concentration in human blood of the dexamethasone, 3 μg/ml, per value in our tissue culture media. Clinically, Nifedipine dose is 30-120 mg per day. The concentration of Nifedipine used in our culture media, 10, 30 μM fall into the clinical dose ranges.

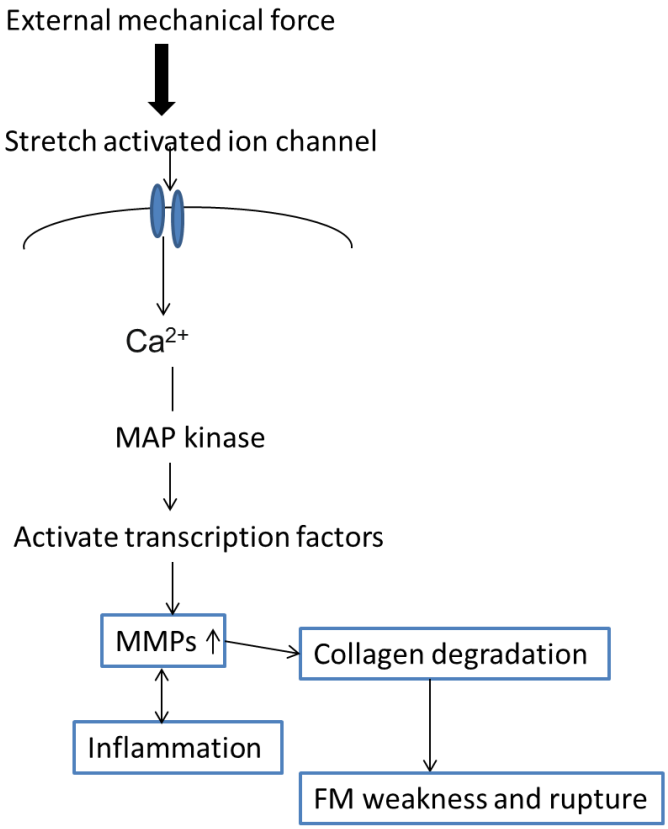


Figure 5.17: Schematic representation of the possible mechanotransduction mechanism. External mechanical force activates the calcium ion channels. Calcium ion influx via intracellular signalling activates the transcription factors which regulate the gene expression and upregulate the MMPs, cytokines etc. This is the potential pathway of fetal membrane rupture and PPROM.

5.5.3 Smoking during pregnancy could lead to FM rupture before term:

The incidence of smoking during gestation despite it is known association with preterm birth and fetal health is still high. Although numerous epidemiological studies and trials have reported the adverse effects of maternal smoking during pregnancy, the pathophysiologic mechanisms behind this correlation remain unclear. The increased expression of MMP 9 and IL-1β in membranes of maternal smokers shown in this study

clearly associates its risk with PROM. Cigarette smoking is proven to be one of the leading causes of lung diseases, asthma and chronic pulmonary diseases. Several studies in pulmonary disease patients indicated that smoking can increase the MMP 9 activity (Watson et al., 2010, Atkinson et al., 2011, Ghosh et al., 2015). Sivaraman et al. found increased concentration of MMP 9 in smokers with acute myocardial infection than non-smokers with acute myocardial infarction (Sivaraman et al., 2014). Kang et.al observed smoking inhibits the concentration ratio of TIMP-1 and MMP 9 (Kang et al., 2004). Later on, Watson et.al confirmed that it can alter the MMP 9: TIMP-1 ratio (Watson et al., 2010). Cigarette smoking is associated with production of reactive oxygen species (ROS) and reduction in nitric oxide activity. These two factors are known to induce MMP 9 secretion (Sivaraman et al., 2014) which possibly is one of the reasons to upregulate the MMP 9 concentration in FM in maternal smoker samples. The activated MMP 9 degrades the collagen fibres and elastin based membranes which lead to membrane weakness and rupture. The other reason for elevated MMP 9 level due to cigarette smoking could be because of the activation of inflammatory factors. As inflammation and oxidative stress is induced by smoking in vascular tissues, these have potential to escalate the levels of MMPs (Perlstein, et al., 2006). F2-IsoP, the only biomarker of oxidative stress was found to activate independently MMPs level in vascular and hepatic cells (Back et al., 2005, Valentin et al., 2005). Menon et.al investigated that oxidative stress can lead to PPROM (Menon et al., 2011). From our study, we can conclude that maternal smoking during pregnancy contributes in activation of MMPs and IL-1β probably due to oxidative stress, ROS, inflammation or apoptosis mediated events which can then degrade the ECM in preterm and induce PPROM and preterm birth. Our immunostaining results also showed the higher expression of IL-1β, which consisted with the result of Ryder et al.'s study who found increased IL-1β and TNF-α level in bloodstream of smokers (Ryder et al., 2002).

There are thousands of different compounds present in cigarette smoke, so it's difficult to elucidate the active agent which is causing the adverse effects during pregnancy. Few known factors could be carbon monoxide, metals and nicotine. (Wickstrom, 2007) The present study shows that upregulation of the matrix degradation proteins and inflammatory cytokine release was induced by external force. We compared samples which were exposed to the nicotine solution in vitro in different concentrations under the influence of external force to show the changes in FM. This was potentially important because of multiple reasons. Mainly, it can provide additional understanding about the adverse impact of cigarette smoking in pregnancy. We were able to compare FM samples from the mothers who smoke and who did not. However, quantifying the exposure was difficult. So, in our study, membrane samples from non-smoking mothers were exposed to different concentration of nicotine solution which helped to describe the mechanism further. Finally, when external force was added to the samples in nicotine solution, the results explained that in some cases smoking along with influence of external force was the likelihood contributor for PPROM. As example, Figure 5.17 showed, IL-1β expressed low in non-ruptured site of membrane. The expression was higher after exposing to 100ug/ml nicotine solution and much higher expression when membranes were exposed to nicotine + external force. The expression was almost similar with the ruptured sites of the membranes.

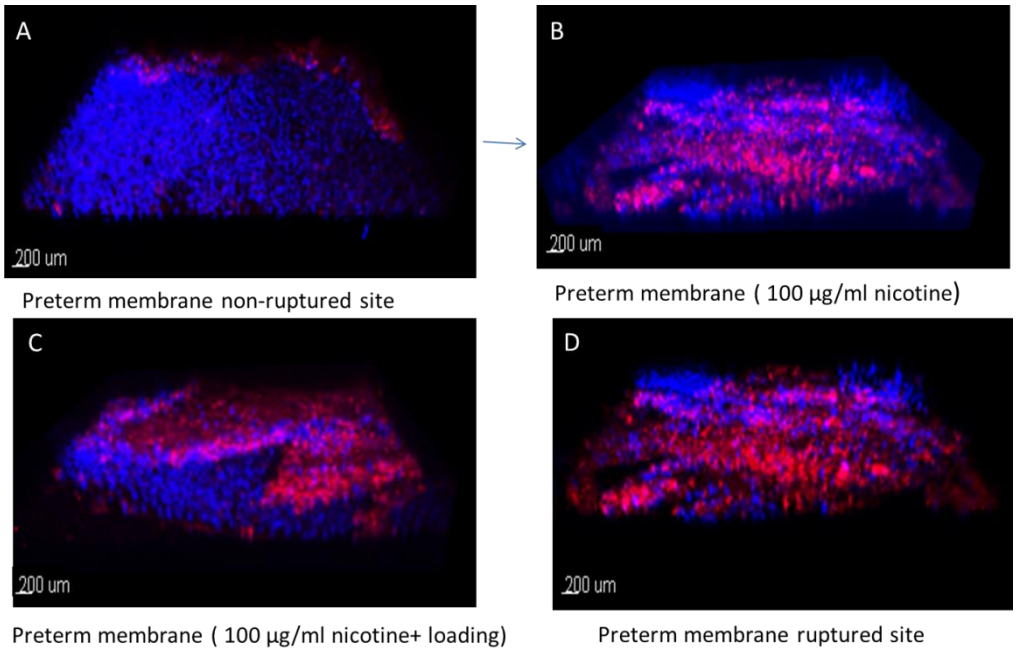


Figure 5.18: Immunofluorescence expression of IL-1β (red) **showing the effect of nicotine.** (A) non-ruptured site of the preterm membrane, (B) preterm membrane non-ruptured site after exposed to the 100 µg/ml nicotine solution, (C) preterm membrane non-ruptured site after exposure to nicotine and loading, (D) ruptured sites of the preterm membrane without loading and nicotine. Scale bar = 200 µm. DAPI (blue) stained for nucleus. (n=3 donors).

The present study suggested that increased nicotine concentration was associated with upregulation of MMPs and IL-1β. One previous study reported that women who smoked more than 20 cigarettes in a day were associated with higher risk of perinatal and neonatal complications compared to the group of women who smoked less than 10 cigarettes in a day (Habek et al., 2002). This can be correlated with our data.

5.5.4 Diabetes in pregnancy:

There are few studies that have reported any direct association of diabetes with preterm birth. In our study, there was barely any difference in expression of biomarkers when membranes were exposed to glucose solutions, even when exposed to higher

concentrations. Some studies observed higher risk of preterm birth in women with diabetes mellitus or gestational diabetes. Both spontaneous and induced preterm delivery incidence was noted to increase in pregnancies with pre-gestational diabetes. However, it was strongly believed that the reason was not directly related to diabetes but due to maternal BMI and gestational weight gain. Diabetes and maternal adiposity during pregnancy is strongly related to increase BMI (Ray et al., 2001, Melamed et al., 2008). Another study confirmed lower rate of spontaneous preterm birth in women with high BMI (Hendler et al., 2005). A cohort study, evaluated maternal and fetal outcomes, comparing gestational diabetes mellitus (GDM) and impaired glucose tolerance (IGT) pregnancy groups respectively. None of the groups found to note any association with significantly increased risk of preterm labour or PROM (Nordin et al., 2006). Further on, Kardesler and his colleagues demonstrated that there was no evidence of increased expression of MMPs (MMP 8 and 13) in gingival cervicular fluid (GCF) among diabetic patients (Kardeşler et al., 2010). The results of our study showed no elevation of biomarkers expression in gestational diabetic mothers which concurs with all the previous studies.

On the other hand, few studies have strongly suggested that increased rate of preterm deliveries. Both spontaneous and induced types were reported among patients with pre-gestational diabetes mellitus compared to healthy women (Sabai et al., 2000). A progressive effect on length of gestation was demonstrated in women with gestational diabetes and it was significantly correlated with spontaneous preterm births. (Lao et al.,2003). Poor maternal and fetal outcomes were found to be strongly affected by Diabetes mellitus. Higher risk of spontaneous preterm birth and related complications in diabetic patients was commonly detected. (Köck et al., 2010) Our study result showed increased expression of MMP 9 and IL-1β only when the samples were exposed to the

external force. We believe these increased expression are triggered because of mechanical stimulation only. Diabetes per se did not trigger early membrane rupture.

5.5.5 Dexamethasone- a potential therapeutic option for preterm rupture of the membrane:

Dexamethasone when used during 23-28 weeks of gestation is known to decrease the incidence of neonatal respiratory distress syndrome and thus reduce neonatal mortality. National institutes of health (NIH, US) reported its beneficial effect on extreme low baby weight (Song et al., 2005). Dexamethasone is a cost effective drug with negligible side effects. The beneficial effects of maternal treatment with dexamethasone are linked to prolonging gestational length and reducing neonatal complications; however, benefit of dexamethasone or corticosteroid on PPROM is not fully evaluated (Magann et al., 2017). Outcomes from our study favoured the beneficial effects of dexamethasone. Similarly, increased duration of pregnancies after dexamethasone treatment has been reported previously although the data was not significant (Chen et al., 2005, Elimian et al., 2007, Urban et al., 2005, Danesh et al., 2012). There are not many studies describing how dexamethasone works to keep the FM intact for longer periods. We found reduced MMP 9 and MMP 13 expression in FMs after being exposed to dexamethasone solution, even after exposure to mechanical loading, indicating that dexamethasone has the capability to alter the expression of MMPs. One pathway could be that dexamethasone was found to upregulate specifically TIMP-1 expression (Carola et al., 2007) which in turn is known to inhibit MMP-9 expression. The secretion of MMP 9 is definitely vital in the pathogenesis of Preterm ruptures of the membrane. Restoration of normal balance of MMP 9 and TIMP 1 ratio is a potential therapeutic angle of dexamethasone. Dexamethasone was found to inhibit the activation of MMP 13 (Caron et al., 1996). It could indirectly be due to Ap-1 transcription factor upregulation. Upregulated AP-1 transcription factor induces multiple

phosphorylation pathways which downregulates the MMP-13 action. (Benbow et al., 1997, Boileau et al., 2005) Also, dexamethasone suppresses the activation of MMPs through inhibition of IL-1β production which is mainly mediated by downregulation of prostaglandin production (Sadowsky et al., 2003). IL-1β expression of the samples in our study after exposure to dexamethasone supported that.

5.6 Conclusion:

In this chapter, an in vitro loading model for the study of biochemical behaviours of FM in the presence of external force was successfully established. Our cyclic force model study revealed that force originating either from the baby or fetal fluid can accelerate the production of matrix degradation enzymes and inflammation markers or trigger apoptosis. From our results, the downstream expressions of these biomarkers by nifedipine suggested that the calcium signalling pathway may be the point of integration of mechanotransduction in FM cells. In addition, maternal smoking correlated with membrane weakness as it responded to elevated biomarkers like MMP 9 and IL-1β. Diabetes did not show direct correlation under the influence of external force, but diabetic maternal membrane can be a risk for PROM. Finally, decreased expressions of membrane weakening biomarkers under the influence of dexamethasone revealed its potentially beneficial effects to reduce the risk of FM rupture. The purpose of this chapter was to better understand FM responses when it is subjected to external force and other risk factors. To our knowledge, this was the first attempt to study risk factors and therapeutic factors with external force simultaneously using an *in vitro* model. Though, further investigation is necessary; this study is definitely contributes useful knowledge for the timely detection and potential prevention of early membrane rupture.

Chapter 6

Correlation of preterm birth and PPROM frequency known potential risk factors

6.1 Introduction:

Despite advances in research and high quality treatment strategies, preterm birth remains a major concern worldwide. There is very little success in reducing the morbidity and mortality of the new born seen in preterm birth. This has not only resulted in negative psychological impact on the individual and the family but also has set a negative impact on the health system and society (Salam, et al., 2000). The main issue is poor understanding of the aetiology and nature of its progression. We have studied the biomechanical and potential biochemical factors which have shown their involvements in preterm rupture of human FM. Subsequently, there are some risk factors thought to be associated with preterm births. Common associations are infection, vaginal bleeding, uterine over distension and other 'immunologically mediated processes', race and low socio economic status (Romero, et al., 2006, Goldenberg, et al., 2008). Obstetric history factors, including previous preterm delivery, multi pregnancy, short gap between pregnancies, maternal age, hypertension and maternal diabetes and lifestyle related factors, such as cigarette smoking and alcohol consumption during pregnancy, mental stress and depression, and extreme physical activity etc have also been assumed to associate with increasing risk of preterm birth (Salam, et al., 2000). However, there has been no consensus on the consistency of these risk factors. Therefore, we believed that exploring risk factors associated with preterm birth will be helpful to improve PROM management and prevent preterm birth in many ways. Identification of risk factors and their associations may be useful for predicting the problem, formatting a risk management strategy and most importantly, can be helpful in recognising PROM aetiology (Goldenberg et al., 2008).

6.2 Objective:

This chapter aimed to correlate the known risk factors of preterm birth and PPROM to the collected FM samples. The selected risk factors include the mode of delivery, maternal smoking, and previous history of preterm delivery, maternal age, birthweight and diabetes during pregnancy, which are thought to be potentially associated with preterm birth. Study the clinical history of the samples, will enhance the understanding of relationship between the risk factors and structural changes in FM and the mechanisms leading to rupturing the membrane early.

6.3 Material and methods:

6.3.1 Sample preparation:

To obtain the human FM samples ethical approval was taken as described previously in chapter 2, section 2.1. Samples from normal vaginal deliveries (n=12) and elective caesarean section (n=10) were collected which were full term (37+ weeks). Similarly, preterm membrane samples (<37 weeks), from spontaneous PPROM (n=10) and elective caesarean section (n=10) were also collected. All were singleton pregnancies. The samples with history of any previous infection were excluded. The Sample preparation method was described previously in chapter 2, section 2.2.

6.3.2 Ball indentation test:

Creep testing on the FM samples was done by ball indentation technique. The detail of the techniques was described at chapter 3, section 3.2.1.

6.3.3 Immunohistochemistry:

Immunostaining was performed following the protocol as discussed previously in chapter 2, section 2.4. Immunostaining was used to identify the IL-6 expression in the FMs. Intact chorioamnion samples were stained and its intensity was observed by confocal microscope. The intensity of immunofluorescences was semi quantified by ImageJ software.

6.3.4 Collagen assay:

Hydroxyproline assay protocol was performed as described at chapter 2, section 2.5.2.

6.4 Result

6.4.1 Mode of delivery:

To investigate the correlation of mode of delivery between the vaginal delivery and caesarean section (C-section), the creeping property of the FM samples (full term and preterm) from two different modes of deliveries has been tested. Ball indentation test was performed on samples taken from different location of membranes including ruptured site (tear zone) and non-ruptured sites, and in combining and separation of the membrane.

Chorioamnion, amnion and chorion were found to have less deformity in samples from C-section deliveries than the normal delivery membranes. The creeping patterns were same in both the locations of the membranes as shown in Figure 6.1.

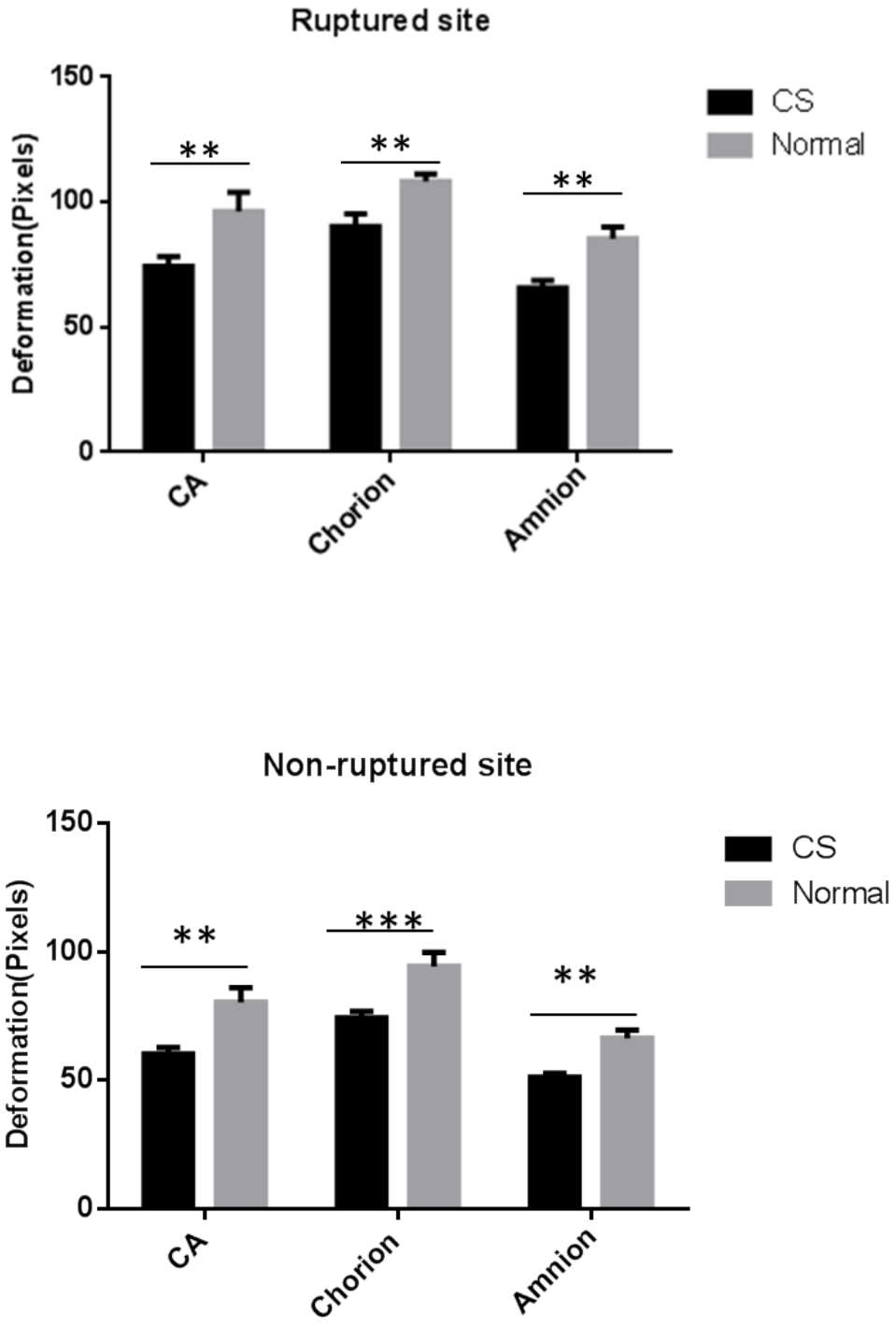
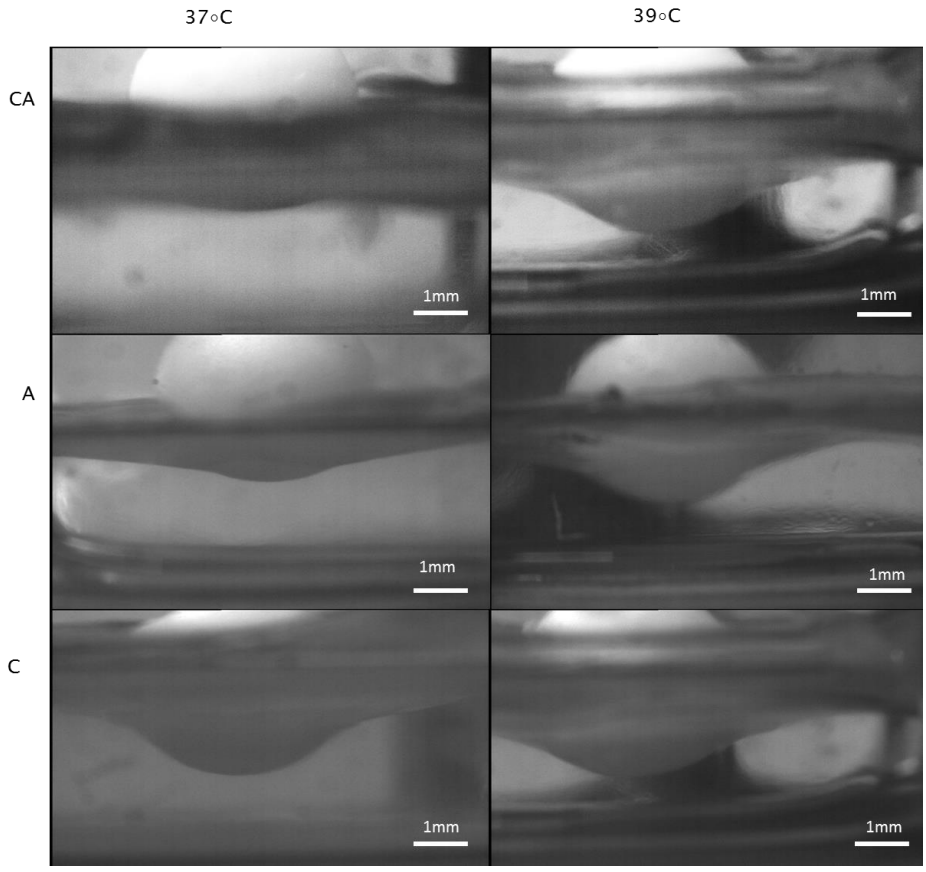


Figure 6.1: The creeping test result of membranes from different delivery modes. The membranes from normal vaginal deliveries (Normal) deformed more than the membranes from the C-section (CS) deliveries. Both ruptured and non-ruptured sites

showed the same pattern of deformations. Data are presented as mean ± SD (n=6 donors and 3 replicates. ** P < 0.01, *** P < 0.001.

6.4.2 Body temperature:

The correlation of the body temperature and membranes' mechanical property was assessed in full term of their creeping behaviour with testing the samples in ball indentation system after the samples were incubated in 37°C and 39°C for 3 hours respectively. When the samples were imaged via ball indentation technique, a clear difference in creeping property was noticed. Chorioamnion, chorion and amnion showed more deformation in the samples subjected to 39°C incubation than the samples incubated at 37°C (Figure 6.2).



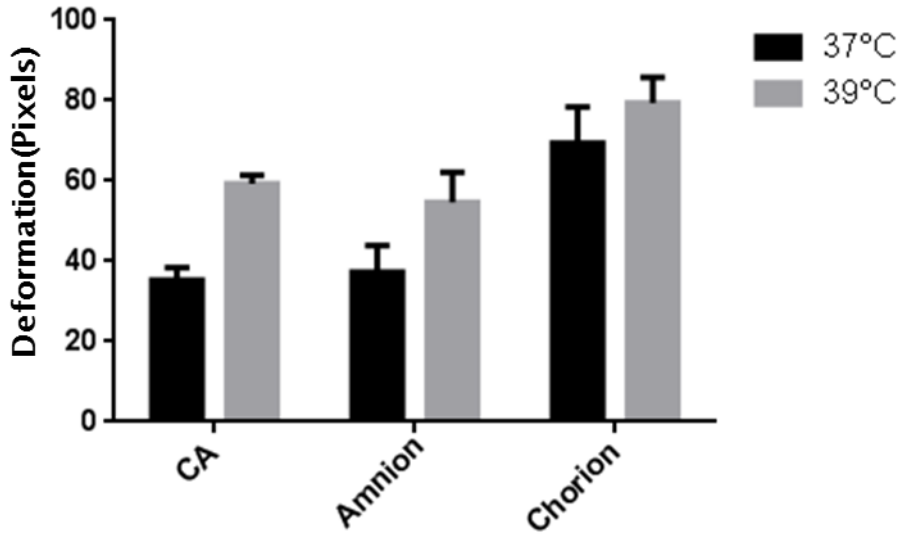


Figure 6.2: Creep properties of the membranes incubated at 37°C and 39°C. Representative images in the ball indentation tests showing that chorion deformed most. All the membranes incubating at 39°C showed more deformation than membranes at 37°C. CA-chorioamnion; A-amnion; C-chorion. Data are presented as mean ± SD (n=3 donors and 3 replicates).

6.4.3 History of previous preterm birth:

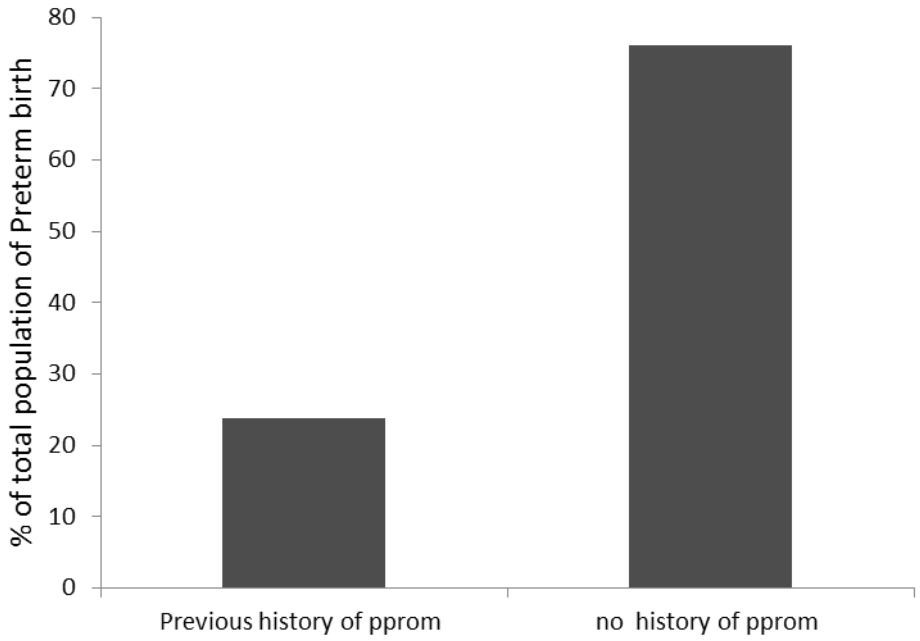
The data analysis of history of previous preterm birth was based on 21 samples. Among them 20 samples were from preterm birth and 1 full term sample which had previous history of PPROM. Approximately 76% of the samples had no previous history of PPROM and the rest 24% had a history of PPROM. (Figure 6.3 A) Among those samples which had previous history of preterm rupture of FM 20% was associated with full term birth while most of the cases were associated with preterm delivery again (80%). (Figure 6.3 C) Though most of the PPROM samples in our study were not associated with any history of previous PPROM, it should be noted that 75% of our PPROM samples were from first time

deliveries, and the rest 25% samples also exhibited PPROM in 2nd or 3rd pregnancy with no previous history of PPROM (Figure 6.3 B).

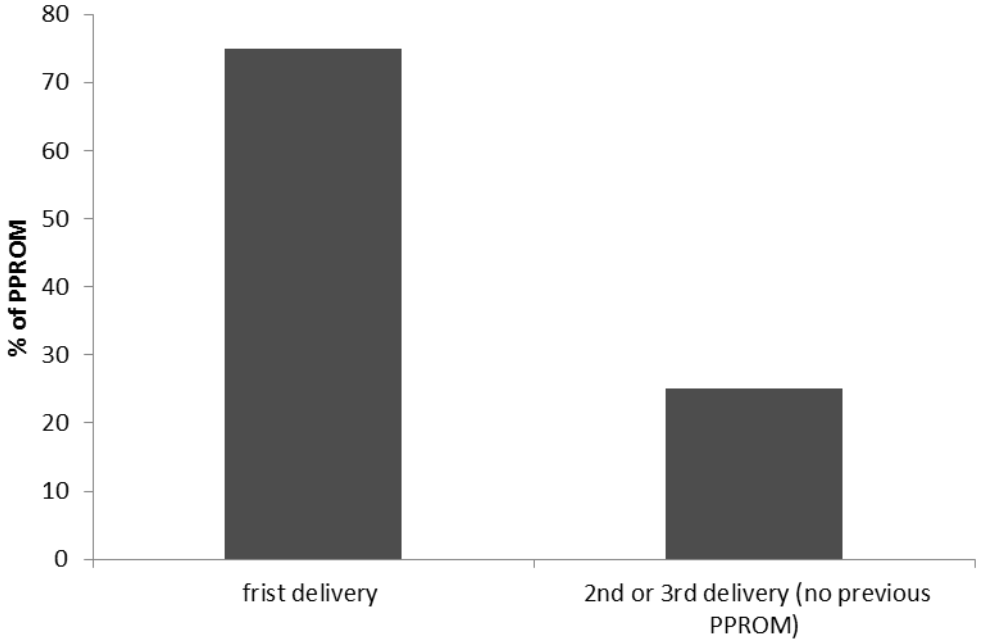
Total samples size 21 (preterm -20 and full term -1)			
Sample which has Previous history of PPROM		Preterm samples which has no previous history of PPROM/preterm birth	
Number	Percentage	Number	Percentage
5 Full term -1 Preterm - 4	23.8% Full term -20% Preterm - 80%	16 First time delivery - 12 Second or third delivery - 4 (After 1 or 2 full term deliveries)	76.2% First time delivery - .(75%) Second or third delivery - (25%)

Table 6.1: History of previous preterm birth studied for recurrent PPROM and preterm birth.

A


80
70
60
50
40
30
20
10
0
% of total population of Preterm birth
Previous history of pprom
no history of pprom

B


80
70
60
50
40
30
20
10
0
% of PPROM
frist delivery
2nd or 3rd delivery (no previous PPROM)

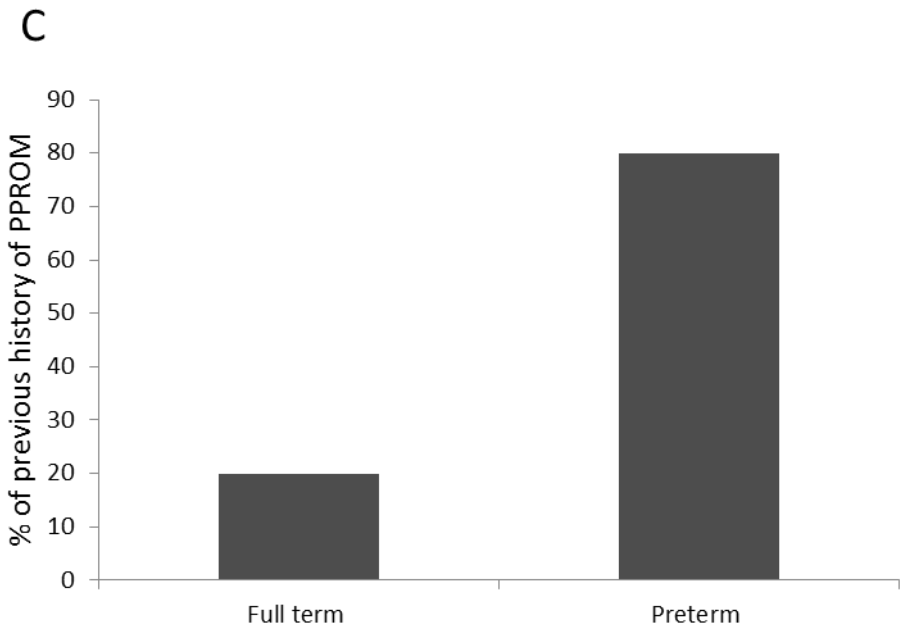


Figure 6.3: The correlation of history of previous preterm birth and PPROM in the current sample cohort. (A) Average percentage of samples from previous history of PPROM versus no PPROM. (B) Percentage of PPROM samples from women in their first delivery (1) versus PPROM samples from women in their 2nd or 3rd delivery with no history of PPROM (2). (C) Full term and pre-term samples in percentage with previous history of PPROM.

6.4.4: Smoking during pregnancy:

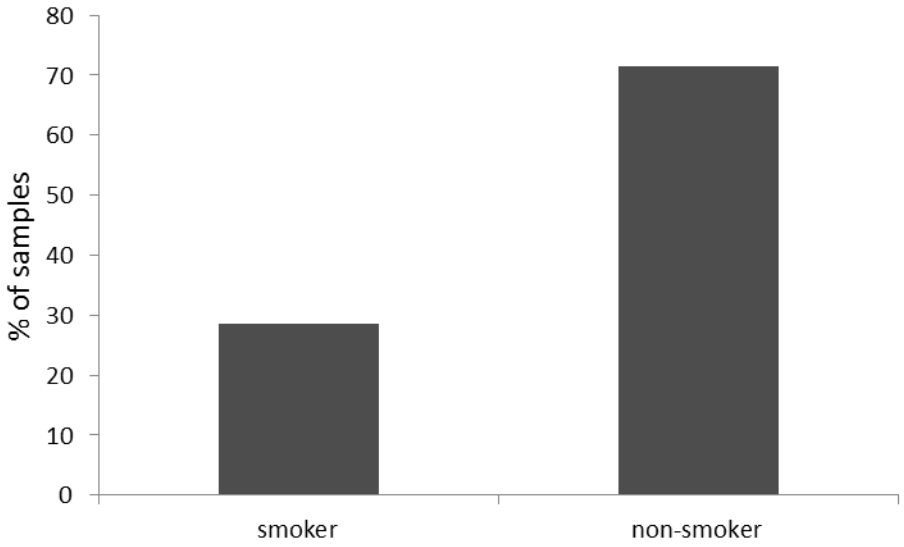
Our sample collection data showed that, 12 samples were from smoker mothers among 42 collected samples. 75% samples of those 12 samples were from preterm deliveries and only 25% were from full term deliveries. Among those mothers who smoked during pregnancy 67% of them had pre-term ruptured of FM and they all had normal vaginal delivery except one. The rest 33% of mothers had elective caesarean section without ruptured membrane (Figure 6.4). Overall a small percentage of sample population was noted to smoke during pregnancy (Figure 6.4 A). The percentages of preterm deliveries

were higher due to smoking compared to the full term deliveries (Figure 6.4 B). Among those pre-term deliveries greater number of PPROM percentage was noticed than elective caesarean section (Figure 6.4 C).

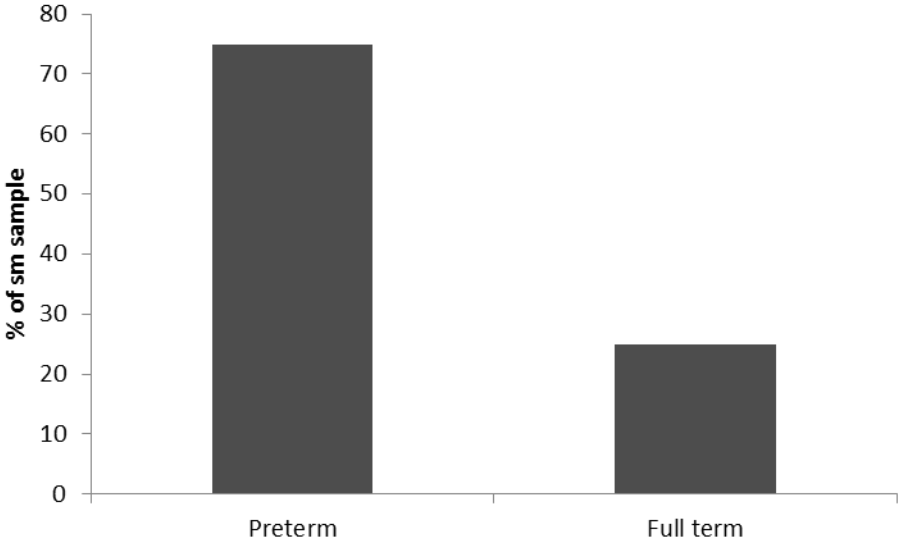
Samples from smoker mothers (Total 42 samples)		Samples from non-smoker mothers (Total 42 samples)	
Number	Percentage	Number	Percentage
12 Preterm-9 Full term -3	28.6% Preterm-75% Full term-25%	30 Preterm-11 Full term-19	71.4% Preterm-37% Full term-63%
Preterm samples from smoker mothers (Total 9 samples)			
PPROM samples -6	67%	CS preterm samples--3	33%

Table 6.2: Studied the association of maternal smoking with risk of preterm birth and PPROM.

A


80
70
60
50
40
30
20
10
0
% of samples
smoker
non-smoker

B


80
70
60
50
40
30
20
10
0
% of sm sample
Preterm
Full term

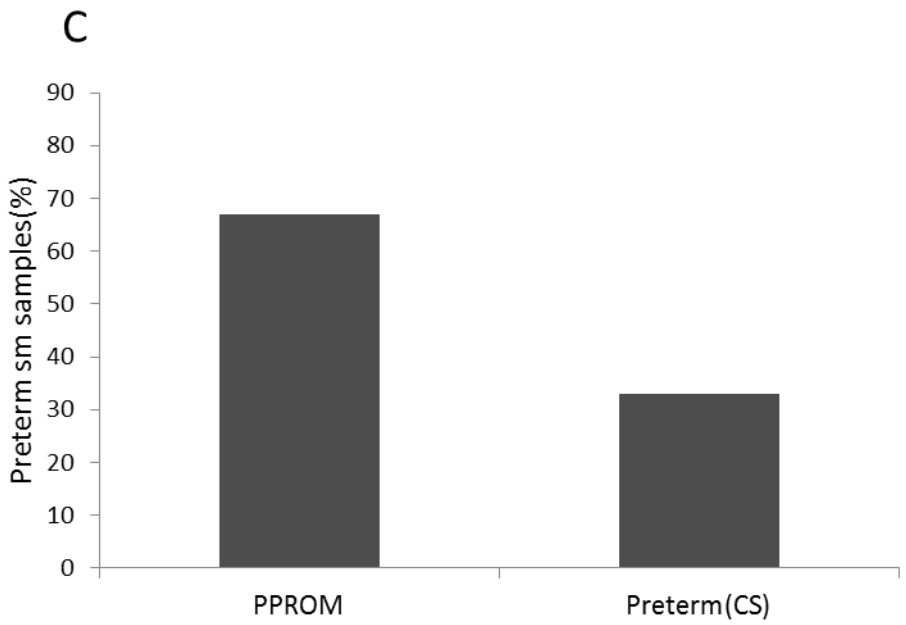


Figure 6.4: The correlation of maternal smoking and PPROM in the current sample cohort. (A) Percentage of samples from smoker (sm) and non-smoker mothers (B) Percentages of full term and pre-term delivery samples from smoker mothers (C) Percentage of the PPROM samples and other preterm samples without PPROM.

Hydroxyproline assay result showed lower total hydroxyproline concentration in samples from women who were smoking during pregnancy than those samples of non-smokers. (Figure 6.5).

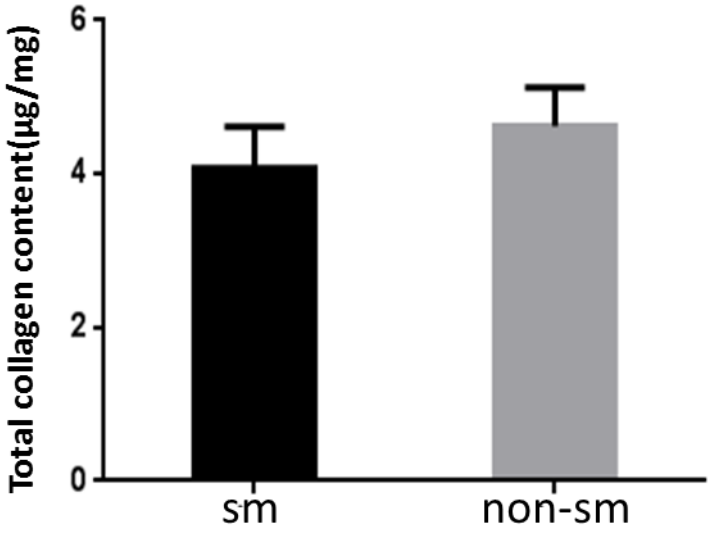


Figure 6.5: The Collagen quantity result of samples from smoker and non-smoker mothers. High intensity was demonstrated in the non-smoker (sm) mothers' samples and low intensity in the smoker(sm) mothers' samples. Data are presented as mean ± SD (n=6 donor membranes).

6.4.5 Diabetes:

4 samples of 42 total samples were from diabetic mother .The percentage of diabetic samples were very low (5%) in our total collected samples. Among those samples, 50% were found to have full term delivery and all were normal vaginal deliveries. The rest 50% of the samples were from preterm deliveries but from elective caesarean sections. None of the samples from diabetic women exhibited spontaneous preterm rupture of membrane (Figure 6.6)

Samples from diabetic mothers		Samples from non-diabetic mothers	
Number	Percentage	Number	Percentage
4 Preterm-2 Fullterm-2	10% Preterm-50% Full term-50%	38 Preterm-18 Fullterm-20	90% Preterm-47% Fullterm-53%

Table 6.3: Data correlated to maternal hyperglycaemia.

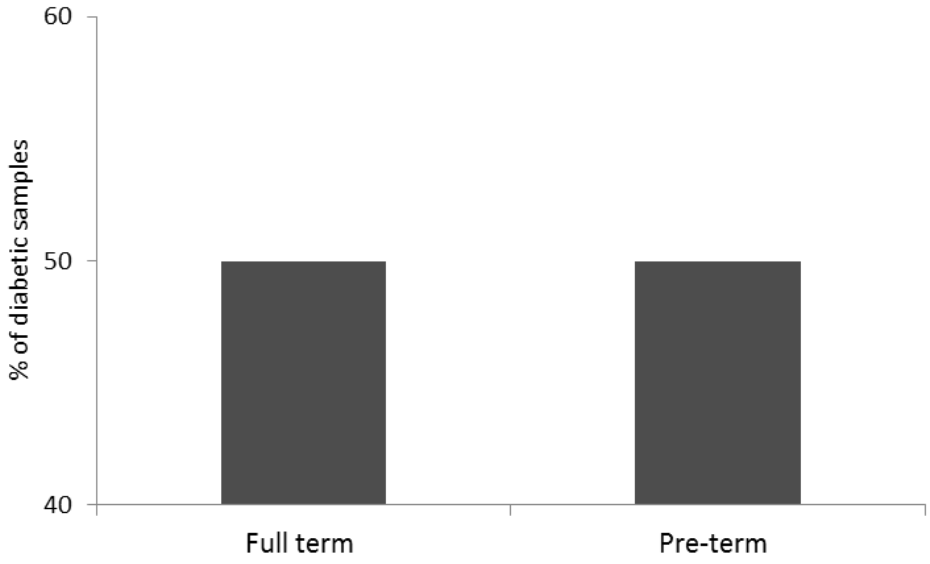
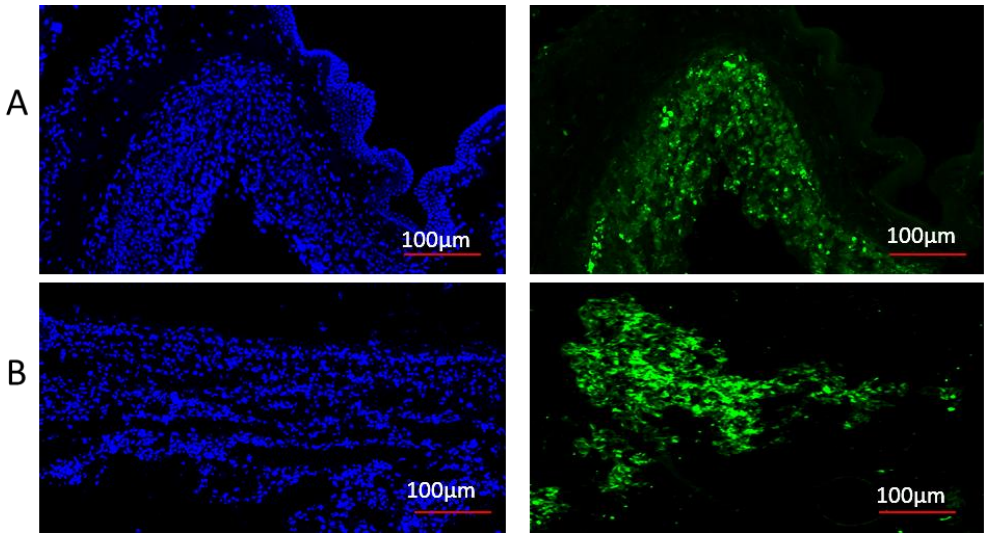


Figure 6.6: Correlation of diabetic population in the sample cohort. A- Percentage of samples from diabetic and non-diabetic mothers. B- Percentages of full term and preterm deliveries of diabetic samples.

Figure 6.7 shows the expression of interleukin-6 by immunostaining. The expression was higher in samples from women who had diabetes during pregnancy than the samples from non-diabetic mothers. The comparable groups were all from preterm birth samples of elective CS.



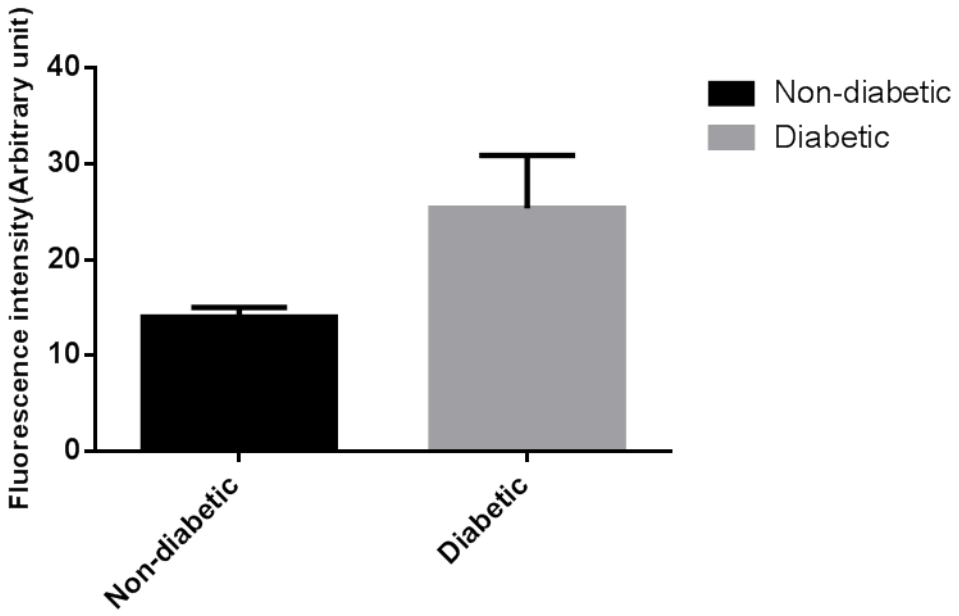


Figure 6.7: IL-6 immunostaining outcome. Representative Immunostaining images of non-diabetic samples (A); diabetic samples (B); the quantitative expression of the immunostaining intensity of IL-6 (C). Green: IL-6; blue: DAPI. Data are presented as mean ± SD, (n=3 donors).

6.4.6 Maternal age:

Samples were collected from 19-42 year-old-mothers. We divided all the samples in two groups, first group was < 30 year old, and the second group > 30 year old. The average age in the first group (19-29 years) was 23.6±3 years, 33.8±4 years in second group (30-42 years). Preterm deliveries were higher in age < 30 years group compared to other age (>30 years) group (Figure 6.8).

Samples from maternal age < 30 (23.6.±3) years		Samples from maternal age >30 (33.4±3) Years	
Number	Percentage	Number	Percentage
24 Preterm- 13 Fullterm-11	57% Preterm-54% Full term- 46%	18 Preterm-7 Fullterm-11	43% Preterm-39% Full term-61%

Table 6.4: Detail of the sample data correlated to maternal age.

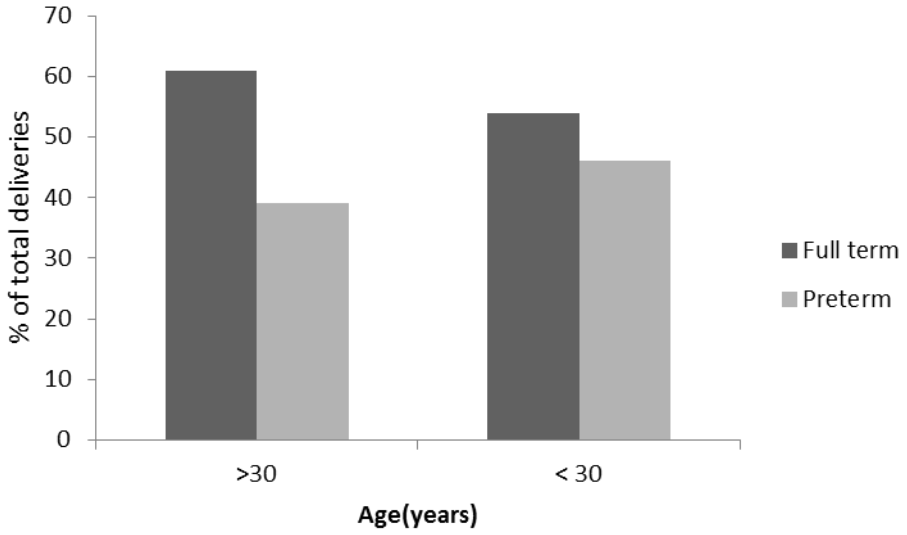


Figure 6.8: Correlation with maternal age. Percentage of full term and preterm samples from mothers with age group < 30 and >30 years**.**

6.5 Discussion

The aetiologies of the FM rupture are multifactorial and unclear. Therefore, it is prudent to study the underlying mechanism. It is also essential to consider the risk factors and understand their involvements in physiological events leading to PROM.

6.5.1 C-section delivery vs normal vaginal delivery:

Present study results revealed that the mechanical properties of the human FM varied depending on the mode of delivery. FMs from normal vaginal deliveries exhibited higher creep compared to the membranes from elective C-sections (in week 37+weeks). The increased rate of mechanical deformation in normal vaginal delivery membranes can be due to the greater changes of ZAM during normal labour compared to the caesarean section samples. According to previous study, the extensibility of amnion and chorion was not found to be the same in vaginal delivery membranes whereas in elective caesarean membranes, it showed similar extensibility between amnion and chorion from the load-strain curve (Helmig et al., 1993). We also noticed separation of amnion and chorion was bit easier in vaginal delivery in ruptured site than the caesarean ruptured site of the membranes. The debouchment of these two layers was accountable of the changes of mechanical properties which we have described before in chapter 3. We assumed that, the collagen concentration and enzymatic change pattern of membranes in healthy individuals throughout the pregnancies should be similar before delivery. So, it is understandable that any alteration of mechanical properties of the membrane (i.e creep property) should have occurred during the delivery. Caesarean delivery is known to be associated with more perinatal complications than the vaginal deliveries (Hussin et la., 2013, Ibishi et al., 2015). It was also reported that a previous history of caesarean section is strongly correlated with FM rupture, especially PPROM (Chandra et al., 2017). On the other hand, according to few study results, caesarean section is a safer delivery mode for

PPROM or pre labour rupture of membrane (Mousiolis et al., 2012, Chakraborty et al., 2013). Recent meta-analysis demonstrated that vaginal delivery is preferable and safer mode of delivery for FM rupture after 28 weeks of gestation. Kayiga and colleagues demonstrated that vaginal delivery is associated with decreased adverse maternal and perinatal outcomes on comparison to caesarean deliveries (Kayiga et al., 2018).

6.6.2 The effect of body temperature:

Higher deformations of membranes were noticed with increased incubation temperature, which mimicked fever or infection in the maternal body. It is well known that elevated temperature is associated with collagen denaturation. Consequently, at 39°C collagen fibre bindings start to loosen up easily and more quickly than at 37°C. Also, in the presence of heat, collagen is vulnerable to upregulated matrix degradation enzymes (Arisa et al., 2003). Placenta plays a vital role in maintaining thermal homeostasis of the fetus. Fetal tissues are exposed to higher temperature than the maternal tissues. Placenta plays a major role in balancing that heat from fetal tissue to maternal tissue. When this equilibrium disrupts, the oxygen level is upregulated and this decreases the blood flow rate in placenta (Schroder et al., 1997). This has a direct or indirect effect on FM. There is barely any study investigated about fetal temperature as risk factor of FM ruptures. However, higher rate of body temperature is known to correlate with infection and inflammation. According to Chandra et al., the body temperature of PPROM group of women was higher than the group where membranes were ruptured in full term. They also reported about increased amount of C - reactive protein in PPROM group. C-reactive protein indicates the presence of infection or inflammation in the body (Chandra et al., 2017). Interlukin-6 was found to trigger synthesis of C-reactive protein which in turn was induced by high body temperature (MARIA et al., 1998). Therefore, it is suggested that high body temperature during pregnancy is a risk factor. More research on it will be helpful in PPROM management.

6.5.3 The association of history of previous preterm birth:

There is an increased risk of PPROM in women in subsequent deliveries following preterm delivery. This phenomenon has been noted by several investigators who have observed an increased risk of PPROM recurrence. A study of 121 women with PPROM showed a 32% risk of recurrence with previous history of PPROM (Asrat et al., 1991). Another study reported that women with previous PROM pregnancy had higher risk of spontaneous preterm delivery because of PPROM compared to those women had no previous history of PROM pregnancy (Mercer et al., 2003). A cohort study with thousands of women with 3 singleton deliveries reported that prior pre term delivery had strong association with preterm PROM. Another study showed an increased incidence of PPROM with more than one previous preterm delivery than a single previous preterm delivery (McManemy et al., 2007). On comparison, our data is consistent with these studies. However, we have also noticed in our study that there have been some cases where there was no instance of preterm rupture of membranes even though these women had preterm PROM history. On the contrary, we have observed PPROM pregnancies with no history of previous PROM. This indicates the association most likely depends on other factors as well. It is suspected that interval duration between pregnancies have an association with increased risk of recurrent PPROM pregnancies. A study concluded that the longer interval between second pregnancy and previous PPROM pregnancy has decreased risk of PPROM compared to the shorter interval (Getahun et al., 2010) Several studies have considered racial factor in association with recurrent PROM or PPROM pregnancies. Non-white women showed higher risk of term or preterm PROM compared to their white counterparts (Kistka et al., 2007, McManemy et al., 2007). Also, in shorter interval duration between pregnancies white women showed lesser incidence of PPROM than the

non-white women (Getahun et al., 2010). Besides that, short cervix (Mazaki et al., 2007), older age (Doody et al., 1997), Body Mass Index-BMI (Marlino et al., 2006), smoking and other environmental factors (Mazaki et al., 2007) were noted as increased risk factors of PPROM among women with previous history of preterm rupture of membranes.

6.5.4 The association of maternal smoking:

According to our outcomes from sample data, cigarette smoking during pregnancy has great impact on Pre-term birth. The association of maternal smoking and early rupture of FMs was noticeable. This outcome was supported by many other previous studies where a significant correlation was found between maternal cigarette smoking and increased risk of preterm birth. Cnattingius et al. reported a strong association with spontaneous preterm birth and very preterm birth (Cnattingius et al., 1998). Castles et al., suggested that smoking is heavily associated with elevated risk of PPROM and its adverse effects on unborn babies (Castles et al., 1999). The risk of Preterm birth was directly proportional to the amount of cigarettes smoked daily. England et al., reported that smoking more than 10 cigarettes everyday can increase the risk of PPROM but they also said smoking less than 10 cigarettes a day was not considered as a significant risk for PPROM (England, et al., 2013). There was another study which reported that women who smoked during their first pregnancy but quit during second pregnancy had a reduced risk of pre term delivery (Cnattingius et al., 2004). On the other hand, women who were not smoking during their first pregnancy but smoked later on during their second delivery faced an increased risk of preterm birth (Wong et al., 2016). Although in our data, factors like the amount of cigarette smoking, ex-smoker or new smoker status are not recorded, the adverse effect of smoking during pregnancy with increased risk of preterm birth and PPROM was definitely consistent with those studies. However, this study contradicts other studies where any correlation of maternal smoking and PPROM was not entertained. Two

studies, Doody in 1997 and Andres in 2013 felt that maternal smoking in the presence any other risk factor accelerates the risk of PPROM (Doody et al., 1997, Andres et al., 2013).

In our study, we found the total collagen level in smoker samples to be lower than the non-smoker FM samples. One study pertaining to wound healing had found that the amount of hydroxyproline was 1.8 times more in non-smokers than smokers (Jorgensen et al., 1998). The synthesis rate of collagen I and III were found to decrease in skin due to smoking (Knuutinen et al., 2002). MacDermott and his colleagues demonstrated that variation of collagen content in FM can affect the membrane properties (MacDermott et al, 2000).

Although, the findings in our study do not directly indicate that maternal smoking triggers PPROM, it certainly provides more reason to study the effect of maternal smoking and its association with PPROM. Therefore, we have tried to investigate this in depth in our previous chapter.

6.5.5 Association with diabetes:

Diabetes during pregnancy has been considered as a clinical complication and its association with high risk of mother's and baby's morbidity is well known (Schwartz et al., 2000). A case control study reported higher risk of PPROM associated with gestational diabetes and diabetes mellitus in pregnancy compared to their normal counterparts who were free from any type of diabetes (Bhat et al., 2012). Another similar study with two groups of patients, 187 women with gestational diabetes and 192 women with no diabetes found a significant tendency of spontaneous pre term membrane rupture in the first group of women (Katharina et al., 2010). Riyami et al., stated a significant correlation existed in gestational diabetes with risk of PPROM and extreme PPROM. (Riyami et al., 2014) Although in our study we have not noticed any significant correlation between diabetes and risk of PPROM, a larger sample size is required to further explore this aspect.

It was also noted in our study that there was elevated expression of IL-6 in diabetic mother's membranes. IL-6, a pre inflammatory cytokine was detected to present in increased level among type 2 diabetes patients and gestational diabetes women (Pradhan et al., 2001, Kuzmicki et al., 2009). Our data was consistent with those studies. Our study outcome was in line with other studies in many aspects. Ramirez et al. suggested that diabetes during pregnancy was associated with increased risk of infection (Ramírez et al., 2005). There was a strong relationship between asymptomatic bacteria and diabetes during pregnancy (Sheiner et al., 2009) and diabetic patients related to higher risk of chorioamnionitis (Kari et al., 2017). These studies indicate an existence of indirect correlation of diabetes with occurrence of PPROM. Further investigation was needed to explore this aspect in detail which influenced to the *in vitro* nicotine study in the last chapter.

6.5.6 Maternal age and its association:

Our analysis has indicated an association of maternal age and risk of preterm birth. The average age of mothers with pre mature FM samples was 24 years. We found pre term deliveries to be comparatively low in mothers with average 34 years of age. This finding is consistent with few recent study results. Fuchs et al, found women aged 30 -34 years old had lowest risk for premature deliveries (Fuchs et al., 2018). A cohort study with different age groups of mothers revealed a relationship between maternal age and preterm births. They reported that at 26-30 years age group of women showed lowest risk preterm delivery. (Lawlor et al., 2011) A nation-wide registered based study reported that the threshold maternal age for preterm delivery was ≥ 35 years (Klemetti et al., 2016). On the other hand, some studies found increased risk of preterm births at 30-34 years of maternal age (Astolfi et al., 1999, Hassan et al., 2009, Koo et la., 2016). High risk of preterm birth was associated with young age (<20 years) and older age (>40 years) too (Fraser et al., 1995, Hediger et al., 1997, Fuchs et al., 2018, Hassan et al., 2009). Its

association with young age delivery could be explained by differences in social economic status and other complications (Fuchs et al., 2018). We did not have enough samples from those age groups to compare with the previous studies. Not many studies investigated occurrence of preterm ruptured preterm birth with maternal age as risk factor. Higher risk of PPROM was reported to correlate with lower maternal age (Noor et al., 2007). Riyami, et al, found that women aged 30 years above were in high risk of extreme PPROM (Riyami et al., 2013). However, none of the studies had confirmed if those were spontaneous rupture or iatrogenic. Based on our finding and previous results, we believe there is a direct association of maternal age with high risk of preterm birth but we feel the correlation with rupture of FM is mostly triggered by other associated risk factors.

6.6 Conclusion:

This chapter found that the association of past obstetric history and factors during gestation increased the risk of preterm birth. Our investigation revealed that mode of delivery, previous history of PROM, maternal body temperature, maternal smoking, diabetes and maternal age were potential risk factors for preterm premature ruptured of membranes. Maternal smoking and high body temperature were associated with increased risk of PPROM. Mode of pregnancies and previous history of preterm birth also indicated a possible link to risk of preterm birth. However, possibly due to small sample size, maternal diabetes did not clearly indicate any direct association with preterm birth especially PPROM. Similarly, not many early maternal age or late maternal age samples were available in cohort, so the correlation with maternal age could not be reliable and large sample size study is required. Maternal smoking and diabetes during pregnancy and their effects on whether they would increase the risk of preterm rupture of membrane or not and the possible pathophysiology has been studied and discussed in detail in our previous chapter (Chapter 5). This chapter has definitely helped in better understanding of pathology of PPROM and preterm birth.

Chapter 7

General discussion, conclusion and future study

7.1 Outline:

Preterm premature rupture of the membrane or PPROM is a leading cause of preterm birth. Preterm birth remains a major health care issue around the world. There are no effective treatment options available as yet. The main reason is felt due to poor understanding of the pathophysiology involved. Although few therapies for preterm labour may be partially effective, they are not robust and hence not globally practiced. Preventative measures are not effective as there is no infrastructure or the expertise to predict PPROM or preterm birth (Norman, et al., 2013). These have led to an interest to aim for better understanding of the mechanisms of preterm birth due to PPROM and offering improved treatment. Though aetiology of failure of FM leading to PPROM is yet to be demarcated it is broadly recognised as a mechanical process (Oyen et al., 2006). To define the mechanical properties of FM, a clear understanding of biomechanical responses of amnion and chorion is essential. There are also some proposed hypotheses on biochemical changes such as imbalanced collagen quantity in the FM, but to date there is minimal understanding of how the biochemical factors lead to early membrane rupture. Investigation of the biomechanical and biochemical effects on the FM of preterm tissues is the most crucial step to understand the pathology and to restore the membrane function following PPROM.

A number of risk factors are thought to have correlation with preterm labours or PPROMs. Although it is assumed that the risk factors interplay to trigger early membrane rupture by instigating systematic inflammation or by influencing to activate infection pathways (Goldenberg et al., 2000), these associated mechanisms have not been established in most cases. Identifying these risk factors and their pathways which may

potentiate the risk of PPROM will provide a better understanding of pathophysiology leading to preterm birth. Further on, defining these risk factors might be helpful for preterm labour management including specific treatment.

Towards the end of the gestation, some programmed metabolic events are normally taking place to disrupt the infrastructure of FM in preparation for the labour process and membrane rupture is fundamental part of normal full term birth (Pressman et al., 2002). When these series of events are accelerated for some reason it can cause rupture of membrane before term (Claire et al., 2007). FM present over cervix is known to alter morphology in normal gestation (Malak et al., 1994). During normal term labour, extreme stretching of membrane from increased pressure on this area might activate some cytokines in amniotic fluid which leads to decrease strength of FM (Kumar et al., 2006). However, in case of PPROM there is no specific ruptured site identified yet (Claire et al., 2007). Characterising the role of such pressure on biochemical changes in the preterm membranes leading to rupture will be helpful in better understanding of the rupture area and the mechanism involved.

Cigarette smoking during pregnancy is associated with increased incidence of premature birth. Numerous studies have approved the association of maternal smoking and PPROM (Shiono et al., 1986, Harger et al., 1990, Castle et al., 1999, Morken et al., 2005) but, a few studies which have not established any such association (England et al., 2013). The contradict opinions and unclear mechanism through which maternal smoking is correlated with preterm birth led to our study of FM on exposure to cigarette smoking and its associated biochemical changes. We hope that this study will establish a relationship between smoking and preterm birth.

Diabetes during pregnancy is another risk factor which is mainly known to associate with maternal and fetal morbidity and neonatal complications (Sharifi et al., 2010). The

association of the maternal diabetes, either pre-existing diabetic mellitus or gestational hyperglycaemia and preterm birth is controversial. The impact of diabetes during gestation on premature ruptured of membrane is not clear yet. Studying this association should provide an improved idea and better understanding whether high glucose level can influence the biochemical changes of FM or not.

Successful treatment of preterm birth is proving to be difficult due to its unclear pathophysiology. Few treatment options are available but, they have failed to show successful outcome (Norman et al., 2012). Generally, therapeutic intervention involves either by delaying the delivery or by improving fetal status before pre labour delivery (Goldenberg et al., 2002). Dexamethasone, a synthetic corticosteroid is widely used as perinatal and neonatal prescribed medicine. However, there are conflicting reports on the efficacy and safety of this drug (Cummings et al., 1989, Mormile et al., 2016). Nifedipine, a calcium channel blocker, is another possible therapeutic option thought to prolong the labour. Limited studies of its effectiveness and conflicting data about the efficacy of calcium channel blocker on preterm rupture of FM has necessitated the need for further exploration.

This thesis aimed to contribute on better understanding and fill the knowledge gap of mechanism of preterm birth and main focus was PPROM. The mechanical characterisations of FM were studied uniquely via new test methods. A consistent outcome from mechanical testing exhibited variations in deformation behaviour and stiffness between membranes from full term and pre term deliveries and between two different areas of same membrane. The effects of biochemical factors such as MMP9, MMP13, IL-1β, Fibronectin, progesterone receptor-A&B on fetal tissue has been compared between membranes from preterm to full term and from different sites within the membranes. Overall outcomes from both biomechanical and biochemical

experiments has drawn a correlation between them. In the new experimental model, physical force stimulating active biomarkers in FM were demonstrated. Later on, a relationship between the risk factors and preterm birth has been discussed in detail. The effect of risk factors like maternal smoking and diabetes during pregnancy was evaluated in vitro and their association with changes in key biochemical molecule expression in the presence of external force on the membrane was noted via our experimental model. The insight into the behaviours of FM from different areas and in different situations was obtained which can contribute in building up the present knowledge of PPROM pathology with an aim to prevent preterm birth.

7.2 Mechanical characterisation of fetal membrane by the two new mechanical tests:

Researches have been searching for decades to find out the cause of preterm rupture of human FM with the aim to reduce preterm birth and related complications. Number of factors has been proposed to involve and play a role in the mechanism of PPROM. Rupture of FM is a unique process and historically biomechanical factor was thought to be responsible for the mechanism. In the past, different types of mechanical test methods have been used to characterise the mechanical properties of the Human FM. However, all the methods had some disadvantages in that they either failed to mimic the in vivo environment or the proposed method was complicated to operate. We utilised a method, ball indentation which has been used for the first time to study human FM's mechanical creep and modulus properties. Ball indentation method is simple, flexible, and can mimic the in vivo environment. Membrane stiffness was studied by another newly utilised technique called Optical Coherence Elastography (OCE). Sadly, there is no proven efficient screening test available at present for PPROM (Nunes et al., 2016). The OCE method in our study indicates that it could be a useful imaging technology for PPROM.

Mechanical characterisation was performed with full term and preterm FM samples from normal vaginal deliveries. Intact membranes along with separated membranes-amnion and chorion were tested. Ball indentation test result revealed lesser deformation and higher modulus in amnion than chorion, which reconfirmed the previously demonstrated findings. Difference in creeping and stiffness was noticed in full term and preterm membranes. Malak et al reported that an excessive and constant morphological alteration of FM took place towards the end of gestation. This could happen in different ways such as basement membrane degradation of the amnion, swelling and shrinking of the trophoblast or membrane thinning all together. This alteration is known as 'zone alter morphology-ZAM' (Malak et al., 1994). El Khwad reported that the strength of membrane in ZAM region is lower in the areas overlying the cervix than in the other areas of the FM rendering them more susceptible for easy rupture. They have also found that the areas of weak zone were observed adjacent to the tear line in full term spontaneously ruptured fetal membranes (El Khwad et al., 2006). Our mechanical test result demonstrated ruptured sites or the membrane sites adjacent to the tear line had higher deformation than the non-ruptured sites or far from tear zone areas, in normal vaginal delivery membranes which seems to be consistent with the previous results. Further investigation on creep and stiffness behaviour with vaginally delivered PPROM membranes showed similar difference between both the sites of FM. So far, there has been no clear information about rupture area of PPROM yet. Both our mechanical test results have suggested strongly that the area overlying the cervix was vulnerable for early rupture in case of PPROM deliveries as this area is mechanically weaker than other areas. The most consistent observation in these test results was the finding of significant difference in mechanical behaviour between ruptured and non-ruptured areas of the same membrane in case of PPROM in comparison to full term membranes. There was a difference in creeping (15%) and stiffness (33%) seen at both the sites in full term membranes as well;

however, in preterm membranes this difference was much higher than their full term counterparts. The OCE results clearly confirmed that the stiffness near non-ruptured sites of preterm membranes were significantly higher than the non-ruptured sites of the full term membranes. We know that the membrane gets weaker with advancing gestational age and our results in full term FM samples seem to be consistent with that. The higher stiffness and lower deformation of non-ruptured sites in preterm membranes also mimics the modulation of membrane, expected at the preterm gestational age. We hypothesize that there were some changes which caused alteration in the normal scheduled membrane activity leading to FM weakness in preterm. This untimely rupture was then easily facilitated during PPROM. Notably, none of the other studies have reported this big difference in mechanical behaviour over various sites in a preterm membrane before.

Thickness measurement of intact membrane samples studied by OCT revealed that the FM of preterm deliveries was thicker than the membranes from full term deliveries. Our data supported the previously done ultrasound measurement study (Severi et al., 2008). The OCT data was correlated with our other two mechanical test outcomes. Perhaps, this gave further support to our explanation that the preterm rupture due to premature local events causes shortening of the gestational age.

7.3 Correlation of biochemical and biomechanical factors in FM rupture:

Furthermore, our study demonstrated a clear association between matrix degradation proteins, apoptosis and inflammation markers and hormone receptors with untimely membrane rupture. For a long time, mechanical stress was believed to be the only responsible factor for rupture of FM in case of delivery with labour. However, the ratio of PPROM (40%) was reported to be much higher compared to the PROM ratio (10%), which made obvious that other factors are also involved in the rupture mechanism (Perry et al., 1998., Joyce 2010). Our mechanical tests results also clearly indicated the same. In term

pregnancy, membranes' weakness is a vital part and it generally goes through several programmed biochemical events (Moore et al., 2006). The microstructural changes such as disorientation of collagen fibres in term membranes in the regions overlying the cervical areas (Malak et al., 1994), degradation of collagen type I in Rat FM during end of the gestation (Joyce et al., 2010), decreased level of total collagen and type III collagen in the ruptured membranes at the end of gestation (Rangaswamy, 2012) were reported as programmed fundamental actions for mechanical weakness of the tissue in the end of the pregnancy to prepare labour. We characterised alignment of collagen fiber by histology method, total collagen concentration via biochemical assay and collagen type I and III level by immunochemistry method. The results were found to corroborate previous studies. Non-ruptured site of PPROM membrane showed better collagen content than full term non-ruptured FMs. Collagen fibres within the FM during and towards the end of labour gestation undergo progressive remodelling and disorientation which leads to high deformation and low stiffness of the FMs. Thus, we found a positive correlation between tissue microstructure and mechanical properties of FMs.

Our study is one of the few studies which have investigated GAG on PPROM. To the best of our knowledge this is the first time it is associated with mechanical properties of FM. Significant difference of GAG levels in the full term and preterm membranes were observed and they also confirmed positive correlation by histology and biochemical assay analysis. GAGs are known to play a role in modulating the bindings of collagen fibres (Rigozzi et al., 2013) which means that it possibly controls the disorientation of collagen fibrils during gestation. So, higher GAG levels in preterm non-ruptured sites correspond to higher collagen level and higher mechanical strength in those areas. Decorin and biglycan are two key regulators for preserving collagen matrix by retaining collagen fibres' alignment and realignment and both are bonded with collagen-I (Robinson et al., 2017). So, change in decorin and biglycan levels in FM, in our study, indicates disorganization of

collagen distribution, low collagen I levels and hence low integrity of extracellular matrix. Our results also further supports other studies who have reported that this alternation between decorin and biglycan takes place in the interphase of amnion and choriodecidua and perhaps they are responsible for membrane detachment and mechanical weakness of FMs (Meinert et al.,2007) which then leads to membrane rupture in normal full term delivery (Strohl et al.,2010). Thus, our study suggests that preterm FM rupture is likely due to the lower content of sGAG, changes in levels of proteoglycans and disruption of collagen networks.

We also noted lower expression of MMPs (MMP 9 & 13) in preterm non-ruptured sites than full term non-ruptured sites. MMP expression at the ruptured sites in both the membranes (preterm and full term) showed similar expression but on comparison, it was higher than their non-ruptured sites. MMPs are known to involve in degradation of collagen structure of FM matrix (Fortunato et al., 2007) and collagen-I degradation are potentially related with MMP9 (Matrisian et al., 1992). The elevated level of MMP 9 in ruptured site is consistent with previous studies and is consistent with the programmed biochemical change for membrane weakness in full term membranes (Bell et al., 1997, Joyce et al., 2010) and preterm membranes (Draper et al., 2001, Yenomoto et al.,2006). Both these studies have not investigated the gap between full term and preterm non-ruptured sites though. There was similar expression of MMP 13 in our study. There are limited studies which have investigated participation of MMP 13 in PPROM. An elevated level of MMP 13 was found in vaginal fluid of PPROM patients (Soydinc et al., 2013). This is consistent with our data; however, they have not studied the membranes.

Fetal fibronectin is one of the known biomarkers for preterm delivery. Fetal fibronectin is located all over the FM and is associated with maintaining contact between the placenta and uterus. It plays a role in structural support of FM (Lockwood et al., 1991).Studies have

shown fetal fibronectin can activate and upregulate the levels of MMP9 (Okamura et al., 2001, Mogami et al., 2013). Our results on fibronectin expressions have supported that and higher expression of it in ruptured sites of preterm than non-ruptured sites has clarified that over expression of Fn can upregulate the MMPs activation and alter the homeostasis of membrane matrix by destructing the collagen bindings. Therefore, we can explain that fetal fibronectin plays a critical role in membrane weakness and rupture.

IL-1β is a pro-inflammatory cytokine found to express in both full term and preterm samples. In our study, the collected samples were free of infection. So, we believe this biomarker has expressed through the alternative pathway when activated through apoptosis. Previous study already has revealed that apoptosis can promote the proteolytic activities (Menon et al., 2011). Another study has confirmed that IL-1β is a great inducer of apoptosis and when it is present in amniotic fluid it can lead to apoptotic cell death of fetal membranes (Fortunato et al., 2003). The association of apoptosis and preterm membrane rupture is well established by many studies (Sagol et al., 2002, Murtha et al., 2002, George et al., 2008, Saglam et al., 2013). Our result is consistent with those findings. However, the minimal expression in non-ruptured sites of PPROM membranes suggest that the increased expression of IL-1β near ruptured sites was induced locally and full tem membranes showed similar expression in both the sites thus suggesting that it was programmed event for full term deliveries.

Progesterone hormone plays a vital role in regulating human pregnancy (Gordon et al., 2000) mediated by specific progesterone receptors. PR-A and PR-B are two proteins expressed by it (Conneely et al., 2002). There is a proven role of progesterone in prevention of preterm birth (Mackenzie et al., 2006) but, very few studies have investigated PR role in preterm labour pathology and for the first time, we have investigated the PR expression in different sites of PPROM and full term membranes.

Progesterone has been noted to diminish the IL-1β effect on placental cell death (Zachariades et al., 2012) and to suppress MMP activities (Sato et al., 1991). In our study, increased expression of PR in non-ruptured sites of preterm membranes than in the ruptured sites was noted. This suggests that because of the declining level of progesterone there was an associated higher expression of IL-1β and higher level of MMP-9 which linked with higher deformation and lower stiffness of the fetal membranes. Our research noted a clear biochemical and biomechanical correlation which might interplay and their untimely interaction could be the potential source of PPROM.

7.4 Understanding the relationship between different risk factors and PPROM:

Furthermore, this study from the case control data has explored some risk factors which could be linked with early FM rupture. Cigarette smoking during pregnancy showed strong association with increased risk of PPROM. Further on, collagen assay result revealed low concentration of collagen quantity in smoker samples compared to the non-smoker mother samples which indicated direct association with membrane weakness. Cigarette smoking is known to be related with inflammatory responses in healthy human being (Chrug et al., 2003) even in the absence of inflammation. Apoptosis rate was found to increase in placental membrane due to cigarette smoking (Menon et al., 2011). The mechanical tests on FM samples from elective caesarean sections revealed that the ruptured sites of these membranes are tougher than the ruptured sites of vaginal normal delivery membranes. Both were full term membranes. It is understandable that the labour process has major effect on mechanical weakness of FM. Correlation between early rupture of the membrane with previous CS delivery has controversial opinion. Our study may not directly address that, but, we can say that mode of delivery has potential influence on membrane weaknesses and ruptures. Higher deformation of membrane was noticed in our study in higher temperature than the normal body temperature.

Temperature is generally connected with infection or inflammation. A direct or indirect correlation of body heat on membrane weakness seemed to be an interesting point to investigate. According to our case-control study, increased risk of PPROM possibly corroborated with previous history of preterm rupture of membranes. Our research also noted that mothers between 30 to 35 years of age got less chance to have premature delivery. There are conflict opinions in literature about maternal age and preterm birth correlation. But, increased risk of preterm birth associated with older age is well established (Fraser et al., 1995, Hediger et al., 1997, Fuchs et al.,2018, Hassan et al., 2009). But that relation is not direct in most of the cases and other complications which generally arise in older age could contribute. We have not noticed any significant correlation with diabetes. However, increased expression of IL6 in the FM from diabetic mothers compared to the non-diabetic mothers hinted that there could be some indirect association. Most of the risk factors we researched showed potential risk to early membrane rupture or preterm birth as its own or in correlation with other factors. The limitation of this study is the small number of each category group. More research is required to confirm these relationships and the risk factors as potential contributors of PPROM pathophysiology gained from our study.

7.5 Abnormal biochemical expression of FM by external mechanical stimulation:

Expressions of biomarkers in the intact FM samples have shown to enhance by the effect of repetitive mechanical force in vitro. To investigate the substantial dissimilar behaviours of two different sites of preterm membranes, we successfully developed an *in vitro* set up. Non-ruptured sites of membranes from elective section deliveries both full term and preterm were exposed to repetitive force. Higher expression of MMP 9, MMP 13, IL-1β, fibronectin and lower expression of PR in FMs as a result of particular force compared to the membranes kept static in the incubator were found. Preterm FM samples were more

sensitive to external force than full term samples. Hence, suggesting that, in situ external stimulation induces the biochemical activation before term which possibly initiates preterm weakening of the FM leading to PPROM. Mechanical force is known to be effective on alternation of collagen orientation and declining collagen content (Perinni et al., 2015, Chowdhury et al., 2014). Our finding further reinforced that there is a strong correlation between the force originated in the womb acting externally on the FM and incidence of preterm membrane rupture. The other possible factors that could govern the sensitivity of preterm membrane could be presence of low amniotic fluid and bigger fetus. Further on, this study has revealed that calcium channel blocker (nifedipine) could be a potential treatment option in early membrane rupture. We have proven that MMP 9 and IL1β expressions were inhibited by calcium channel blocker even in the presence of mechanical force. Additionally, it has indicated that the calcium signalling pathway is the possible mechanism through which external force works. Calcium influx is activated by external stimuli and this targets the downstream of biomarkers and upregulates the biochemical activities in FM prior to term (Figure 5.17).

We further studied two other risk factors, maternal smoking and diabetes during pregnancy in detail. In vitro exposure of nicotine solution elevated the expression of MMP 9 and IL-1β and this has confirmed the association with increased risk of PPROM. The biomarkers showing higher expression with the higher concentrations of nicotine solution denoted an increasing risk with increase in number of inhaled cigarettes. Finally, when external force was added to the samples in nicotine solution, the in vitro study results revealed that smoking along with influence of external force might be the one of the greatest risk for membrane rupture prematurely. Figure below can explain it in a better way.

When membranes were exposed to the glucose solution and compared to the membranes that were incubated without any glucose solution, it was noted that higher glucose level during pregnancy has not shown any significant association with FM weakness. Case control studies have reported the possible correlation of diabetes with preterm birth and neonatal complications. But, that is most likely related to high BMI which is triggered by hyperglycaemia during pregnancy or a pre-existing condition. In present study, MMP 9 and IL-1β were found to Increase their expression once they were exposed to the mechanical force along with glucose solution. Thus, maternal diabetes singlehandedly may not be associated with risk of membrane rupture prematurely but could be incorporated with any other factors especially with the mechanical stimulation perhaps to increase the risk of PPROM.

Reduced expression of MMP 9 and IL-1β in the samples after exposure to the dexamethasone solution, confirmed the potential therapeutic role for prevention of early membrane ruptures along with the prolonging the preterm birth. After loading and mechanical stimulation with dexamethasone solution also exhibited lower biochemical expressions than the membranes that were loaded without dexamethasone solution indicating that it has capability to uphold the membrane strength by diminishing the biochemical activities. Current understanding from the present research with dexamethasone and calcium channel blocker can put insights to the future therapy of PPROM.

7.6 Conclusion:

The epidemiology of preterm birth associated with PPROM and its related complications are well defined, but the underlying mechanism is poorly understood. Numerous factors have been proposed and there are ongoing studies to uncover the aetiology behind PPROM. However, the combined efforts so far have failed to produce a clear

pathophysiology. PPROM aetiology is multifactorial. In this project, biomechanical, biochemical and risk factors associated with premature FM rupture have been assessed and studied in comparison with the full term delivery membranes. Some new insights into the involvement of these factors in PPROM aetiology have been generated.

Two techniques, the ball indentation test and OCE were explored and used for the first time to study the mechanical behaviours of FM. The ball indentation test not only resulted in a better understanding of the change of mechanical behaviour of FM in different layers and areas from the same membrane sample, but also overcome some of the operational difficulties associated with previous techniques. The OCE device showed heterogenic biomechanical changes within the membrane between ruptured and non-ruptured sites. The OCE device could be identified as a potential screening tool. Preterm membrane had a significant heterogenic difference in mechanical property between ruptured and non-ruptured sites (creep property 36%, modulus 55% and thickness 48%) in comparison to its full term counterpart (creep property 15%, modulus 33% and thickness 23%), indicating that a weak zone existed in preterm membrane ahead of the programmed rupture time.

The FM samples from PPROM also revealed dissimilar microstructural and histological configuration. A disorganised collagen network and lower concentration of collagen I and III were observed in the tear zone sites in comparison to sites near the placenta which were consistent with membranes deformation, thickness and elasticity results. The outcomes of sGAG quantity and the expression of decorin and biglycan suggested that in PPROM membrane, there was loss of collagen preservation and decreased retaining attachment capacity of two layers-amnion and chorion till the term. The influence of MMP 9, MMP 13, IL-1β, and fibronectin along with progesterone receptor on membranes weakness was noticed. A direct correlation has been found for the first time between

mechanical and biochemical changes in both the sites of the PPROM membranes. A greater heterogeneity in two sites of PPROM membranes (MMP 9-57%, MMP 13-74%, Fn-53% and IL-1β-97%) than the two sites of full term membranes (MMP 9-23%, MMP 13-19%, Fn-29% and IL-1β-14%) has been demonstrated.

We were able to successfully establish a new *in vitro* study model which observed biochemical changes within the intact preterm membranes in response to external forces and revealed enhanced expression. Preterm membranes were found to be more sensitive to external force leading to expression of membrane weakening molecules which trigger the premature membrane rupture.

The *in vitro* culture study system (*ex vivo* models) provided a convenient tool to study the association of certain risk factors with increased risk of preterm deliveries. The ex vivo study of the two risk factors such as smoking and diabetes has revealed that nicotine is one of the largest contributors of FM weakness and with force stimulation presented even more risk while high glucose does not seem to be a direct threat to FM rupture although indirectly or with the presence of external force it could be a potential risk. Overall, the findings have contributed to the current understanding of PPROM mechanism and hopefully will lead to future research.

The outcomes from this research can guide future studies and aid to develop new clinical approaches. Techniques like the OCE method could explore the mechanical characterisation further and this device could be useful as a screening tool for preventing PPROM. Early detection techniques are limited in PPROM. The proven correlation between biochemical factors with mechanical factors and their interplay to cause membrane weakness found from this study can explain that fibronectin, Progesterone receptors and proteoglycans could all be attractive options to explore. The treatment options for PPROM are not promising yet. Progesterone hormone therapy may be helpful

as it has showed suppression ability of MMPs and inflammatory marker activities in this study. The results of dexamethasone and nifedipine could also be considered as good potential future treatment options for PPROM.

7.7 Future study:

For future study, further confirmation of the heterogeneity of preterm membrane with a larger sample size is needed. Studying more biomarkers would be useful in investigating the correlation between biomechanical and biochemical properties.

Exploring the *in vitro* model at the cellular level and study the genetic factors to find out the reason of the sensitivity of the preterm membranes in the response to external force would be beneficial. More investigation is needed on fetal movement force responses. Therefore, the investigation of FM biomechanics and mechanotransduction (e.g. multiple targets, FM models, animal versus in vitro studies, mechanisms, cell/tissue mechanics in the different layers and cell types) to improve understanding and clinical performance with a combined pharmacological and bioengineering approach will be explored in future.

Although dexamethasone and nifedipine are shown to prolong preterm birth by downregulating biochemical factors, there is room for further research on this aspect. The aim of this study was to investigate the role of external force in triggering different biochemical expression and the effectiveness of these drugs on the activation of these biochemical syntheses. We only used clinically relevant dose in this study, but not dose-response effects. Investigation of the cellular and inflammatory pathways and their interactions with the pharmacological agents at dose-dependent manner with our loading system can be our future study.

Due to time limitations and difficulty to get PPROM samples regularly, the sample size was small to explain the risk factors clearly. However, the chapter 6 data gives good

foundation for future prospective and provides a beneficial detailed study of these risk factors. More samples should be studied in future work.

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Appendix

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13 October 2014

Dr Pensee Wu
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Keele University
University Hospital of North Staffordshire, Maternity Centre
Newcastle Road, Hartshill
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ST4 6QG

Dear Dr Wu

Study Title:	**Preterm Premature Rupture of Membranes: Strength and structure of the fetal membrane**
REC reference:	**14/WM/1169**
Protocol number:	**N/A**
IRAS project ID:	**147559**

The Research Ethics Committee reviewed the above application at the meeting held on 08 October 2014. Thank you for attending to discuss the application.

Provisional opinion

The Committee is unable to give an ethical opinion on the basis of the information and documentation received so far. Before confirming its opinion, the Committee requests that you provide the further information set out below.

Authority to consider your response and to confirm the Committee's final opinion has been delegated to the Chair.

Further information or clarification required

1. Please change sentence five of the 'Aims of the Research' section of the Patient Information Sheet to read 'We believe that women with PPROM may have weaker and structurally abnormal membranes.'

2. Please change the last sentence of the 'If I take part, what do I have to do' section of the Patient Information Sheet to read 'We will test whether the swab contains bacteria linked to pre-term labour or infection.'

3. Please rewrite the Patient Information Sheet to use more lay-friendly language and to include the revisions in points 1 and 2.

If you would find it helpful to discuss any of the matters raised above or seek further clarification from a member of the Committee, you are welcome to contact the REC Manager at nrescommittee.westmidlands-solihull@nhs.net.

When submitting a response to the Committee, the requested information should be electronically submitted from IRAS. A step-by-step guide on submitting your response to the REC provisional opinion is available on the HRA website using the following link: http://www.hra.nhs.uk/nhs-research-ethics-committee-rec-submitting-response-provisional-opinion/

Please submit revised documentation where appropriate underlining or otherwise highlighting the changes which have been made and giving revised version numbers and dates. You do not have to make any changes to the REC application form unless you have been specifically requested to do so by the REC.

The Committee will confirm the final ethical opinion within a maximum of 60 days from the date of initial receipt of the application, excluding the time taken by you to respond fully to the above points. A response should be submitted by no later than 12 November 2014.

Summary of the discussion at the meeting

Social or scientific value; scientific design and conduct of the study

The Committee asked whether in fact the study is a pilot due to the sample size.

The applicants confirmed that 120 participants were needed due to the complexity of the study: 20 for the study group and 20 for each of 5 control groups.

Care and protection of research participants; respect for potential and enrolled participants' welfare and dignity

The Committee asked about the process for advising participants of any incidental findings resulting from their participation in the study.

The applicants confirmed that should there be any clinically relevant findings these will be reported to the participant and the participant's GP. The participant will be invited to see Dr Wu, a consultant obstetrician, as any findings may be relevant to a future pregnancy.

Informed consent process and the adequacy and completeness of participant information

The Committee noted that the Participant Information Sheet will be given to the potential participant when they are in labour and enquired whether this could be done at an earlier stage.

The applicants explained women who would be eligible for this study would probably not be in a lot of pain when admitted but suggested that women who are likely to be eligible could be targeted at an earlier stage in their care. It was also suggested that information on the study could be included in the pack which is sent to all women ante-natally.

The applicants said that the ladies' midwives would be consulted for an opinion prior to any approach being made. Only women who are not in distress would be spoken to.

Alternatively, it may be possible to approach women who have already given birth.

The applicants acknowledged that this is a very sensitive time for women and recruitment will be flexible for this reason and suggested that women may be interested in taking part if it is a problem which is likely to recur.

Suitability of supporting information

The Committee asked for sentence five of the 'Aims of the Research' section of the Patient Information Sheet to be changed to read 'We believe that women with PPROM may have weaker and structurally abnormal membranes.'

The applicants agreed.

The Committee asked for the last sentence of the 'If I take part, what do I have to do' section of the Patient Information Sheet to be changed to read 'We will test whether the swab contains bacteria linked to pre-term labour or infection.'

The applicants agreed.

The Committee requested that the Patient Information Sheet be simplified and rewritten in more lay-friendly language. This is particularly important given the circumstances in which some women will be expected to read it.

The applicants agreed.

The Committee asked whether participants could opt out of being contacted regarding future research as noted on the Consent Form.

The applicants confirmed that participants were free to opt out of any part of the Consent Form by not ticking the relevant section.

Documents reviewed

The documents reviewed at the meeting were:

Document	*Version*	*Date*
RAS Checklist XML [Checklist_09092014]		09 September 2014
Participant consent form	Control group	

Participant consent form	Study group	
Participant information sheet (PIS)	Control group	
Participant information sheet (PIS)	Study group	
REC Application Form [REC_Form_09092014]		09 September 2014
Referee's report or other scientific critique report		19 August 2014
Referee's report or other scientific critique report [Scientific critique amendment]		03 September 2014
Research protocol or project proposal		
Summary CV for Chief Investigator (CI)		19 August 2014

Membership of the Committee

The members of the Committee who were present at the meeting are listed on the attached sheet

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

14/WM/1169	**Please quote this number on all correspondence**

Yours sincerely

pp Dr Rex J Polson
Chair

Email: nrescommittee.westmidlands-solihull@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments.*

Copy to: *Dr Darren Clement*