Doctoral Thesis

Mechanical characterization and modeling of human fetal membrane tissue

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Mechanical characterization and modeling of human fetal membrane tissue

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presented by

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Abstract

The bilayer fetal membrane (FM), composed of amnion and chorion, surrounds and protects the developing fetus during pregnancy. Rupture of the FM is part of normal term delivery but has serious complication when it happens prior to term. The spontaneous preterm premature rupture of the membrane (PPROM) is associated with 30% to 40% of all preterm birth and related to high mortality and morbidity of the newborn. Moreover, minimally invasive fetoscopy has become a therapeutic option for the treatment of severe or live-threatening birth defects on the fetus inside the pregnant uterus. However, the potentially beneficial prenatal interventions are limited by the high frequency of subsequent iatrogenic preterm rupture (iPPROM). The present work is dedicated to a better understanding of the mechanical properties of human fetal membrane tissue. The main focus is on the mechanical characterization of the tissue, i.e. measurements and model formulations, under consideration of particular aspects of its microstructural composition.

Several experimental studies were conducted for the characterization of the mechanical response of intact FM tissue and the separate amnion and chorion layers under uniaxial and equibiaxial loading. In addition, the contents of microstructural constituents of the same membranes were determined by the use of biochemical assays. Combination of these results allowed investigating the correlations between parameters characterizing the mechanical response under close to physiologic conditions and parameters quantifying the microstructural constituents: There is a relation between membrane’s strength as well as stiffness at large deformations and the collagen content as well as its cross-linking. The results also revealed large differences between the uniaxial and equibiaxial mechanical response, i.e. the FM tissue is stiffer and less extensible under equibiaxial loading. Moreover, the results showed that amnion is stiffer and stronger than chorion, caused by its twofold collagen content, and can therefore be considered the mechanically dominant layer of the FM.

A further study investigating the separate layer behavior under uniaxial loading with particular focus on the kinematic response revealed amnion’s unique and highly reproducible
in-plane contraction behavior. Maximum values of incremental Poisson’s ratio in the range of 5 to 8 were calculated for these tests, which are higher than any previously reported value for biological or synthetic materials. This behavior was attributed to mechanisms of fiber reorientation, stretching, and buckling of the underlying collagen network. These findings allowed the formulation of a transversely isotropic constitutive model, which includes microstructural information. Representation of the averaged uniaxial response and model predictions for other loading conditions were found to be excellent. Implementation of this model in a commercial finite element code made it available for numerical simulations.

Failure of FM tissue was mainly investigated in terms of the rupture sequence. Observations in tensile tests on intact FM showed separation of amnion and chorion during uniaxial extension. This separation is expected to happen at the interface layer and is caused by a difference in the layer specific Poisson’s effect. Observations in inflation experiments showed that in most cases failure of the FM tissue is characterized by amnion rupturing first. However, this was in contrast to observations reported in the literature based on puncture testing and motivated the development of an in-house puncture test setup as well as a general analysis of this test method in terms of its application to FM characterization. Tests performed with different clampings as well as results from numerical simulations showed that the ex vivo rupture sequence of FM is mainly a characteristic of the clamping type. Besides that, puncture testing allows simple and fast determination of membrane strength if the prerequisites on suitable sample fixation as well as adequate ratios of plunger to clamping diameter are fulfilled.

Membrane puncturing for diagnostic or surgical reasons leads to local defects in the tissue. Ex vivo tests were performed for the qualification of a new hydrogel glue, denoted as “mussel glue”, as a possible sealant for membrane defects. Tests on elastomeric and fetal membranes as well as the mechanical characterization of the glue point to mussel glue’s suitability for membrane repair. Analytical and numerical investigations of the stress redistributions around circular holes revealed large stress concentration factors even for small deformations. Such defects could be repaired by the application of a mussel glue patch on the amnion side, which effectively reduces the stress concentrations.

The insights gained within this thesis contribute to current research and enable future developments of methods to prevent preterm rupture after fetoscopic interventions, and thus reducing the risk related to iPPROM.
Zusammenfassung


Die gewonnenen Erkenntnisse dieser Arbeit tragen zur aktuellen Forschung bei und ermöglichen die zukünftige Entwicklung von Methoden zur Vorbeugung eines vorzeitigen Blasensprungs nach fetoskopischen Eingriffen und daher eine Reduzierung des Risikos verbunden mit iPPROM.
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Chapter 1

Introduction

1.1 Preterm rupture of the fetal membranes

The fetal membrane (FM) is the membranous structure that surrounds and protects the developing fetus during gestation. It is composed of two layers, called amnion and chorion. During pregnancy the membrane deforms as a consequence of internal pressure as well as fetal movements. The rupture of the FM is an integral part of term delivery but has serious complications when it happens prior to term. The premature rupture of the fetal membrane (PROM) is defined as the rupture of the membrane before the onset of labor. However, it is followed in almost all cases by spontaneous or induced labor and is therefore considered a variant of normal delivery (Calvin and Oyen, 2007). Premature rupture before the 37th week of gestation is usually referred to as preterm premature rupture of the membranes (PPROM) (Parry and Strauss, 1998). In 2010, approximately 15 million babies were born preterm, which corresponds to 11% of all live births worldwide, ranging from about 5% in several European countries to 18% in some African countries (Blencowe et al., 2012). The complications related to preterm birth are severe and are estimated to be responsible for 35% of the world’s 3.1 million annual neonatal deaths. Preterm birth complications do not only contribute to mortality but also to lifelong effects on neurodevelopmental functioning such as increased risk of cerebral palsy, impaired learning, and visual disorders as well as an increased risk of chronic disease in adulthood (Blencowe et al., 2012). The preterm rupture of the membrane (PPROM) is associated with 30% to 40% of all preterm births (Parry and Strauss, 1998; Mercer, 2003). Several clinical risk factors for the premature rupture of the FM (term and preterm) are well-documented. These include population based epidemiological factors such as low socioeconomic status, low body mass index, previous preterm birth and others as well as factors with obvious mechanical implications like cervical insufficiency and
amniotic fluid abnormalities (Calvin and Oyen, 2007). Cervical insufficiency is related to the problem that for some women the cervix opens prior to contractions, which leads to a loss of the pregnancy. Also nonphysiologic conditions in the uterus like excessive amniotic fluid pressure (polyhydramnios) can cause significant stretching of the membrane, thus increasing the risk of failure (Calvin and Oyen, 2007). Also acute inflammation was shown to be related to PROM (Moore et al., 2006).

Traditionally, membrane rupture was thought to be a consequence of stresses due to contractions only. However, in 8% to 10% of term pregnancies and up to 40% of preterm deliveries, rupture of the membrane precedes contractions (Parry and Strauss, 1998). Thus, it is obvious that rupture cannot be a consequence of physical stresses only. Failure of biological tissues such as bone, skin, or ligaments is a pathological process. Failure of these tissues is typically caused by weakening of the tissue due to diseases, infections or other medical conditions as well as by high force impact or stress. On the contrary, rupture of the FM is a unique event in human physiology. Recent research shows that FM rupture is related to a programmed, biochemically mediated weakening process (Joyce, 2009). Fetal membranes rupturing spontaneously at term show a zone of altered morphology (ZAM), characterized by marked swelling and disruption of the fibrillar collagen network of the compact, fibroblast, and spongy layer (Malak and Bell, 1994). The same region was also confirmed to be present in term membranes prior to contractions (McLaren et al., 1999a) and was characterized by increased matrix metalloproteinase activity, which is the main mediator for extracellular matrix degradation (McLaren et al., 2000). The supracervical region of the FM was also shown to exhibit increased cellular apoptosis at term (McLaren et al., 1999b; Reti et al., 2007). So far, El Khwad et al. (2005) were the first and only ones who demonstrated that the biochemical changes are related to physical weakening of the FM, characterized by reduction of strength and deformation capacity in the ZAM. Moreover, the discrete zone of weakness, present in term prelabour FMs, has characteristics consistent with tissue remodeling and apoptosis. These recent findings lead to the view that premature rupture, term or preterm, represents an acceleration or exaggeration of the mechanisms leading to spontaneous rupture during labor (Parry and Strauss, 1998).
1.2 Mechanical characteristics of fetal membrane tissue

In the last decades many experiments were conducted to understand FM mechanics and failure and its relation to membranes structure and morphology (Moore et al., 2006; El Khwad et al., 2005). Some studies were aimed at the determination of the rupture strength to get insight into the mechanisms leading to PROM. However, Artal et al. (1976) did not find a difference in the rupture strength of prematurely and non-prematurely ruptured membranes. The studies of Lavery and Miller (1979); Al-Zaid et al. (1980a); Oyen et al. (2004b) compared the rupture properties of term membranes with those of preterm ruptured membranes and found that preterm membranes have a higher strength. Differences in the rupture properties between vaginally delivered membranes and membranes from cesarean sections were analyzed by Lavery et al. (1982) and Oyen et al. (2004b) and it was shown that contractions or the process of vaginal delivery lead to a weakening of the membrane. Other studies were performed in order to determine the different mechanical properties of amnion and chorion, where Oxlund et al. (1990) found that amnion is stronger than chorion but less extensible and therefore breaks first.

Three types of mechanical test setups were used for these purposes: (i) uniaxial tensile testing (Oxlund et al., 1990; Helmig et al., 1993; Jabareen et al., 2009), which is rather simple to perform and to analyze but does not represent the physiological conditions of loading, (ii) puncture testing (Schober et al. (1994b); Arikat et al. (2006)), where a spherical metal probe is used to deflect the clamped circular membrane specimen, and (iii) inflation or burst testing (Polishuk et al., 1962; Lavery and Miller, 1979; Al-Zaid et al., 1980a; Wittenberg, 2011), where a circular membrane is deformed with the aid of pressurized water or air. Puncture testing allows characterizing a large number of samples in each FM, but the local force application leads to a state of deformation that differs from the in vivo loading condition. Nevertheless, Schober et al. (1994b) have shown that results obtained by puncture testing can be related to results from inflation tests when tests with different ratios of plunger to clamping size are performed. Membrane inflation best mimics the physiological loading situation and the in vivo mechanical deformation. Burst tests were accomplished to acquire data on rupture properties of FM such as burst pressure (MacLachlan, 1965) or elevation at rupture (Parry-Jones and Priya, 1976). Most investigations provide system parameters (e.g. burst pressure, which depend on sample geometry) instead of specific properties of FM. Fewer studies also determined material specific rupture properties such as critical membrane tension or critical stress (Lavery
Chapter 1. Introduction

and Miller, 1979; Lavery et al., 1982; Polishuk et al., 1962). Schober et al. (1994a) pro-
vided also information on the deformation behavior in inflation experiments, in that they
calculated the slope (tangent stiffness) at the end of the pressure-elevation curve. Apart
from the foregoing mentioned three types of mechanical testing, planar biaxial testing is a
further common method for multiaxial characterization of materials. Joyce (2009) has re-
cently applied it to fetal membranes and determined nonlinear stress-strain curves as well
as the maximum tangent modulus and the maximum membrane tension. Surprisingly,
despite all the research that has been done in the past decades, the component of the FM
that ruptures first in mechanical tests is not consistently documented in the literature.
Different test configurations lead to contradicting results. For example Artal et al. (1976)
(uniaxial), Lavery and Miller (1979) (inflation), and Arikat et al. (2006) (puncture) state
that chorion ruptures first, whereas Helmig et al. (1993) (uniaxial), and Schober et al.
(1994b) (puncture) found that amnion ruptures first.

Also the time and history dependence of fetal membrane’s deformation behavior was an-
alyzed. Lavery and Miller (1979) performed creep tests and Oyen et al. (2004a, 2005)
performed uniaxial relaxation tests. Moreover, Oyen et al. (2006) have shown that am-
nion is more sensitive to chemical and mechanical changes that occur during gestation.
The mechanical properties of FM after cyclic loading were investigated by several stud-
ies. Toppozada et al. (1970) demonstrated that repeated ex vivo stretching progressively
weakens the membrane, which is in agreement to clinical observations that the rupturing
contraction was not the strongest one to which the membranes were subjected. Further-
more, Oyen et al. (2005) performed uniaxial and biaxial cyclic tests and observed a large
hysteresis in the first cycle as well as a dependency of the energy dissipation on the num-
ber of cycles and the strain level. In contrast, Pandey et al. (2007) found an increased
rupture strength and work to rupture after repetitive stretching of FM samples.

Miller et al. (1979) were the first who determined parameters for a nonlinear constitu-
tive model (Mooney material) based on inflation testing. Prevost (2004) (uniaxial and
inflation tests), Joyce (2009) (planar biaxial tests), and Jabareen et al. (2009) (uniaxial
tensive tests) proposed different nonlinear constitutive models for the rationalization of
the mechanical response of FM. While Miller et al. and Jabareen et al. utilized isotropic
continuum models, the studies of Prevost and Joyce approached modeling of FM behav-
ior by using phenomenological structural models that include some features of the planar
network structure of collagen. No other quantitative information characterizing the non-
linear stress-strain response of FM in a uniaxial or biaxial state of stress can be found in
the literature.
1.3 Current status of research and open questions

Despite a lot of research done in the past decades for a better understanding of the biochemical and mechanical behavior of the fetal membrane tissue, its nonlinear mechanical characterization, compared to other mammalian tissues such as bone, aortic tissue, ligaments and tendons, is still in an early stage of development. Most studies covering the mechanical behavior of FM tissue focused on the determination of membrane strength. Only very few studies determined the nonlinear stress-strain behavior, but characterization of these curves by determination of representative parameters as well as the formulation of a suitable constitutive model is almost absent. Numerous constitutive models for the modeling of the mechanical behavior of soft tissues are available to date and have been successfully applied to a broad range of tissues. Basic features like the exponential strain stiffening are included in rather simple isotropic (Demiray, 1972) or orthotropic formulations (Fung, 2004). Other models include partial microstructural information by embedding nonlinear fibers (representing the collagenous component) in specific directions in an isotropic matrix (Holzapfel et al., 2000). Modifications of these models were made by including spatial dispersion as well as gradual recruitment of the collagen fibers (Gasser et al., 2006; Hill et al., 2012). The desire of including more microstructural information into the models and improvements in the computing power opened up the territory for simulations of random fiber networks, which are more and more used for simulations of biological tissues (Picu, 2011; Koh and Oyen, 2012).

**Objective 1** There is a need for a better understanding of the nonlinear mechanical behavior of FM tissue as well as its relation to the microstructural composition and architecture. Determination of the key features for mechanical functioning enables the development of a constitutive model, able to represent the mechanical response of FM to different loading conditions.

Failure of FM tissue is part of normal functioning, but has serious consequences when it happens prior to term. Most previous research focused solely on the investigation of membrane strength and the biochemical weakening process. Well defined mechanical analysis investigating the failure of intact FM samples as well as samples containing an initial defect are missing. In fact, there are still questions concerning the FM component (amnion or chorion) to rupture first. With the aim of minimizing the risk of failure when defects are present, fracture toughness becomes more important. Fracture toughness characterizes the ability of a material to resist the growth of initial cracks. Fracture mechanics theories were introduced for engineering materials, but were recently also applied to soft
elastic materials at large deformations (Krishnan et al., 2008). Although there exists some literature about the fracture toughness and crack-growth resistance of bone and dentin (Norman et al., 1995; Vashishth, 2004), corresponding applications to soft biological tissues are just coming up. Oyen-Tiesma and Cook (2001) analyzed neocartilage and introduced a novel technique to estimate the fracture resistance from a hysteresis loop. Xu et al. (2008) studied the fracture mechanical properties of rabbit aorta and found that the circumferential direction is more crack tolerant than the axial direction due to anisotropy. Special techniques like a needle insertion test (Gokgol et al., 2012) or a guillotine test (Chu et al., 2013) were applied for the experimental determination of the fracture toughness of bovine liver and porcine aorta. Moreover, Horgan and Smayda (2013) have recently analyzed the so called “trousers test” by means of anisotropic hyperelastic models. Taylor et al. (2012) summarize most of the previous work of fracture mechanics tests on biological tissues and argue that most of the previous studies measured material’s strength rather than fracture toughness due to too small sample sizes or crack lengths. Despite the problems discovered, Taylor et al. (2012) conclude that soft tissues are highly defect tolerant. Recent studies about the fracture mechanics of random fiber networks (Stachewicz et al., 2011; Koh and Oyen, 2012; Koh et al., 2013) support this finding by the detection of a zone of high fiber rearrangement in front of the crack tip.

**Objective 2** The fetal membrane is probably the only tissue where failure is part of normal functioning. Therefore, analysis of the failure behavior in terms of the rupture sequence in mechanical tests as well as determination of fracture mechanics properties and the relationship between tissue strength and microstructure is essential.

In the last decades, progress was also made in the field of medical interventions in the uterine cavity. Fetal surgery characterizes a broad range of surgical techniques to treat severe or life-threatening birth defects on the fetus inside the pregnant uterus. Interventions that were traditionally performed by open surgery or were treated postnatal can nowadays be done by minimally invasive fetoscopy. However, needle and fetoscopic punctures of the fetal membrane for diagnostic or surgical interventions cause local defects that carry a significant risk for subsequent iatrogenic preterm premature rupture of the FM (iPPROM). The potentially beneficial prenatal interventions are limited by the high occurrence of iPPROM, which is in the range of 4% to 100% (Deprest et al., 2010). Thus, iPPROM is the Achilles’ heel for developments in the field of minimally invasive fetal surgery. Healing of the FM tissue was observed to be very limited or even absent (Devlieger et al., 2006). Therefore, attempts were made for artificial sealing or repair such as plugging, stimulation of biological repair, or sealing by surgical glues (Mallik et al.,
1.3. Current status of research and open questions

2007; Ochsenbein-Kölble et al., 2007; Bilic et al., 2010). However, none of these methods has made it into clinical practice. It is known from clinical studies that there is a relation between the type of surgery as well as the size of the instruments and the frequency of iPPROM. However, experimental (ex vivo) studies investigating FM failure as a consequence of membrane puncturing are missing.

The solution of the corresponding mechanical problem of the stress concentrations and redistributions around circular holes in plates within the realm of linear elasticity has been known for a long time and is part of many text books (Timoshenko and Goodier, 1951). Extensions of this solution to nonlinear materials under large deformations (Rivlin and Thomas, 1951) as well as considerations of orthotropy (Konish and Whitney, 1975) were proposed. Only one study covers the same problem under consideration of aspects of biological tissues (exponential stiffening, orthotropy): David and Humphrey (2004) found a particular influence of the anisotropy on the stress concentrations and concluded that a circumferentially stiffer material can reduce the stress concentrations. Moreover, the role of anisotropy is greater for smaller holes, which is in conflict with medical efforts to minimize the fetoscopic entry site.

Objective 3 The aim of reducing the risk related to iPPROM requires investigation and optimization of the surgical procedures for membrane puncturing. Recreation of the mechanical integrity of FM tissue after minimally invasive surgery asks for the development of methods to seal and repair the fetoscopic entry site.

Although, operative fetoscopy has become a therapeutic option, iatrogenic preterm rupture of the fetal membranes is a complication with severe consequences for the newborn. Development of future treatments to prevent (iatrogenic) premature rupture requires a better understanding of the mechanical properties fetal membrane tissue.

The present thesis contributes to the research on human fetal membrane tissue. The main focus is on the mechanical characterization of the tissue under consideration of particular aspects of its microstructural composition. Mechanical characterization in this context includes experimental investigations and model formulations for the description of the nonlinear subfailure deformation behavior, the failure behavior, and fracture properties. The thesis is structured as follows: Chapter 2 introduces the anatomy and formation of the fetal membrane and its structural composition. Chapter 3 provides a comprehensive experimental characterization of the nonlinear mechanical response of intact FM tissue as well as of the separate layers under uniaxial and equibiaxial loading. The corresponding microstructural constituents are analyzed by biochemical assays. Combination of the re-
results from both disciplines enables the investigation of the correlations between mechanical and histological parameters. Also aspects of FM failure in different experimental configuration are covered in this chapter. Chapter 4 continues with a mechanical characterization of the separate layer deformation behavior under uniaxial loading, with particular focus on the in-plane kinematic response. The experimental results enable the formulation of an anisotropic constitutive model for the mechanical response of FM, which is subsequently implemented in a commercial FE package. Although chapters 3 and 4 cover the same topic and are both related to the fulfillment of objective 1, the present division into two separate chapters reflects their individual publication in Buerzle et al. (2013) and Buerzle and Mazza (2013). Chapter 5 covers a detailed mechanical analysis of puncture testing applied for the characterization of FM tissue. Experiments utilizing different methods of sample fixation and corresponding numerical simulations are performed and analyzed with particular focus on the failure behavior of intact FM as well as on the possibility of data extraction for mechanical characterization to address parts of objective 1 and 2. Chapter 6 qualifies the mechanical behavior of a synthetic hydrogel glue (the so called “mussel glue”) as a possible sealant for the repair of punctured membranes by inflation tests on elastomeric and fetal membranes. Moreover, the stress concentrations around circular defects in FM tissue as well as general methods of repair are analyzed by numerical simulations to cover relevant aspects of objective 3. Chapters 5 and 6 are written as stand alone reports, similar to a scientific paper, with an extended introduction.
The human fetal membrane

The fetal membrane is the compliant membrane surrounding the developing fetus inside the uterus. It is composed of two main layers, referred to as amnion and chorion. The fetal membrane facilitates gas and waste exchange and transports essential nutrients to and from the fetus, similar to the placenta. The membrane also acts as a barrier to protect the fetus against infections ascending from the reproductive tract (Parry and Strauss, 1998). The fetal membrane consists of several cell types including epithelial cells, mesenchymal cells, and trophoblast cells, embedded in a collagenous matrix. These cells retain the amniotic fluid and secrete substances into the amniotic fluid and toward the uterus (Parry and Strauss, 1998). The amniotic fluid is a dynamic milieu that changes as pregnancy progresses. It contains nutrients and growth factors that facilitate fetal growth and provides mechanical cushioning of the fetus (Underwood et al., 2005). The fetal membranes (amnion and chorion are initially separated) and the placenta develop and grow simultaneously with the developing fetus. During pregnancy, the fetal membrane has to deform as a consequence of internal pressure as well as fetal and maternal movements. Integrity of the fetal membrane is essential for the maintenance of pregnancy. However, rupture of the membrane is part of and required for normal term delivery.

2.1 Formation of the fetal membrane

Human fertilization begins with the union of an immature egg cell (oocyte) and a sperm, usually occurring in the uterine tube. The result is the formation of a zygote (fertilized egg), which initiates prenatal development. Within the first two to three days of development, a cleaving zygote proceeds along the uterine tube (Joyce, 2009), see Figure 2.1. Mitotic division occurs at a rate of about one per day, increasing the number of cells. The first cell divisions create a ball of undifferentiated cells called morula (Cross,
Chapter 2. The human fetal membrane

Figure 2.1: Schematic representation of the development of the fertilized egg to the blastocyst during the first week of pregnancy. Source: Stem Cells Information, NIH USA 2006. Reprinted with permission. © 2001 Terese Winslow.

1998). The fertilized egg enters the uterine cavity after about four days, when about 8-16 cells are present (Joyce, 2009). At the stage of 16-32 cells, a cavity can be detected within the morula, which is now denoted as blastocyst. The blastocyst is characterized by separation of the undifferentiated cells into surrounding trophoblast cells, a blastocystic cavity, and the inner cell mass (embryonic cells, embryoblast) accumulated on one side of the blastocyst (Carlson, 2004). Cells of the inner cell mass give rise to the body of the embryo itself plus several extraembryonic structures, whereas cells of the trophoblast form only extraembryonic structures, including the outer layers of the placenta (Carlson, 2004). Typically 6-8 days after ovulation, the fertilized egg attaches itself to the uterine wall. This process is called implantation and marks the beginning of pregnancy. The blastocyst invades the connective tissue of the uterus (endometrium) and is covered by a generated epithelial layer, see Figure 2.2. Thus, it is truly an implantation and not only an attachment. Once the blastocyst begins to penetrate the uterine epithelium, the endometrium is termed as decidua (Joyce, 2009).

Soon after beginning of implantation the inner cell mass of the blastocyst forms the embryonic disc composed of epiblast and hypoblast cells. The amniotic epithelium develops from the epiblast. Amniogenic cells (amnioblasts) separate from the epiblast and organize to form a thin membrane, the amnion, which encloses the amniotic cavity within 7-8 days after fertilization (Ilancheran et al., 2009; Moore et al., 2013). The process of implantation lasts up to 14 days after ovulation.
2.1. Formation of the fetal membrane

Figure 2.2: Major stages of blastocyst implantation around 5-6, 7-8, and 9-10 days. The blastocyst invades the endometrium, while the inner cell mass forms the embryonic disc composed of epiblast and hypoblast cells (a). The amniotic epithelium forms from the epiblast and begins to build the amniotic cavity. Around 9-10 days after fertilization the extraembryonic mesoderm is appearing (b). Reprinted from Carlson (2004) and Ilancheran et al. (2009) with permission. Copyright Elsevier.

The hypoblast forms the exocoelomic membrane that surrounds the blastocystic cavity and lines the internal surface of the cytotrophoblast. The exocoelomic membrane and cavity soon become modified to form the primary umbilical vesicle (the primary yolk sac). The outer layer of cells from the umbilical vesicle forms a layer of loosely arranged connective tissue, the so called extraembryonic mesoderm (Moore et al., 2013). The combination of the two trophoblast layers (cytotrophoblast, syncytiotrophoblast) and the extraembryonic mesoderm form the chorion. The embryo, amniotic sac, and umbilical vesicle are suspended in the chorionic cavity, which is surrounded by the chorionic sac (Moore et al., 2013). The process of gastrulation starts on day 9 after fertilization where the epiblast gives rise to the three primary germ layers (ectoderm, mesoderm, and endoderm) of the embryo (Ilancheran et al., 2009). Gastrulation is the beginning of morphogenesis, i.e. the development of the form and structure of various organs and parts of the body (Moore et al., 2013). The ectoderm develops into the central nervous system, inner ear, the cornea and lens of the eye, while the mesoderm builds structures like the skeleton, the heart, muscles, and the dermis of skin. The endoderm forms organs such as the liver, lungs, and the digestive tract (Carlson, 2004).

Early placental development is characterized by the rapid proliferation of the trophoblast and development of the chorionic sac and chorionic villi (Moore et al., 2013). The entire surface of the early conceptus is covered by chorionic villi. By expansion of the gestational sac, villi in the lower part of the sac are compressed reducing the blood supply and start to degenerate, which forms the chorionic layer, visible by 6-8 weeks of gestation (Ilancheran...
et al., 2009). Those villi associated with the decidua basalis (the part of the decidua underlying the conceptus) rapidly increase in number, branch, and enlarge. This part of the chorionic sac is known as villous chorion and builds the fetal part of the placenta, see Figure 2.3. It is connected to the maternal part of the placenta (decidua basalis) by the cytotrophoblastic shell (Moore et al., 2013). The intervillous space between the fetal and maternal components of the placenta is occupied by freely circulating maternal blood (Carlson, 2004). The placenta and umbilical cord function as a transport system for substances passing between the mother and the fetus. Nutrients and oxygen pass from the maternal blood through the placenta to the fetal blood, and waste materials and carbon dioxide pass from the fetal blood through the placenta to the maternal blood (Moore et al., 2013).

### 2.2 Structural composition of the fetal membrane

The structural composition of the human fetal membrane distinguishes two main layers, i.e. amnion on the inner side toward the fetus, and chorion on the outer side. Amnion and chorion can be divided into different sublayers composed of different structural components and cellular content. Both layers are composed of three types of tissues: epithelium, basement membrane, and connective tissue (Malak and Bell, 1994). Figure 2.4 provides a schematic representation of the FM structure at term. Human amnion is composed of five distinct sublayers and it contains no blood vessels or nerves. The innermost layer of amnion is the amniotic epithelium. Epithelial cells secrete collagen types III and IV.
that form the adjacent basement membrane (Parry and Strauss, 1998). The next layer is the compact layer of connective tissue. The compact layer is made predominantly of collagen types I and III that form a dense network of thin fibers (Malak et al., 1993). Collagen types V and VI form the filamentous connections between the collagen types I and III in the compact layer and the basement membrane (Malak et al., 1993; Parry and Strauss, 1998). The adjacent fibroblast layer is the thickest layer of amnion and is characterized by collagens forming a looser network. The outermost layer of amnion is the intermediate or “spongy” layer, which contains a nonfibrillar network of mostly type III collagen (Parry and Strauss, 1998). Although the spongy layer lies between amnion and chorion it is usually attributed to amnion and originates from the fusion of amnion and chorion around 17 - 20 weeks of gestation (Ilancheran et al., 2009). The structure of the spongy layer permits a certain amount of relative movement between amnion and chorion (Bourne, 1962).

Chorion resembles a typical epithelial membrane (Parry and Strauss, 1998). The reticular layer of chorion builds its connective tissue. It is made up of a network of reticular fibers in which fibroblasts are embedded (Malak et al., 1993). The chorionic basement membrane underlies the trophoblast layer and contains collagen type IV. The outermost layer, in contact with the maternal decidua, is the trophoblast layer. Chorion is firmly adherent to the maternal decidua. At delivery, when the membrane separates from the uterus, some material from the maternal decidua remains attached to chorion (Joyce, 2009). Therefore, the resulting layer is often denoted as choriodecidua.
Chapter 3

Mechanical characterization of human fetal membrane tissue

3.1 Introduction

The fetal membrane is composed of several sublayers of different structural and cellular content as described in the foregoing chapter. Many studies were performed to investigate the mechanical behavior of FM by the use of different experimental configurations. Most of those studies determined only system parameters (e.g. maximum pressure, maximum force) which depend on the sample geometry instead of specific material properties. Fewer studies also determined material specific rupture properties, such as critical membrane tension or critical stress. Other studies focused on the biochemical characterization of the FM by the measurement of the collagen or elastin content. Despite the large amount of literature available, there is a lack in the field of characterization of the nonlinear mechanical response and its direct relation to microstructural constituents.

This study aims at the mechanical and biochemical characterization of the fetal membrane tissue. Uniaxial tension tests and inflation experiments are performed to characterize the uniaxial and equibiaxial mechanical response of intact FM. Furthermore, inflation tests on separate amnion and chorion layers are performed to determine their individual contributions. Biochemical assays are performed to quantify the contents of collagen and elastin, and collagen cross-links are measured by high performance liquid chromatography. The biochemical assays are also applied to intact FM and to the separate layers.

The data will provide a comprehensive characterization of the mechanical response of intact FM tissue as well as its constitutive layers. Moreover, combination of the results from mechanical and biochemical tests allows the investigation of correlations between the mechanical behavior and the microstructural constituents.
3.2 Sample collection and ethical aspects

All fetal membrane samples within the scope of this thesis were collected from patients with single child pregnancies who underwent elective cesarean sections between 37 and 40 weeks of gestation. Patients were recruited for this study with informed written consent according to the protocol approved by the Ethical Committee of the District of Zurich (study Stv22/2006). The patients were randomly selected for this study after negative testing for HIV, hepatitis B, and streptococcus B as well as chlamydia and cytomegaly. The selected pregnancies had no history of diabetes, connective tissue disorders or chromosomal abnormalities. The membranes were cut approximately 2 cm away from the placental border and stored in saline solution until mechanical testing within a few hours after delivery. It should be noted that the FM samples included parts of the maternal decidua. The main cellular layer might thus be referred to as choriodecidua.

Obstetric parameters of the tested membranes can be found in the corresponding sections. To clearly distinguish between membrane samples used for uniaxial tension testing, inflation testing of intact FM and inflation testing of separate amnion and chorion layers, the membrane samples are numbered according to the scheme XN, where X is a capital letter specifying the test type (U for uniaxial tension, I for inflation tests of intact membrane, and L for inflation tests of separate layers) and N is an integer number indicating the membrane number. If data are reported as sample specific values, then the abbreviation $- Sn$ is added, where S indicates the sample specific nature and n the sample number.

3.3 Uniaxial tension testing

3.3.1 Experimental setup

In order to test the FM samples in a uniaxial stress configuration, a custom built experimental setup was used. This setup consists mainly of four computer controlled hydraulic actuators equipped with 20 N force sensors (100 N capacity), see Figure 3.1. Custom made clampings were attached to the load cells and equipped with sandpaper P320 to improve soft tissue gripping. A CCD camera system with a telecentric lens (field of view $30 \times 30$ mm, resolution $1000 \times 1000$ pixel) was mounted above the testing area and allows to record image series during the experiment. A Plexiglas chamber with a heating source was mounted on the testing area, which allows to test the samples in a physiologic
3.3. Uniaxial tension testing

![Experimental setup and sample preparation for the uniaxial tension tests. Test setup consists of four hydraulic actuators equipped with force sensors, a Plexiglas chamber filled with saline solution, and a CCD camera system above the test area (a). A rectangular piece is cut out from the membrane (b), brought into a cutting device to create samples with the dimension of $15 \times 60$ mm (c), and finally mounted in the clamping (d).](image)

Figure 3.1: Experimental setup and sample preparation for the uniaxial tension tests. Test setup consists of four hydraulic actuators equipped with force sensors, a Plexiglas chamber filled with saline solution, and a CCD camera system above the test area (a). A rectangular piece is cut out from the membrane (b), brought into a cutting device to create samples with the dimension of $15 \times 60$ mm (c), and finally mounted in the clamping (d).

The experimental data consist of the force and displacement data of each actuator and the recorded image series. The raw force signal is sampled at 2 kHz and contains large noise. Therefore, a filtered force signal is typically used which is defined as moving average along 100 data points.

3.3.2 Sample preparation and experimental protocol

The experimental protocol for the tension tests corresponds mainly to the one used in Jabareen et al. (2009). The fetal membrane samples were spread out on a plastic mat and rectangular pieces of approx $10 \times 12$ cm were cut. Those membrane pieces were supported with a sheet of paper and placed on a dedicated cutting device, which allows obtaining samples of dimensions $15 \times 60$ mm, see Figure 3.1(c). After clamping of the top plate of
the cutting device, FM samples were cut out by careful incision along the grooves of the
top plate with the aid of a surgical scalpel. During preparation, the FM samples were
sprayed frequently with saline solution to avoid dehydration. The sample strips were
mounted in the clampings outside of the testing machine, the supporting paper carefully
removed and then brought into the tension test machine and clamped with an initial free
length of 40 mm. The tests were performed in a bath filled with saline solution and the
temperature of the bath was held constant at 37 °C.

The mechanical testing consisted of five cycles of preconditioning between 0 and 20 % nom-
inal strain and a subsequent monotonic tension to failure test. The tests were performed
under a displacement controlled regime at a constant elongation rate of 0.5 % nominal
strain/s, according to Jabareen et al. (2009). This elongation rate was determined in
preliminary tests and shown to be slow enough to measure the quasi-static response of
fetal membrane tissue.

In order to obtain a robust and repeatable definition of the preconditioning cycles, special
considerations had to be taken into account with respect to the setup used. After mount-
ing the clamping levers on the force sensors, the clamps were displaced 1 mm in negative
direction, so that the sample was slack and the force signal was zeroed in this position.
A displacement ramp in positive direction was applied afterward with simultaneous ob-
servation of the tension force. When the force signal reached the force threshold value,
0.01 N in this case, the current length of the sample was registered as reference length
and the preconditioning cycles were performed with respect to the reference length. The
preconditioning cycles were defined as loading ramp up to 20 % nominal strain while the
unloading ramp stopped when the tension force fell below the force threshold. After pre-
conditioning the clamps were displaced to the initial position from where the monotonic
tension to failure test started.

### 3.3.3 Normalization and analysis of tension tests

In order to repeatably define a common reference configuration, a low force threshold of
0.01 N is introduced. Values of stress and strain are calculated with respect to this ref-
erece configuration. The longitudinal stretch $\lambda$ and engineering strain $\varepsilon_n$ follow directly
from the clamping displacement,

$$\lambda = \frac{l}{L_0}, \quad \varepsilon_n = \frac{l - L_0}{L_0}$$

(3.1)
3.4. Inflation testing

where \( l \) indicates the current sample length and \( L_0 \) is the sample length at the reference configuration. The loading of the membrane can either be assessed by the use of the membrane tension \( T \) or the stress \( \sigma \). Both values can be expressed as nominal values (subscript \( n \)) or with respect to the current configuration (subscript \( c \)). The membrane tension is defined as force \( F \) acting per unit width \( W \).

\[
T_n = \frac{F}{W}, \quad T_c = \frac{F}{W\lambda_2} \quad (3.2)
\]

The Cauchy stress \( \sigma \) in axial direction can be calculated from the measured tensile force and the current cross section area \( A_c \) of the sample.

\[
\sigma_n = \frac{F}{A_0}, \quad \sigma_c = \frac{F}{A_c} = \frac{F}{A_0\lambda} \quad (3.3)
\]

The fetal membrane tissue exhibits large deformations and shows pronounced nonlinearity in the stress-strain behavior often denoted as bilinear behavior. Two scalar parameters are used to characterize this behavior: the small strain stiffness \( E_1 \) (Young’s modulus) and the high strain stiffness \( E_2 \). These values are evaluated as the slope of a linear regression through the first and last ten percent of the stress-strain curves. The same definition is used for the slopes \( K_1 \) and \( K_2 \) of the tension-stretch curves. Further scalar parameters are the maximum stress \( \sigma_{max} \) and strain \( \varepsilon_{max} \). Values of membrane tension reported within this chapter are evaluated as nominal values due to difficulties in the optical analysis of the lateral contraction \( \lambda_2 \) within these tests.

Note, the definition of the Cauchy stress in this section (Eqn 3.3) is based on the assumption of isotropic and incompressible material behavior (\( \lambda_2 = \lambda_3 = 1/\sqrt{\lambda} \)). A detailed analysis of the in-plane kinematic response can be found in chapter 4.

3.4 Inflation testing

3.4.1 Experimental setup and protocol

With the aim of measuring the mechanical response of FM in a biaxial, close to physiologic loading configuration, a dedicated inflation device was developed, see Figure 3.2(a). The circular sample, supported through the sandpaper rings, is placed, with the amnion side facing downwards, on a fluid filled cylinder and fixed using a clamping ring with 50 mm inner diameter, see Figure 3.3(a). Saline solution is pumped into the cylinder with
the aid of a peristaltic pump (type 314VBM, four rollers, Watson-Marlow Ltd., Zurich, Switzerland). During the test, the pressure (digitale manometer, LEX 1, accuracy 0.05 %, Keller, Winterthur, Switzerland) within the cylinder is measured and pictures from the side view profile (Model Dragonfly2, resolution 640 × 480 pixel, 1/3” CCD, Point Grey, Vancouver BC, Canada) of the deformed membrane are simultaneously registered. To improve the contrast of the images, a red LED backlight (Moritex, MEBL CR7050, Fuji-film AG, Dielsdorf, Switzerland) was mounted behind the cylinder. The system for image acquisition and analysis is calibrated at the beginning of each measurement series. The test is executed under a pressure controlled regime with a rate of pressure increase of 0.65 mbar/s. This leads to a low strain rate (comparable to the tensile tests) and allows to measure the long time response. Saline solution is at room temperature and no temperature control is included in the present system. Monotonic pressure increase continues until membrane bursting. The typical duration of each experiment is 4 minutes. During this time the outer surface of the membrane is sprayed with saline solution to avoid dehydration. The whole system is controlled by a LabView code (LabView 8.5, National Instruments, Austin, USA). The experimental data used for further analysis consist of the pressure time history and the corresponding image series of the deformed membrane profile.

![Experimental setup](image)

**Figure 3.2:** Experimental setup. (a) A circular membrane sample is inflated with the aid of a peristaltic pump which conveys saline solution from the fluid reservoir. The pressure inside the cylinder is measured and controlled by a LabView code to follow a desired pressure profile. A CCD camera records images and a red LED backlight is installed behind the sample to improve the contrast of the images. (b) Schematic drawing of the cylinder with the inflated membrane. The measure $h$ indicates the height of the fluid column with respect to the level where the membrane is clamped and the pressure is zeroed at the beginning of the measurement.
3.4. Inflation testing

Table 3.1: Obstetric parameters: patient’s age, gestational age (GA) and number of samples (N) for mechanical testing per membrane. * indicates membranes that were frozen at $-20^\circ$C for one day before mechanical testing.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Age [year]</th>
<th>GA [week + day]</th>
<th>N [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1*</td>
<td>20</td>
<td>38 + 4</td>
<td>4</td>
</tr>
<tr>
<td>I2*</td>
<td>32</td>
<td>38 + 2</td>
<td>6</td>
</tr>
<tr>
<td>I3</td>
<td>36</td>
<td>38 + 6</td>
<td>4</td>
</tr>
<tr>
<td>I4</td>
<td>36</td>
<td>38 + 2</td>
<td>4</td>
</tr>
<tr>
<td>I5</td>
<td>28</td>
<td>39 + 2</td>
<td>3</td>
</tr>
<tr>
<td>I6</td>
<td>27</td>
<td>37 + 4</td>
<td>5</td>
</tr>
<tr>
<td>I7*</td>
<td>41</td>
<td>37 + 2</td>
<td>4</td>
</tr>
<tr>
<td>I8</td>
<td>34</td>
<td>37 + 3</td>
<td>4</td>
</tr>
<tr>
<td>I9</td>
<td>29</td>
<td>37 + 0</td>
<td>5</td>
</tr>
<tr>
<td>I10</td>
<td>34</td>
<td>38 + 2</td>
<td>5</td>
</tr>
<tr>
<td>I11</td>
<td>42</td>
<td>38 + 2</td>
<td>3</td>
</tr>
<tr>
<td>I12</td>
<td>46</td>
<td>38 + 4</td>
<td>4</td>
</tr>
</tbody>
</table>

3.4.2 Sample preparation

Twelve fetal membranes were collected for the mechanical characterization by inflation testing and the corresponding histological measurements, the obstetric parameters can be found in Table 3.1. Due to logistic reasons, three membranes were frozen at $-20^\circ$C for one day before mechanical testing (indicated by * in Table 3.1). Analysis of the mechanical and histological tests revealed no significant difference to the fresh samples for which reason also the frozen membrane samples were included in the analysis of this campaign. To generate samples for mechanical testing, FM were gently spread out on a plastic mat and sandpaper rings (3M, wetordry P400), with an inner diameter of 50 mm and an outer diameter of 70 mm, were glued (UHU, super glue) on each side of the membrane, see Figure 3.3(a). The sandpaper rings allowed to maintain the tissue in a relaxed state during handling and considerably improved gripping so to avoid slippage within the clamping device of the inflation experiment. After preparation, the samples were stored in saline solution until mechanical testing to prevent dehydration. For the testing of separate amnion and chorion layers sandpaper rings were also glued on each side of the membrane but then separated manually by careful blunt dissection.
Chapter 3. Mechanical characterization of human fetal membrane tissue

Figure 3.3: (a) sandpaper rings are glued on both side of the FM sample to stabilize the sample during handling and to improve soft tissue gripping. (b) the sample is fixed using a clamping ring with 50 mm inner diameter. (c) schematic drawing of an inflated membrane with indication of geometrical quantities.

3.4.3 Image analysis

Analysis of the recorded calibration images and experimental image series is used to quantify the deformation of the membrane. The image analysis procedure has three main goals: (i) determination of the mm-pixel calibration ratio, (ii) measurement of the apex displacement, and (iii) extraction of the radius of curvature of the inflated membrane. To analyze the images the MATLAB (The MathWorks, Inc., Natick, MA, USA) intrinsic function `edge`, using the so called Canny method (Canny, 1986) for edge detection is applied. A ball of known diameter is placed in the cylinder to generate a calibration picture. The edge extraction algorithm delivers the contour of the reference sphere. Comparison of the sphere dimensions with the corresponding data in the pixel space allows determining the mm-pixel ratio, typically in the range of 0.1 mm/pixel.

The apex position is obtained by measuring the position of the highest point of the inflated membrane with respect to the upper surface of the fluid filled cylinder. Since the clamping ring has a thickness of 1 mm, displacements below this value cannot be detected. The radius of curvature $R$ is determined by the analysis of a region of $\pm 100$ pixel from the axis of the cylinder, see Figure 3.3(c). The edge contour points within this region are used to fit a circular arc, thus providing $R$, the radius of this arc. For each experiment the image analysis procedure determines the time histories of displacement and radius, $d(t)$ and $R(t)$, respectively. Typical image sampling rates are in the order of 8 Hz.
3.4.4 Normalization

The pressure $p_{tot}$ measured inside of the fluid filled cylinder during membrane inflation results from two contributions: the hydrostatic pressure $p_{hydr}$ due to the increasing fluid column and the pressure due to the resistance of the stretched membrane $p_{memb}$.

$$p_{tot} = p_{hydr} + p_{memb}$$

(3.4)

The hydrostatic pressure contribution is given by the law of Bernoulli:

$$p_{hydr} = \rho gh$$

(3.5)

where $\rho$ is the density of the fluid, $g$ the gravitational constant, and $h$ the height of the fluid column, see Figure 3.2(b). Since the pressure is zeroed at the beginning of the measurement, the height $h$ refers to the level where the sample is clamped. The clamped membrane sample is initially slack with irregular wrinkles, and the initial resistance to inflation is negligible. Thus, we define the beginning of the measurement at the time point for which the membrane starts to oppose measurable resistance to elevation, i.e. when the difference between the measured total pressure and the hydrostatic pressure

$$P_{diff} = p_{tot} - p_{hydr}$$

(3.6)

reaches a threshold value. For the present setup and pressure sensor resolution, a threshold value of 1 mbar was selected. Similar to Myers et al. (2010), this procedure was introduced in order to obtain a repeatable definition of the reference configuration for the analysis of each experiment. This so called “preinflation state” corresponds to a slightly distended condition of the membrane and differs considerably from the slack state of the initially clamped membrane. All measured data are set to zero with respect to this reference state and the pressure difference $p_{diff} = 1$ mbar is the preload. The reference geometry is characterized by the corresponding “preinflation displacement” $D_0$, see Figure 3.3(c). Note that none of the previously reported experimental investigations using inflation experiments included such an analysis for determination of the reference state, which was shown to have a significant influence on the measurement results, see section 4.6.
3.4.5 Tension calculation

The membrane is stretched in radial and circumferential directions and contracts in the thickness direction with respect to the axisymmetric reference configuration of the inflation test. For a homogeneous membrane, the deformation field ranges from a so called “pure-shear” configuration (Ogden, 1972), at the clamping site to an equibiaxial state at the apex position. This study focuses on the characterization of FM behavior under equibiaxial stress. Thus, only the apex region is considered for the analysis of tension-stretch response. Since the membrane thickness is much smaller than the sample size, a plane stress state is assumed. Further, the membrane is considered as isotropic in its plane (in fact, no anisotropy could be observed in uniaxial or multiaxial experiments in our laboratory as well as in planar biaxial tests by Joyce et al. (2009)). The membrane tension $T$ at the apex can be calculated based on force equilibrium (Laplace’s law) as a function of the internal pressure $p$ (membrane’s pressure load $p_{memb}$) and the radius of curvature $R$ for each timestep $t$ of the recorded image series:

$$T = \frac{p(t)R(t)}{2}$$

(3.7)

It is well-known that the two major constituent layers of the fetal membrane, amnion and chorion, possess different mechanical properties (Oxlund et al., 1990). Amnion is known to be stiffer, stronger and thinner than chorion. Testing intact FM samples causes an inhomogeneous stress distribution along the sample thickness. For this reason stress is not an adequate measure for the mechanical characterization of intact FM samples. Correspondingly, in the present work we characterize FM as a structure (membrane) and not as a material.

3.4.6 Stretch estimation

With the current experimental setup no direct measurement of the stretch in the equibiaxial region is performed. Comparison between reference and current images taken from the side provides an integral measure of membrane’s profile deformation. An approach based on the finite element (FE) method was chosen to assess the time history of biaxial stretch $\lambda_{plane}(t)$ in the apex region. So called “stretch curves”, describing $\lambda_{plane}(t)$ as a function of the apex displacement $d(t)$, were calculated based on FE simulations and parameterized to get a mathematical representation to assign the equibiaxial stretch to the measured apex displacement.
3.4. Inflation testing

The FE simulation consists of two steps. The first step, called “preinflation”, starts from an initially flat membrane. The pressure load is applied and the maximum pressure magnitude varied to achieve the deformed reference configuration, characterized by the apex displacement value $D_0$. Once this target displacement is achieved, the nodal stresses and strains are set to zero. In this way the nodal position at the end of the first step represents the reference configuration (“preinflation state”) for the following membrane inflation simulation. In this second step, the pressure magnitude is increased, leading to growing apex displacement up to $d = 15\, \text{mm}$.

The FE software ABAQUS 6.9-1 (Abaqus Inc. RI, USA) in combination with MATLAB 2010a was used for these simulations. The FE model consists of 270 axisymmetric 4 node solid elements (element type CAX4RH). Geometric and material nonlinearities are considered. Dissipative behavior is neglected for the present analysis (assuming that the observed FM deformation corresponds to the long term response of the material). Different (incompressible) hyperelastic constitutive model formulations are implemented to represent the nonlinear material behavior. In fact, evaluation of the FM response in one single experimental configuration does not provide sufficient information to uniquely identify a representative strain energy functional form. Calculations using different model equations were performed in order to investigate the influence of the constitutive model formulation on the extraction of FM mechanical parameters as well as on the corresponding correlations with microstructural data. The implemented constitutive models are (i) a three parameter reduced polynomial form (Rivlin and Saunders, 1951), with the strain energy expressed as a function of the second invariant $I_2$ of the right Cauchy-Green deformation tensor $\mathbf{C}$ (i.e. the coefficients of $I_1$ are set to zero), (ii) the Rubin-Bodner model (Rubin and Bodner, 2002), based on the first invariant $I_1$ of $\mathbf{C}$, and (iii) the Ogden model (Ogden, 1972), with the strain energy as a function of the principal stretches. The mathematical formulation of the strain energy associated with each model as well as the used set of parameters can be found in Table 3.2. Note that the Rubin-Bodner model formulation when restricted to isotropic elastic incompressible behavior is akin to the formulation proposed in Demiray (1972).

Simulations were carried out for a set of preinflation displacements, i.e. $D_0 = \{0, 4, 8, 12, 16, 20, 24\} \, \text{mm}$. The plane stretch $\lambda_{\text{plane}}$ as a function of the apex displacement is determined from the FE calculation and represented as polynomial function:

$$\lambda_{\text{plane}}(d, D_0) = 1 + C_1(D_0)d + C_2(D_0)d^2 + C_3(D_0)d^3. \quad (3.8)$$
Table 3.2: Implemented constitutive models and corresponding set of parameters, given in [N/mm] (except $\alpha$ and $q$ which are dimensionless). The model formulations have been adapted for a uniform expression. Model parameters are reported for a membrane model formulation $\Psi_m$: strain energy per unit reference area of the membrane midplane surface.

<table>
<thead>
<tr>
<th>Model Formulation</th>
<th>Reduced Polynomial</th>
<th>Rubin-Bodner</th>
<th>Ogden</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Psi_m$</td>
<td>$\sum_{j=1}^{3} C_{0j} (I_2 - 3)^j$</td>
<td>$\frac{\mu_0}{2q} \left[ e^{qm_2(I_1 - 3)} - 1 \right]$</td>
<td>$\sum_{p=1}^{3} \frac{\mu_p}{\alpha_p} (\lambda_1^{\alpha_p} + \lambda_2^{\alpha_p} + \lambda_3^{\alpha_p} - 3)$</td>
</tr>
<tr>
<td></td>
<td>$C_{01} = 2.24 \cdot 10^{-6}$</td>
<td>$\mu_0 = 2.88 \cdot 10^{-3}$</td>
<td>$\mu_1 = 3.15 \cdot 10^{-6}$, $\alpha_1 = 28.3$</td>
</tr>
<tr>
<td></td>
<td>$C_{02} = 1.30 \cdot 10^{-2}$</td>
<td>$q = 1.75$</td>
<td>$\mu_2 = 8.53 \cdot 10^{-4}$, $\alpha_2 = 10.5$</td>
</tr>
<tr>
<td></td>
<td>$C_{03} = 0.402$</td>
<td>$m_2 = 3.43$</td>
<td>$\mu_3 = 3.39 \cdot 10^{-4}$, $\alpha_3 = 15.1$</td>
</tr>
</tbody>
</table>

Table 3.3 lists the expressions of the corresponding coefficients $C_1(D_0)$, $C_2(D_0)$ and $C_3(D_0)$, and Figure 3.4 illustrates the corresponding stretch curves for all implemented constitutive models.

### 3.4.7 Analysis of tension-stretch curves

The fetal membrane tissue undergoes large deformations and exhibits distinct nonlinearity in the pressure-displacement as well as in the tension-stretch relation, often denoted as bilinear behavior. Two scalar parameters, the low stretch modulus $K_1$ and the high stretch modulus $K_2$, are determined in order to characterize this bilinear behavior in each curve of tension versus plane stretch. The values are calculated from the initial and final 10% of the curves obtained from calculations based on measured time histories of pressure and apex displacement. The strength of the membrane tissue is determined as the membrane tension at rupture. The rupture of the membrane is characterized by a sudden decrease of the pressure value due to fluid leakage through a local defect. In case of a gradual rupture, membrane failure is identified by the first distinct pressure drop. Thus in both cases failure coincides with the time point of the value $p_1$ in Figure 3.13. Careful visual inspection allows identification of the rupture position and sequence. In addition, the corresponding pressure histories are analyzed to associate characteristic $p(t)$ patterns to
### 3.4. Inflation testing

**Table 3.3:** Constitutive models and coefficients for the parameterization of the stretch curves, see equation 3.8.

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameterization</th>
<th>Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced Polynomial</td>
<td>$C_1$</td>
<td>$1.41 \cdot 10^{-2} + 1.92 \cdot 10^{-3} D_0 - 4.32 \cdot 10^{-5} D_0^2$</td>
</tr>
<tr>
<td></td>
<td>$C_2$</td>
<td>$6.67 \cdot 10^{-4} - 4.34 \cdot 10^{-5} D_0 + 5.92 \cdot 10^{-7} D_0^2$</td>
</tr>
<tr>
<td></td>
<td>$C_3$</td>
<td>$0$</td>
</tr>
<tr>
<td>Rubin-Bodner</td>
<td>$C_1$</td>
<td>$8.25 \cdot 10^{-4} + 2.21 \cdot 10^{-3} D_0 - 5.65 \cdot 10^{-5} D_0^2$</td>
</tr>
<tr>
<td></td>
<td>$C_2$</td>
<td>$8.20 \cdot 10^{-4} - 4.41 \cdot 10^{-5} D_0 + 8.48 \cdot 10^{-7} D_0^2$</td>
</tr>
<tr>
<td></td>
<td>$C_3$</td>
<td>$0$</td>
</tr>
<tr>
<td>Ogden</td>
<td>$C_1$</td>
<td>$-1.55 \cdot 10^{-3} + 2.68 \cdot 10^{-3} D_0 - 9.09 \cdot 10^{-5} D_0^2$</td>
</tr>
<tr>
<td></td>
<td>$C_2$</td>
<td>$1.26 \cdot 10^{-3} + 2.51 \cdot 10^{-5} D_0 + 2.16 \cdot 10^{-6} D_0^2$</td>
</tr>
<tr>
<td></td>
<td>$C_3$</td>
<td>$1.34 \cdot 10^{-6} - 2.62 \cdot 10^{-6} D_0 - 6.31 \cdot 10^{-8} D_0^2$</td>
</tr>
</tbody>
</table>

**Figure 3.4:** Stretch curves obtained from different constitutive models as a function of total displacement $= \text{preinflation displacement } D_0 + \text{further apex displacement } d$. The corresponding model formulations and parameters can be found in Table 3.2 and 3.3.
the observed rupture sequence. For the determination of the strength only the samples which ruptured in the free area (no rupture at or close to the clamping) are taken into account. In summary, four mechanical parameters were extracted from the tension stretch curves: low and high stretch stiffness $K_1$ and $K_2$, critical membrane tension $T_{crit}$, and failure stretch $\lambda_{crit}$.

### 3.5 Determination of histological properties

Biochemical assays enable the quantitative determination of microstructural constituents such as elastin, collagen, and collagen cross-links, which are expected to contribute determining the mechanical behavior of the tissue. Results from three different studies are summarized in the current chapter. Determination of the histological parameters was performed sample or membrane specific, depending on the study type, see Table 3.4. If the histological properties were determined as membrane specific values, the biochemical analyses were performed on three samples per membrane from the material left over after extraction of the samples used for mechanical testing. The biochemical assays for the determination of the collagen and elastin content as well as the measurement of the thickness were performed at the Department of Obstetrics at the University Hospital Zurich.

<table>
<thead>
<tr>
<th>Study type</th>
<th>Collagen</th>
<th>Elastin</th>
<th>Thickness</th>
<th>Cross-links</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniaxial tension</td>
<td>m</td>
<td>m</td>
<td>m</td>
<td>-</td>
</tr>
<tr>
<td>Inflation, intact FM</td>
<td>m</td>
<td>m</td>
<td>s</td>
<td>m</td>
</tr>
<tr>
<td>Inflation, separate layers</td>
<td>s</td>
<td>s</td>
<td>s</td>
<td>-</td>
</tr>
</tbody>
</table>

### 3.5.1 Thickness measurement

For the measurement of the sample thickness the procedure previously published in Jabareen et al. (2009) was followed. Briefly, tissue samples of intact FM as well as separate membrane layers were preserved in 4% formalin and later processed for Hematoxylin and Eosin (H&E) staining. The tissue samples were embedded in paraffin and 4µm thick slices were cut using a microtome. The measurement of the thickness was performed optically by the use of a Zeiss microscope (Axiovert 200M, Carl Zeiss AG, Jena, Germany)
3.5. Determination of histological properties

with the Axio Vision 4.5 software. The thickness of the amnion and chorion layer was measured with the aid of a software measuring tool, as illustrated in Figure 3.5. The reported thickness value usually represents an average of six measurements per tissue sample. If the thickness is reported sample specific, then the measurement was performed after mechanical testing. Otherwise, thickness was measured on the material adjacent to the sample used for mechanical testing.

Figure 3.5: Histological sections of intact fetal membrane samples. Images show the H&E stained cross section of two FM samples used for the thickness measurement.

3.5.2 Collagen assay

For the estimation of the total collagen, an acid hydrolysis method is applied using 6 N hydrochloric acid (HCl) to determine the hydroxyproline. 10 mg lyophilized tissue is hydrolyzed with hydrochloric acid (120 °C and for 20 h) and 50 µl hydrolyzed collagen is converted with 450 µl Chloramine T at room temperature for 25 min to pyrrole-2-carboxylic acid. Further reaction with 4-dimethylaminobenzaldehyde in propan-2-ol and perchloric acid at 60 °C for 20 min gives the amount of hydroxyproline, a red discolored product, whose absorbance is measured at 540 nm. Hydroxyproline is a major component of the collagen protein and important for the stability of collagen. It is found only in few proteins other than collagen, for which reason it is used as indicator of the collagen content.

3.5.3 Elastin assay

The procedure for determination of elastin content has been described in Jabareen et al. (2009). In short, insoluble elastin is quantified from membrane samples as soluble cross-linked polypeptides by oxidation with oxalic acid. Approximately 50 mg of each lyophilized
tissue was extracted three times in 2 ml of 0.25 M oxalic acid by boiling at 100 °C for 1 h. The three extracts were pooled and the amount of soluble elastin was determined colorimetrically using a Fastin Elastin assay kit (Biocolor Ltd., Newtonabbey, Northern Ireland) following the manufacturer’s instruction.

3.5.4 Determination of collagen cross-links
Measurement of the two collagen cross-links pyridinoline (PYD) and deoxypyridinoline (DPD) was performed at The Institute for Clinical Chemistry and Hematology, University Hospital St. Gallen, Switzerland. Cross-links were determined by high performance liquid chromatography (HPLC). The first step of the analysis is the hydrolyzation of the tissue samples. After dilution the mixture is loaded on an extraction tube. After a washing step, the pyridinium cross-links PYD and DPD are eluted from the extraction tube. The separation of PYD and DPD on the HPLC system is based on the ion pair chromatography on a reversed phase cartridge with isocratic elution. Detection is achieved by their natural fluorescence.

3.6 Statistical analysis
Data of collagen and elastin content, amnion and chorion thickness as well as the mechanical parameters are presented as mean ± standard deviation. Coefficients of correlation are calculated using the built in function of MATLAB. Statistical significance of the coefficients of correlation is evaluated by a two-tailed t-test. Results are considered to be statistically significant if the $p$ value is smaller than 0.05.

3.7 Mechanical properties of human fetal membrane tissue

3.7.1 Uniaxial data
Uniaxial tension tests were performed on a total of 35 samples from ten different membranes. Figure 3.6(b) shows the averaged nominal tension-stretch curves for each of the tested membranes. The tension-stretch curves in Figure 3.6 correspond to the monotonic tension to failure test that was performed after five cycles of preconditioning. It can be
3.7. Mechanical properties of human fetal membrane tissue

seen in Figure 3.6(b) that the variability between different membranes is somewhat larger than the scatter between samples from one membrane, as illustrated in Figure 3.6(a). The tension-stretch curves show a typical nonlinear behavior characterized by a low initial stiffness and a following transition into a region of higher stiffness.

The scalar parameters $K_1$, $K_2$, and $T_{\text{crit}}$ are evaluated as nominal values since the evaluation of the in-plane deformation is uncertain due to difficulties involved in the image analysis when the tension tests are performed in saline solution. Table 3.5 shows the membrane specific averages of the scaler parameters used to characterize the nonlinear mechanical response. Overall averaged values are calculated in order to characterize the mechanical response within a uniaxial state of stress: stiffness values of $K_1$ and $K_2$ are $(2.8 \pm 1.7) \cdot 10^{-2}$ N/mm and $(1.15 \pm 0.37)$ N/mm, respectively. The strain at rupture of the membrane is $(32 \pm 9)\%$ and the maximum nominal membrane tension is $(0.19 \pm 0.06)$ N/mm. Rupture of the membrane sample occurs in almost all tension tests near the clamping site. Therefore, the given values of maximum membrane tension and strain at rupture might not represent the real critical values of fetal membrane tissue.

**Effect of preconditioning**

The cyclic behavior during preconditioning was analyzed on the 35 samples used for uniaxial tension testing. Five cycles of preconditioning up to nominal strain of 20% were applied prior to the tension to failure test. Figure 3.7(a) shows the tension-stretch curve of one sample for the whole tension test and Figure 3.7(b) illustrates the corresponding
behavior during preconditioning. The cyclic behavior of the fetal membrane tissues is characterized by a distinct hysteresis, as can be seen in Figure 3.7(b). The biggest change between cycles happens between the first and the second cycle and decreases with an increasing number of cycles and reaches an almost stable response after five cycles. The maximum tension in the fifth cycle is in average 63% of the one in the first cycle. The stress softening observable in Figure 3.7(b) indicates the presence of the Mullins effect (Mullins, 1969). The preconditioning cycles lead to a shift of the reference configuration \( F_0 = 0.01 \text{N} \) between the first loading ramp, the virgin response, and the subsequent tension to failure test. Corresponding values of the reference length are \((45.8 \pm 3.3) \text{mm}\) for the virgin response and \((50.1 \pm 4.1) \text{mm}\) for the subsequent tension to failure test. Correspondingly, the initial stiffness increases with increasing cycle number. The initial stiffness of the first loading cycle \( K_{1st} \) is on average \((1.0 \pm 0.5) \cdot 10^{-2} \text{N/mm}\) and the initial stiffness of the tension test is on average \((2.8 \pm 1.7) \cdot 10^{-2} \text{N/mm}\), as determined in the previous section.

### 3.7.2 Inflation data of intact fetal membrane

**FM response to biaxial inflation**

A total of 51 samples from 12 membranes have been tested using the inflation device. Figure 3.8(a) shows the pressure vs. displacement curves for the samples of one membrane after normalization according to section 3.4.4. The averaged pressure-displacement curves of all 12 membranes are illustrated in Figure 3.8(b). As can be seen in Figure 3.8 there...
3.7. Mechanical properties of human fetal membrane tissue

Figure 3.7: Mechanical response of one fetal membrane sample in the uniaxial tension test as defined in section 3.3.2. Tension-stretch response for the whole test (a) and behavior during preconditioning (b).

is a pronounced variability within the samples of the same membrane and a somewhat larger variability among averaged curves of the membranes from different donors. All curves are characterized by a low initial stiffness followed by a transition region leading to an almost linear pressure-displacement relation for large displacements.

Overall averaged data were determined in order to characterize the present inflation experiments: $D_0$ is $(15.3 \pm 2.7)$ mm, the maximum pressure and displacement at rupture of the membrane are $(153 \pm 51)$ mbar and $(9.0 \pm 1.6)$ mm, respectively (note that for the latter values only the 38 samples which ruptured away from the clamping were considered).

Influence of model formulations

Due to the non-homogeneous strain field within the FM samples, the biaxial tension-stretch parameters extracted from the experiments depend on the constitutive model formulation assumed for the inverse analysis. Different hyperelastic constitutive equations were applied and their influence on the correlations between mechanical and microstructural parameters investigated. Figure 3.4 illustrates the parameterized curves for determination of $\lambda_{\text{plane}}$, as obtained using the different constitutive models. It is evident that the calculated stretch values converge for small deformations and moderate preinflation displacements, thus the model formulation does not influence the low stretch stiffness value $K_1$. On the contrary, for large displacements the $\lambda_{\text{plane}}$ values predicted from the different models differ significantly, thus affecting $K_2$. 

33
Figure 3.8: Pressure-displacement graphics for the four samples of membrane I5 plus average curve (a) and average pressure-displacement curves for all tested membranes (b).

Figure 3.9 illustrates the dependence of the experimental high stretch stiffness values $K_2$ on the model formulations. There is a strong correlation between the values obtained from different model equations. The same is valid for $\lambda_{crit}$. Due to the proportionality between the corresponding mechanical parameters, the choice of the constitutive model influences the absolute values but not the existence of a correlation between mechanical and microstructural parameters. For the analyses of section 3.4.7, the reduced polynomial model, based on the second invariant $I_2$, was chosen.

### 3.7.3 Inflation data of separate amnion and chorion layers

A total of 13 samples from five different membranes were used for the separate layer characterization by inflation testing. The samples were prepared according to section 3.4.2 and manually separated in single amnion and chorion samples so that both samples correspond to the same initial intact fetal membrane region. Inflation tests were performed within a pressure controlled regime as described in section 3.4.1. In order to obtain a repeatable reference configuration the measurements were normalized according to section 3.4.4, but to account for the smaller initial stiffness of the single layers a pressure threshold $p_{diff}$ of 0.2 mbar was used.

Figure 3.10 illustrates the resulting tension-stretch curves for the single amnion and chorion samples. The extracted scalar parameters which quantify the stiffness, strength, and failure stretch are summarized in Table 3.7. Averaged data of amnion and
3.7. Mechanical properties of human fetal membrane tissue

Figure 3.9: Influence of constitutive model on the mechanical parameters determined for each FM sample, at the example of $K_2$.

Table 3.6: Mechanical parameters extracted from the tension-stretch curves. Values are membrane specific averages. The number of samples per membrane can be found in Table 3.1. * The absolute values of these parameters depend on the constitutive model adopted for the inverse analysis.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>$K_1 [\cdot 10^{-2} \text{ N/mm}]$</th>
<th>$K_2^* [\text{N/mm}]$</th>
<th>$T_{\text{crit}} [\text{N/mm}]$</th>
<th>$\lambda_{\text{crit}}^* [-]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I1</td>
<td>7.2 ± 1.8</td>
<td>3.19 ± 1.17</td>
<td>0.40 ± 0.10</td>
<td>1.25 ± 0.02</td>
</tr>
<tr>
<td>I2</td>
<td>7.3 ± 4.0</td>
<td>3.46 ± 1.00</td>
<td>0.30 ± 0.08</td>
<td>1.23 ± 0.04</td>
</tr>
<tr>
<td>I3</td>
<td>13.9 ± 7.8</td>
<td>2.79 ± 0.95</td>
<td>0.27 ± 0.14</td>
<td>1.18 ± 0.01</td>
</tr>
<tr>
<td>I4</td>
<td>12.9 ± 5.4</td>
<td>2.62 ± 0.68</td>
<td>0.20 ± 0.04</td>
<td>1.15 ± 0.02</td>
</tr>
<tr>
<td>I5</td>
<td>15.3 ± 1.3</td>
<td>3.13 ± 1.01</td>
<td>0.27 ± 0.10</td>
<td>1.17 ± 0.01</td>
</tr>
<tr>
<td>I6</td>
<td>13.4 ± 12.8</td>
<td>2.17 ± 0.69</td>
<td>0.24 ± 0.07</td>
<td>1.18 ± 0.02</td>
</tr>
<tr>
<td>I7</td>
<td>6.1 ± 1.4</td>
<td>2.38 ± 0.10</td>
<td>0.26 ± 0.08</td>
<td>1.25 ± 0.04</td>
</tr>
<tr>
<td>I8</td>
<td>8.4 ± 2.6</td>
<td>2.15 ± 0.19</td>
<td>0.15 ± 0.01</td>
<td>1.16 ± 0.01</td>
</tr>
<tr>
<td>I9</td>
<td>10.3 ± 4.2</td>
<td>2.19 ± 0.23</td>
<td>0.22 ± 0.05</td>
<td>1.19 ± 0.02</td>
</tr>
<tr>
<td>I10</td>
<td>11.4 ± 3.2</td>
<td>2.33 ± 0.36</td>
<td>0.21 ± 0.02</td>
<td>1.18 ± 0.01</td>
</tr>
<tr>
<td>I11</td>
<td>7.6 ± 0.9</td>
<td>2.10 ± 0.25</td>
<td>0.20 ± 0.04</td>
<td>1.19 ± 0.03</td>
</tr>
<tr>
<td>I12</td>
<td>11.7 ± 3.7</td>
<td>3.20 ± 0.46</td>
<td>0.34 ± 0.10</td>
<td>1.21 ± 0.01</td>
</tr>
</tbody>
</table>
chorion for the specified parameters are as follows: the initial stiffness \( K_1 \) of amnion is \((21.9 \pm 14.4) \times 10^{-2} \text{N/mm}\) and of chorion \((10.1 \pm 4.3) \times 10^{-2} \text{N/mm}\). Similarly, the high strain stiffness \( K_2 \) of amnion and chorion is \((2.14 \pm 0.84) \text{N/mm}\) and \((1.45 \pm 0.84) \text{N/mm}\), respectively, thus demonstrating that amnion is stiffer than chorion. Amnion is also stronger than chorion, i.e. the maximum membrane tension is \((0.20 \pm 0.09) \text{N/mm}\) for amnion and \((0.12 \pm 0.05) \text{N/mm}\) for chorion. The strain at rupture also shows that amnion is less extensible than chorion. The corresponding values are \((14 \pm 3)\%\) and \((19 \pm 5)\%\), respectively. All four mechanical parameters differ significantly \((p < 0.05)\) between amnion and chorion. Due to the limited database all samples were considered for the determination of critical values of tension and stretch.

\[\begin{array}{cc}
\text{Tension [N/mm]} & \\
\text{Stretch \( \lambda \) [-]} & \\
0 & 0.1 \quad 0.2 \quad 0.3 \quad 0.4 \\\n1 & 1.1 \quad 1.2 \quad 1.3
\end{array}\]

(a)

\[\begin{array}{cc}
\text{Tension [N/mm]} & \\
\text{Stretch \( \lambda \) [-]} & \\
0 & 0.1 \quad 0.2 \quad 0.3 \quad 0.4 \\\n1 & 1.1 \quad 1.2 \quad 1.3
\end{array}\]

(b)

**Figure 3.10:** Tension-stretch curves of the samples used for the separate layer characterization by inflation testing. (a) amnion layer and (b) chorion layer.

### 3.7.4 Comparison of uniaxial, equibiaxial, and separate layer data

Three different studies were performed in order to characterize the mechanical behavior of fetal membrane tissue under uniaxial and equibiaxial loading, as well as the mechanical behavior of the separate amnion and chorion layers. The current section summarizes and compares the results of the mechanical tests from the previous sections. Overall averaged data of the extracted scalar parameters are reported in Table 3.8.

A comparison of mechanical parameters is only meaningful when the corresponding reference configuration is known, and when the tests were performed with a similar strain rate.
### Table 3.7: Mechanical parameters extracted from the tension-stretch curves of the samples used for the separate layer characterization by inflation testing. Values are reported as sample specific data. * The values of these parameters depend on the used constitutive model.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_1$ [·10^{-2} N/mm]</th>
<th>$K_2$ [N/mm]</th>
<th>$T_{crit}$ [N/mm]</th>
<th>$\lambda_{crit}$ [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amnion</td>
<td>Chorion</td>
<td>Amnion</td>
<td>Chorion</td>
</tr>
<tr>
<td>L1-S1</td>
<td>22.5</td>
<td>8.0</td>
<td>2.82</td>
<td>2.63</td>
</tr>
<tr>
<td>L1-S2</td>
<td>5.9</td>
<td>9.6</td>
<td>3.67</td>
<td>2.70</td>
</tr>
<tr>
<td>L2-S2</td>
<td>48.1</td>
<td>4.7</td>
<td>2.59</td>
<td>1.71</td>
</tr>
<tr>
<td>L2-S3</td>
<td>32.2</td>
<td>5.8</td>
<td>2.74</td>
<td>1.74</td>
</tr>
<tr>
<td>L2-S4</td>
<td>20.7</td>
<td>14.3</td>
<td>2.42</td>
<td>2.80</td>
</tr>
<tr>
<td>L3-S1</td>
<td>8.5</td>
<td>15.5</td>
<td>2.70</td>
<td>0.99</td>
</tr>
<tr>
<td>L3-S2</td>
<td>24.2</td>
<td>12.8</td>
<td>2.56</td>
<td>1.10</td>
</tr>
<tr>
<td>L4-S1</td>
<td>24.0</td>
<td>15.0</td>
<td>1.76</td>
<td>0.64</td>
</tr>
<tr>
<td>L4-S2</td>
<td>8.9</td>
<td>14.6</td>
<td>1.55</td>
<td>0.90</td>
</tr>
<tr>
<td>L4-S3</td>
<td>7.6</td>
<td>13.5</td>
<td>0.65</td>
<td>1.79</td>
</tr>
<tr>
<td>L5-S2</td>
<td>15.0</td>
<td>5.9</td>
<td>1.07</td>
<td>0.64</td>
</tr>
<tr>
<td>L5-S3</td>
<td>17.3</td>
<td>5.9</td>
<td>1.99</td>
<td>0.49</td>
</tr>
<tr>
<td>L5-S4</td>
<td>50.5</td>
<td>5.2</td>
<td>1.28</td>
<td>0.73</td>
</tr>
</tbody>
</table>

All tests within this work were performed with a low strain rate (0.5 % nominal strain/s or less) to measure the quasi-static response of the tissue. In order to enable a comparison of the data reported in Table 3.8, also the corresponding initial membrane tensions were evaluated. The raw pressure and displacement data resulting from inflation tests were normalized by the use of a pressure criterion as defined in section 3.4.4. Since this criterion is formulated in terms of a threshold between the theoretical and measured hydrostatic pressure, the absolute pressure at which the criterion is fulfilled changes from sample to sample. Because the membrane tension is a function of pressure and curvature, the initial membrane tension at the reference configuration changes. The overall averaged initial membrane tension for the inflation tests on intact FM samples is $(21 \pm 4) \cdot 10^{-4}$ N/mm. The corresponding value for the inflation tests on amnion is $(9 \pm 6) \cdot 10^{-4}$ N/mm and for chorion $(11 \pm 6) \cdot 10^{-4}$ N/mm. Since the uniaxial tension tests were normalized by the use of a force criterion, the initial membrane tension is constant with a value of $7 \cdot 10^{-4}$ N/mm. Further aspects concerning the importance of the reference configuration for characterization of soft biological tissues can be found in section 4.6.
### Table 3.8: Comparison of mechanical parameters obtained from uniaxial tension tests on intact fetal membranes, inflation tests on intact FM samples and inflation tests on single amnion and chorion layers. * The values of these parameters depend on the constitutive model used for the inverse analysis of the inflation tests.

<table>
<thead>
<tr>
<th>Study</th>
<th>$K_1 \cdot 10^{-2}$ [N/mm]</th>
<th>$K_2^*$ [N/mm]</th>
<th>$T_{crit}$ [N/mm]</th>
<th>$\lambda_{crit}^*$ [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniaxial</td>
<td>2.8 ± 1.7</td>
<td>1.16 ± 0.37</td>
<td>0.19 ± 0.06</td>
<td>1.32 ± 0.09</td>
</tr>
<tr>
<td>Inflation</td>
<td>10.5 ± 3.1</td>
<td>2.65 ± 0.78</td>
<td>0.25 ± 0.07</td>
<td>1.20 ± 0.03</td>
</tr>
<tr>
<td>SL, amnion</td>
<td>21.9 ± 14.4</td>
<td>2.14 ± 0.84</td>
<td>0.20 ± 0.09</td>
<td>1.14 ± 0.03</td>
</tr>
<tr>
<td>SL, chorion</td>
<td>10.1 ± 4.3</td>
<td>1.45 ± 0.83</td>
<td>0.12 ± 0.05</td>
<td>1.19 ± 0.05</td>
</tr>
</tbody>
</table>

### Table 3.9: Microstructural data of the samples used for uniaxial tension testing: collagen and elastin content in percentage per dry weight (DW) and sample thickness. All quantities are given as membrane specific averages.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Collagen [%DW]</th>
<th>Elastin [%DW]</th>
<th>Thickness [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1</td>
<td>10.9 ± 0.6</td>
<td>13.9 ± 5.3</td>
<td>385 ± 27</td>
</tr>
<tr>
<td>U2</td>
<td>9.3 ± 0.2</td>
<td>15.5 ± 1.6</td>
<td>471 ± 88</td>
</tr>
<tr>
<td>U3</td>
<td>5.9 ± 0.9</td>
<td>19.3 ± 5.1</td>
<td>464 ± 55</td>
</tr>
<tr>
<td>U4</td>
<td>12.5 ± 2.3</td>
<td>12.1 ± 2.0</td>
<td>593 ± 20</td>
</tr>
<tr>
<td>U5</td>
<td>9.8 ± 0.7</td>
<td>17.1 ± 3.4</td>
<td>421 ± 22</td>
</tr>
<tr>
<td>U6</td>
<td>10.5 ± 2.6</td>
<td>14.5 ± 4.0</td>
<td>448 ± 25</td>
</tr>
<tr>
<td>U7</td>
<td>8.9 ± 0.5</td>
<td>11.3 ± 5.2</td>
<td>698 ± 91</td>
</tr>
<tr>
<td>U8</td>
<td>10.5 ± 0.5</td>
<td>23.2 ± 4.1</td>
<td>369 ± 83</td>
</tr>
<tr>
<td>U9</td>
<td>12.2 ± 3.1</td>
<td>20.3 ± 6.7</td>
<td>485 ± 44</td>
</tr>
<tr>
<td>U10</td>
<td>11.6 ± 1.0</td>
<td>14.0 ± 6.3</td>
<td>383 ± 40</td>
</tr>
</tbody>
</table>

### 3.8 Histological properties

#### 3.8.1 Data of membranes used for uniaxial tension tests

The histological parameters of the ten membranes used for uniaxial mechanical characterization are summarized as membrane specific averages in Table 3.9. The quantities were obtained from three tissue samples (for each membrane) left over after extraction of the samples used for mechanical testing. The overall averaged collagen content is $(10.9 ± 4.6)\%$ per dry weight (DW) and the average elastin content is $(15.8 ± 5.4)\%$DW. Average thickness of the tissue samples is $(472 ± 114)\mu m$. 
3.8. Histological properties

Table 3.10: Microstructural data of the membranes used for inflation testing on intact FM samples: collagen and elastin content in percentage per dry weight (DW), and concentrations of pyridinium cross-links PYD and DPD. Collagen, elastin, and cross-link measurements were performed on three samples for each membrane from the material left over after extraction of the samples used for mechanical testing. Thickness was measured sample specific after mechanical testing.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I1</td>
<td>21.9 ± 0.2</td>
<td>19.0 ± 2.3</td>
<td>10976 ± 410</td>
<td>677 ± 108</td>
<td>608 ± 152</td>
</tr>
<tr>
<td>I2</td>
<td>25.6 ± 0.0</td>
<td>16.6 ± 2.8</td>
<td>9569 ± 2589</td>
<td>457 ± 146</td>
<td>263 ± 117</td>
</tr>
<tr>
<td>I3</td>
<td>21.7 ± 1.3</td>
<td>17.0 ± 1.8</td>
<td>7071 ± 1144</td>
<td>359 ± 50</td>
<td>451 ± 288</td>
</tr>
<tr>
<td>I4</td>
<td>9.9 ± 0.2</td>
<td>16.9 ± 0.7</td>
<td>3509 ± 808</td>
<td>131 ± 37</td>
<td>644 ± 101</td>
</tr>
<tr>
<td>I5</td>
<td>18.5 ± 0.2</td>
<td>16.9 ± 1.7</td>
<td>7071 ± 1062</td>
<td>364 ± 73</td>
<td>453 ± 19</td>
</tr>
<tr>
<td>I6</td>
<td>16.9 ± 0.8</td>
<td>15.6 ± 0.9</td>
<td>3179 ± 1228</td>
<td>153 ± 105</td>
<td>545 ± 167</td>
</tr>
<tr>
<td>I7</td>
<td>17.3 ± 1.1</td>
<td>18.0 ± 1.1</td>
<td>3556 ± 255</td>
<td>187 ± 18</td>
<td>561 ± 255</td>
</tr>
<tr>
<td>I8</td>
<td>17.1 ± 1.2</td>
<td>16.2 ± 2.2</td>
<td>2654 ± 544</td>
<td>89 ± 22</td>
<td>437 ± 139</td>
</tr>
<tr>
<td>I9</td>
<td>15.1 ± 0.9</td>
<td>17.4 ± 2.2</td>
<td>2266 ± 25</td>
<td>97 ± 19</td>
<td>541 ± 117</td>
</tr>
<tr>
<td>I10</td>
<td>12.6 ± 0.8</td>
<td>21.4 ± 0.8</td>
<td>1502 ± 582</td>
<td>39 ± 14</td>
<td>697 ± 195</td>
</tr>
<tr>
<td>I11</td>
<td>18.0 ± 1.0</td>
<td>15.8 ± 2.3</td>
<td>2070 ± 804</td>
<td>87 ± 27</td>
<td>487 ± 139</td>
</tr>
<tr>
<td>I12</td>
<td>26.2 ± 3.5</td>
<td>20.7 ± 2.8</td>
<td>3774 ± 886</td>
<td>162 ± 42</td>
<td>430 ± 108</td>
</tr>
</tbody>
</table>

3.8.2 Data of membranes used for inflation testing

Table 3.10 summarizes the microstructural data of the 12 membranes which underwent inflation testing. Resulting from the histological assays, the contents of the microstructural constituents are given as mg of collagen or elastin per g dry weight of the tissue sample and converted to % per dry weight (DW). Average contents of collagen and elastin (N=12) are (18.4 ± 4.9) %DW, and (17.6 ± 1.9) %DW, respectively. Note that the difference in the collagen content between the membranes used for inflation testing and the membranes used for tensile testing is statistically significant (p < 0.05). The concentrations of the pyridinium cross-links PYD and DPD was determined by HPLC method. Average concentration (N=12) of PYD is (4766 ± 3125) nmol/l and DPD (233 ± 191) nmol/l, giving an average PYD/DPD ratio of (23.8 ± 5.7)

3.8.3 Data of samples used for inflation testing on separate layers

A total of 13 FM samples subdivided in single amnion and chorion layers were used to analyze the single layer mechanical behavior as well as corresponding microstructural
Table 3.11: Microstructural data of the samples used for inflation testing on separate amnion and chorion layers: collagen and elastin content in percentage per dry weight (DW) and individual sample thickness. All quantities are given as sample specific values. Table entry “-” denotes that the measurement failed and entry “*” denotes that a tissue sample adjacent to the original one was used for the extraction of the histological parameters.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Collagen [%DW]</th>
<th>Elastin [%DW]</th>
<th>Thickness [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amnion</td>
<td>Chorion</td>
<td>Amnion</td>
</tr>
<tr>
<td>L1-S1</td>
<td>60.4</td>
<td>29.2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>L1-S2</td>
<td>28.7</td>
<td>12.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>L2-S2</td>
<td>26.4</td>
<td>8.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>L2-S3</td>
<td>30.9</td>
<td>7.5</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>93</td>
</tr>
<tr>
<td>L2-S4</td>
<td>33.4</td>
<td>11.9</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>63</td>
</tr>
<tr>
<td>L3-S1</td>
<td>31.8</td>
<td>13.5</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>L3-S2</td>
<td>32.0</td>
<td>13.2</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>66</td>
</tr>
<tr>
<td>L4-S1</td>
<td>32.4</td>
<td>23.4</td>
<td>21.8*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>56</td>
</tr>
<tr>
<td>L4-S2</td>
<td>21.1</td>
<td>7.7</td>
<td>15.3*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>82</td>
</tr>
<tr>
<td>L4-S3</td>
<td>7.1</td>
<td>18.9</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>122</td>
</tr>
<tr>
<td>L5-S2</td>
<td>19.8</td>
<td>10.6</td>
<td>22.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>L5-S3</td>
<td>22.6</td>
<td>11.3</td>
<td>23.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>L5-S4</td>
<td>31.6</td>
<td>11.7</td>
<td>19.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>140</td>
</tr>
</tbody>
</table>

constituents. Table 3.11 summarizes the data of the histological analysis of the separate layers. All measurements were performed sample specific, for which reason only one measurement per sample was possible. As can be seen in Table 3.11 the set of histological data is not complete. Due to experimental difficulties, extraction of biochemical data failed for some samples. However, the corresponding data set of mechanical parameters is complete, which was considered to be more important for the current work. The content of the microstructural constituents collagen and elastin is reported as % per dry weight (DW). The overall averaged collagen content is $(29.1 \pm 12.0)\%$DW for amnion and $(13.8 \pm 6.4)\%$DW for chorion. Similarly, the averaged elastin content of amnion and chorion is $(21.4 \pm 5.4)\%$DW and $(20.9 \pm 4.9)\%$DW, respectively. The averaged thickness of the amnion layer is $(72 \pm 31)\mu$m and the one of chorion $(320 \pm 131)\mu$m.

3.9 Correlations between mechanical parameters and microstructural constituents

Coefficients of correlation were calculated between the microstructural data and the mechanical parameters of the intact FM samples used for inflation testing, see Table 3.12.
Table 3.12: Coefficients of correlation $R$ between microstructure and mechanical parameters. Values in bold are statistically significant ($p < 0.05$).

<table>
<thead>
<tr>
<th></th>
<th>Collagen</th>
<th>Elastin</th>
<th>PYD</th>
<th>DPD</th>
<th>Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_1$</td>
<td>-0.207</td>
<td>-0.004</td>
<td>-0.099</td>
<td>-0.140</td>
<td>0.119</td>
</tr>
<tr>
<td>$K_2$</td>
<td>0.689</td>
<td>0.263</td>
<td><strong>0.824</strong></td>
<td><strong>0.764</strong></td>
<td>-0.460</td>
</tr>
<tr>
<td>$T_{crit}$</td>
<td>0.705</td>
<td>0.390</td>
<td><strong>0.760</strong></td>
<td><strong>0.801</strong></td>
<td>-0.160</td>
</tr>
<tr>
<td>$p_{max}$</td>
<td><strong>0.808</strong></td>
<td>0.432</td>
<td><strong>0.741</strong></td>
<td><strong>0.740</strong></td>
<td>-0.314</td>
</tr>
<tr>
<td>$\lambda_{crit}$</td>
<td>0.527</td>
<td>0.242</td>
<td>0.439</td>
<td>0.514</td>
<td>-0.098</td>
</tr>
</tbody>
</table>

The corresponding data of FM samples that underwent uniaxial tensile testing can be found in appendix A. The small stretch stiffness $K_1$ correlates neither with the elastin content nor with the collagen content. There is a distinct correlation between the high stretch stiffness $K_2$ and the collagen content as well as between the critical membrane tension and the collagen content, Figure 3.11. Furthermore, there is a pronounced proportionality between the mechanical data of high stretch stiffness, critical tension as well as maximum pressure and the amount of PYD as well as DPD cross-links, as illustrated in Figure 3.12. No correlation is present between elastin content and the values of membrane stiffness, critical tension, and critical stretch.

3.10 Failure behavior of fetal membrane tissue

3.10.1 Rupture sequence and position for inflation testing on intact FM

Different rupture sequences (amnion first vs. chorion first) and different positions of initiation of rupture were observed during the inflation tests. In 37 cases amnion ruptured before chorion (in 11 cases with fluid leaking through amnion and separating the two layers). Chorion ruptured before amnion in only eight cases. For six samples both layers ruptured simultaneously. In addition to the visual assessment of the rupture sequence, the pressure histories were also analyzed. Figure 3.13 illustrates two characteristic pressure courses. Rupture of the membrane is characterized by a sudden decrease of the pressure value which reached a first maximum $p_1$. If only one layer of the membrane ruptured, a subsequent increase of the pressure can be observed which reaches a second maximum $p_2$. Two characteristic cases can be distinguished: if amnion ruptures first, the second maximum is clearly smaller than the first $p_1 > p_2$. In contrast, there is a pronounced
Figure 3.11: Correlations between microstructure and mechanics. (a) correlation between collagen content and high stretch stiffness $K_2$, (b) correlation between collagen content and critical membrane tension. Solid line: regression line, dashed line: 95% confidence interval.

Figure 3.12: Correlations between microstructure and mechanics. (a) correlation between content of PYD cross-links and high stretch stiffness $K_2$, (b) correlation between PYD cross-links and critical membrane tension. Solid line: regression line, dashed line: 95% confidence interval.
increase of the pressure after the first drop up to almost the same pressure level \( p_2 \approx p_1 \), if chorion ruptures first. These pressure versus time characteristics were in 75\% of the cases in agreement with the visual observations. As to the rupture position, it was distinguished if the sample ruptured in the center, 27 cases, away from the clamping cover, 10 cases, or very close to or at the clamping ring, 14 cases.

![Figure 3.13: Characteristic pressure vs. time curves. (a) Amnion rupture first, characterized by \( p_1 > p_2 \). (b) Chorion rupture first, characterized by \( p_1 \approx p_2 \).](image)

**Figure 3.13:** Characteristic pressure vs. time curves. (a) Amnion rupture first, characterized by \( p_1 > p_2 \). (b) Chorion rupture first, characterized by \( p_1 \approx p_2 \).

### 3.10.2 Separation of amnion and chorion in tensile tests

Assessment of rupture properties in terms of maximum tension or stress is difficult to obtain in uniaxial tension tests, since rupture often occurs close to the clamping site and is therefore influenced by boundary effects. Nevertheless, a consistent separation of the amnion and chorion layer prior to rupture was observed during the tension tests. The fetal membrane is composed of two main layers, amnion and chorion, which are interconnected by an intermediate layer, as described in section 2.2. Recorded image series during the tension test show that both layers contract together at the beginning of the tension test. At a certain level of longitudinal deformation amnion starts to separate from chorion. This separation is caused by a difference in the layer specific contraction behavior (Poisson’s effect) with amnion displaying a much greater lateral contraction than chorion, as illustrated in Figure 3.14.

Accurate analysis of the onset of the separation process is difficult due to difficulties involved in the image analysis for tests performed in saline solution. Furthermore also bad light conditions and insufficient contrast of the sample surface structure complicate
the image analysis. Four additional samples were tested with improved light conditions. Analysis of these samples revealed that the separation of the layers starts already at a level of approximately 5% of longitudinal strain with respect to the common reference configuration.

Figure 3.14: Image of an intact FM sample during a tension test. Amnion and chorion start to separate at a level of approximately 5% longitudinal strain. The image indicates that the separation is caused by a difference in the layer specific contraction behavior.

3.11 Discussion

This work aims at characterizing the mechanical behavior of the human FM as well as its microstructure. In fact, several measurements were performed to quantify the mechanical response and the corresponding microstructural constituents of intact fetal membranes and separate amnion and chorion layers. The corresponding data are reported in various tables and illustrate the variability and uncertainty of the measurements. Due to these uncertainties, it is important to compare parameter values obtained in the present work with corresponding findings reported in the literature.

3.11.1 Mechanical parameters of intact FM under uniaxial tension

Uniaxial tension testing was applied several times to characterize the mechanical behavior of the FM tissue. However, most of the studies report only values that characterize the failure strain and the maximum tension and not the nonlinear behavior of the stress-strain curve. For parameters comparable to the present high stretch stiffness $K_2$, values range from $0.87 \text{ N/mm}$ (Oxlund et al., 1990) to $0.92 \text{ N/mm}$ (Jabareen et al., 2009) up to
3.11. Discussion

1.35 N/mm (Helmig et al., 1993). These values are in good agreement to the present $K_2$ of $(1.15 \pm 0.37)$ N/mm. Maximum uniaxial membrane tension (nominal values) has often been characterized, leading to values of 0.15 N/mm (Jabareen et al., 2009), 0.25 N/mm (Oxlund et al., 1990), 0.36 N/mm (Helmig et al., 1993), 0.44 N/mm to 0.85 N/mm (Artal et al., 1976) and 0.98 N/mm (Artal et al., 1979). Our finding of the average critical tension of $(0.20 \pm 0.06)$ N/mm is within the reported ranges. However, the initial stiffness of $(0.9 \pm 0.2) \cdot 10^{-2}$ N/mm from our previous uniaxial study (Jabareen et al. 2009) was lower than the present value of $(2.8 \pm 1.7) \cdot 10^{-2}$ N/mm. In the same way, the average critical deformation or strain at rupture from this study with a value of $(32 \pm 9)$ % is significantly smaller compared to uniaxial studies, obtaining values around 50 % (Prevost, 2004; Helmig et al., 1993; Jabareen et al., 2009) or even up to 70 % to 100 % (Artal et al., 1979, 1976). Differences in the strain at rupture can be related to differences in the definition of the reference state, the sample fixation, and possible mechanisms of sample slippage. In fact, rupture of the FM sample in the present tests often happened at the clamping site.

Large scatter in data characterizing the mechanical behavior is common for biological tissues. Nevertheless, there is a pronounced difference between the current study and our previous uniaxial study (Jabareen et al., 2009) for parameters quantifying the initial stiffness and the strain at rupture. The reason for the deviation might be related to the implementation of the preconditioning cycles. In the current work a meticulous definition of the strain amplitude within the preconditioning was used, since the maximum strain of 20 % refers to a repeatable reference configuration which is defined by a force threshold value. On the other hand, the strain amplitude during preconditioning in the previous study might have been determined according to the initial free sample length. Furthermore, the data in section 3.7.1 indicate that there is a large change in the initial stiffness during preconditioning and afterward. The initial stiffness of the virgin response (first loading ramp of preconditioning) is with a value of $(1.0 \pm 0.5) \cdot 10^{-2}$ N/mm very close to the value of $(0.9 \pm 0.2) \cdot 10^{-2}$ N/mm of our previous study. This indicates that the effective strain amplitude during precondition of our previous study was smaller than the specified 20 % nominal strain.

3.11.2 Mechanical parameters of intact FM under equibiaxial tension

A direct comparison of the determined mechanical parameters with the corresponding values reported in the literature is often difficult due to differences in the experimental
setup and procedure. Most of the previous work with inflation experiments focused on the determination of the strength of the FM, so that often only values of burst pressure (MacLachlan, 1965) or maximum deflection (Parry-Jones and Priya, 1976; Al-Zaid et al., 1980a) were determined. These values cannot be compared with the present data, since they depend on the size of the FM sample. Using Laplace’s law, Polishuk et al. (1962) and (Lavery and Miller, 1979; Lavery et al., 1982) determined the critical stress at rupture and the critical membrane tension in their inflation experiments. The membrane’s “stress tolerance” in Lavery et al. (1982) can be converted, with the assumption of an average membrane thickness of 0.5 mm, to a critical membrane tension, providing values between 0.37 and 0.45 N/mm. Polishuk determined the critical membrane tension to be 0.21 N/mm which is in good agreement to the herein determined value of (0.25 ± 0.07) N/mm, but which in contrast is considerably higher than the maximum membrane tension of 0.04 N/mm determined by Joyce (2009), based on planar biaxial tests. Joyce (2009) has also evaluated a maximum tangent modulus within planar biaxial testing of (0.66 ± 0.09) N/mm, which is lower than the present average value of the high stretch stiffness of (2.64 ± 0.49) N/mm. In agreement with the study of Schober et al. (1994a) no correlation between the sample thickness and the mechanical parameters characterizing strength and ductility could be found in the present work.

Comparison of the data characterizing the uniaxial and biaxial behavior from the present work reveals pronounced differences between the two load cases. The initial stiffness of (10.5 ± 3.1) · 10⁻² N/mm was found to be considerably higher than the corresponding uniaxial stiffness of (1.0 ± 0.5) · 10⁻² N/mm (virgin response). Also the high stretch stiffness of (2.64 ± 0.49) N/mm is higher than the corresponding uniaxial value of (1.15 ± 0.37) N/mm. In the same way the strain at rupture within equibiaxial loading with a value of (19.5 ± 3.4) % is smaller compared to the uniaxial value of (32.9 ± 9.0) %. An improved setup for inflation experiments is currently being developed using an additional camera placed above the sample and a light source within the cylinder, with and without additional markers on the membrane. Extraction of local strain fields in preliminary experiments proved difficult due to membranes inhomogeneity leading to a complex displacement field. Digital image correlation was used to determine radial and circumferential displacement at the boundaries of a “central region”, from which an “average” biaxial strain can be extracted for the apex. The failure strain measured at the apex in these preliminary investigations was around 20 %. Joyce (2009) determined the maximum areal strain in planar biaxial tests to be (0.60 ± 0.03), which corresponds to 26 % in-plane strain according to our definition. This difference between uniaxial and biaxial initial stiffness and critical deformation is related to the larger compliance ob-
served in uniaxial tensile tests, which might be associated with significant reorientation of collagen fibers. In contrast, as demonstrated by (Joyce, 2009), a biaxial state of stress causes only a slight increase in fiber alignment in the amnion layer for small pressure loads and no change in fiber orientation thereafter, thus leading to a reduction in the deformation capacity and to an initially stiffer response. Moreover, differences in the reference configuration can also contribute to an initially stiffer response as well as to smaller values of critical deformations. As shown in section 3.7.4 the averaged initial membrane tension used for the normalization of the raw data of the inflation tests is a value of \((21 \pm 4) \cdot 10^{-4} \text{N/mm}\), significantly larger than the corresponding threshold of \(7 \cdot 10^{-4} \text{N/mm}\) used for the uniaxial tests.

As shown in section 3.7.2, the membrane specific values of the mechanical parameters \(K_2\) and \(\lambda_{\text{plane}}\) in Table 3.6 depend on the constitutive model formulation used for the analysis of the inflation experiment. Table 3.13 reports the averages of these parameters for each of the model equation investigated. The differences are of the order of 15% of the respective parameters, except the high stretch stiffness which shows the strongest dependence on the constitutive model formulation, with a deviation > 50% between the Ogden model and the other formulations. The side view profile of selected membranes was analyzed and the contour (as well as the area enclosed within the contour) compared with corresponding finite element model predictions. Good agreement between calculations and measurements were obtained with the reduced polynomial and with the Rubin-Bodner model, whereas the predictions using the Ogden model did not match the data well. The correlation coefficients reported in Table 3.12 were calculated for the reduced polynomial \(I_2\) model, but corresponding evaluations using the other models lead to similar results. Inhomogeneity in the tissue causes variations in the measurements within one membrane as well as between membranes from different donors, as illustrated in Figure 3.8. A quantitative measure of the variation is the coefficient of variation (COV). Calculation of the intra (within one membrane) and inter (within averages from different membranes) coefficient of variation enables the assessment of the accuracy of the measurements, see Table 3.14. For the present study the intra COV of the mechanical parameters characterizing the strength, stiffness, and failure stretch ranges from 10% to 26% and the corresponding inter COV from 18% to 33%. For the study of Schober et al. (1994a), the intra COV is 16% to 34% and the inter COV 10% to 60%, evaluated for the same mechanical parameters. For our previous uniaxial study (Jabareen et al., 2009), the corresponding variations are 7% to 21% and 55% to 63%, respectively. The variations of the present study are at the lower limit of the reported ranges, confirming the reliability of the experimental setup and the procedures used for data analysis.
Table 3.13: Comparison of the mechanical parameters from the stress-strain curves for different constitutive models.

<table>
<thead>
<tr>
<th>Model</th>
<th>$K_2$ [N/mm]</th>
<th>$\lambda_{crit}$ [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced Polynomial</td>
<td>2.64 ± 0.49</td>
<td>1.20 ± 0.03</td>
</tr>
<tr>
<td>Rubin-Bodner</td>
<td>2.24 ± 0.39</td>
<td>1.22 ± 0.04</td>
</tr>
<tr>
<td>Ogden</td>
<td>1.42 ± 0.25</td>
<td>1.30 ± 0.07</td>
</tr>
</tbody>
</table>

Table 3.14: Evaluation of the intra and inter coefficient of variation between different studies for the assessment of the reliability of the present inflation setup.

<table>
<thead>
<tr>
<th>Study</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>intra</td>
</tr>
<tr>
<td>Inflation, intact FM</td>
<td>10%</td>
</tr>
<tr>
<td>Schober et al. (1994a)</td>
<td>16%</td>
</tr>
<tr>
<td>Jabareen et al. (2009)</td>
<td>7%</td>
</tr>
</tbody>
</table>

3.11.3 Mechanical parameters of separate amnion and chorion layers (inflation experiment)

Several studies were performed to analyze the individual contributions of the separate amnion and chorion layers. But, to the best of the author’s knowledge, only one used the method of inflation testing. Polishuk et al. (1962) report values for the critical membrane tension of amnion and chorion as 0.17 N/mm and 0.12 N/mm, respectively. Manabe et al. (1991) analyzed only the inflation response of the amnion layer and report values of the critical membrane tension of 0.1 N/mm to 0.15 N/mm. These values are in good agreement to the herein determined values of (0.20 ± 0.09) N/mm for amnion and (0.12 ± 0.05) N/mm for chorion.

Studies based on uniaxial tensile tests on separate layers report values of the critical membrane tension of amnion from 0.19 N/mm (Oxlund et al., 1990) to 0.31 N/mm (Helmig et al., 1993) and of chorion from 0.08 N/mm (Helmig et al., 1993) to 0.11 N/mm (Oxlund et al., 1990). Also these data are in the same range as the values determined in the current work. Values characterizing the high stretch stiffness are in the range of 0.89 N/mm to 1.39 N/mm for amnion and 0.36 N/mm to 0.41 N/mm (Oxlund et al., 1990; Helmig et al., 1993) for chorion, significantly smaller than the present values of (2.14 ± 0.80) N/mm and (1.45 ± 0.80) N/mm for amnion and chorion. Deviations between these parameters might be related to the general differences between uniaxial and equibiaxial loading, as discussed in the previous section.
3.11. Discussion

Comparison of the mechanical parameters characterizing the inflation response of intact FM and the separate layers (see Table 3.8) shows a remarkable high initial stiffness and low strain at rupture for amnion. On the contrary, corresponding values of chorion agree well with the values of the intact FM. The low values of critical strain of amnion can be explained by the fact that eight out of 13 samples ruptured at the border of the clamping, which might have caused a premature failure. The high initial stiffness of the separate amnion layer leads to the hypothesis that the initial stiffness of intact FM is mainly determined by chorion. The fact that the initial membrane tension of amnion is comparable to the corresponding values of chorion and intact FM (see section 3.7.4) supports this interpretation.

The critical membrane tension of amnion and chorion is approximately in the same range. Nevertheless, if it is considered that chorion is approximately four times thicker than amnion it follows that the critical stresses differ by approximately one order of magnitude. Therefore, amnion is considered as the mechanically dominant layer of the fetal membrane. Different studies followed the fundamental works of Polishuk et al. (1962); Oxlund et al. (1990); Helmig et al. (1993) and focused only on the amnion layer. MacDermott and Landon (2000) and Wilshaw et al. (2006) performed tensile tests on fresh amnion and reported values of the critical membrane tension of $(0.11 \pm 0.05) \text{ N/mm}$ to $(0.19 \pm 0.14) \text{ N/mm}$. The corresponding strains at rupture range from 30% to 37%, the smallest that can be found in the literature, and are the closest to the values determined in the current work. Deviations to the present values of strain at rupture can be explained by the different test methods (inflation vs. tensile testing) and the corresponding detection of failure. It is worth mentioning that the study of Wilshaw et al. (2006) is one of the few that reports a value of a force threshold $(0.01 \text{ N})$ utilized for the definition of the reference configuration.

3.11.4 Mechanical behavior of fetal membrane tissue

The model formulations used for the analysis of the present data can be fitted also to corresponding uniaxial stress experiments (Jabareen et al. 2009). Both the $I_1$ and the principal stretch based model equations (with parameters selected to fit the average uniaxial response) largely underestimate the resistance to deformation in inflation experiments, see Figure 3.15. Better predictions are obtained when the model formulation is based on the second invariant $I_2$ of the right Cauchy-Green deformation tensor $C$. It can be shown that $I_1$ is proportional to the average (squared) stretch of the sides of an infinitesimal volume element whereas $I_2$ is proportional to the average area stretch of the faces of an
Chapter 3. Mechanical characterization of human fetal membrane tissue

infinitesimal volume element. The fact that fetal membrane behaves as an $I_2$ material might be associated with the network-like arrangement of the collagen, suggesting a response similar to that of a textile, with much higher resistance to changes in the area (in the plane of the membrane) than to elongations in a uniaxial state of stress.

Miller et al. (1979) optimized a Mooney material, which includes by definition the first and second invariants of $C$, on the response of FM. They reported that each value of $C_2$, the multiplier of $I_2$, was 0.1 times the value of $C_1$. Based on the corresponding strain energy formulation and the assumption of an average membrane thickness of 0.5 mm, the initial slope of the tension-stretch curve can be calculated. The corresponding values range from 0.066 N/mm to 0.99 N/mm with a population peak at 0.33 N/mm. Our finding of an average low stretch stiffness $K_1$ of $(0.105 \pm 0.031)$ N/mm is in line with these data.

Prevost (2004) and Joyce (2009) optimized structural constitutive models on the biaxial response of FM. Prevost worked with a square unit cell model consisting of four individual chains, where each chain represents a collagen fibril and has a simple force-stretch relationship. He achieved a good prediction of the uniaxial response but failed to capture the initial compliant response under biaxial stress. The structural constitutive models used by Joyce (2009) are based on the tissue behavior on the fiber level and include a statistical distribution of the fiber recruitment. Those models fitted well the biaxial response of FM. It is interesting to note that both Prevost and Joyce worked with experimental stress-strain curves which were characterized by no initial stiffness, since they have chosen a non pre-stretched reference configuration in their experiments. As a consequence, Prevost’s simulation could only be related to the experimental data after a corresponding horizontal shift. Joyce overcomes this problem by the introduction of a so called “slack strain”, which characterizes the initial undulation of the tissue.

3.11.5 Histological and biochemical data

Reported values for the collagen content of intact fetal membranes range from 4% to 20% (Hampson et al., 1997; Jabareen et al., 2009) of dry weight, which is in line with the present values of $(10.9 \pm 4.6)\%\text{DW}$ to $(18.4 \pm 4.9)\%\text{DW}$. The amount of elastin in the present work was determined to be in the range of $(15.8 \pm 5.4)\%\text{DW}$ to $(17.6 \pm 1.9)\%\text{DW}$. This result agrees with the one obtained in Jabareen et al. (2009) in terms of average and variability if the present values are expressed as percentage of wet weight ($(2.50 \pm 0.44)\%\text{WW}$ vs. $(2.10 \pm 0.72)\%\text{WW}$), but differs evidently from the 0.08% of fat free dry weight (Hieber et al., 1997) or 36% of (total) wet weight of fresh amnion (Wilshaw et al., 2006). Note that accounting for scatter in water content significantly increases the variability of
Figure 3.15: Pressure vs. apex displacement curves from inflation experiments on fetal membrane samples (membrane specific averages plotted as continuous lines), and model predictions based on average stress-strain data of uniaxial tension tests. The simulation results have been obtained by the use of the parameters in Table 3.2. The Ogden and the Rubin-Bodner model underestimate the initial stiffness in inflation tests.

The data. The large deviations in the reported values of the elastin content point to the general difficulty of its extraction and measurement using biochemical methods. Although the values determined in the present work are consistent within our studies, a systematic error in the assay cannot be excluded and thus the present findings cannot be validated. Micrographs of FM samples taken by a multi photon microscope show no elastin within the FM structure. However, this might be caused by the fact that the size of the elastin fibers is below the optical resolution. The fact that the content of elastin might be very low was confirmed by Dr. M. Oyen (personal communication). In conclusion, the role of elastin in contributing to the mechanical behavior of FM remains unclear.

The average collagen content of amnion was determined as \((29.1 \pm 1.2)\%\text{DW}\) and of chorion as \((13.8 \pm 6.4)\%\text{DW}\). These values are also in agreement to values reported in the literature. Halaburt et al. (1989), Meinert et al. (2001), and Prevost (2004) report values for the collagen content of amnion in the range of \(31\%\text{DW}\) to \(42\%\text{DW}\) and for chorion of \(11.7\%\text{DW}\) to \(15.5\%\text{DW}\), thus concluding that amnion contains approximately twice the amount of collagen as chorion. To the best of the author’s knowledge, apart from the above mentioned studies of Hieber et al. (1997), Wilshaw et al. (2006), and Jabareen et al. (2009) which measured the elastin content of intact FM or amnion, there is no study reporting values of the elastin content of chorion. Also the averaged thickness values, \((72 \pm 31)\,\mu\text{m}\) of amnion and \((320 \pm 131)\,\mu\text{m}\) of chorion, match well the values reported in the literature (Halaburt et al., 1989; Meinert et al., 2001; Prevost, 2004).
Differences mainly in the thickness of the chorion layer can be attributed to different measurement methods. There is only one study by Stuart et al. (2005) reporting values of pyridinoline and deoxypyridinoline cross-links in human amnion. The reported pyridinoline concentrations range from 7.9 nmol/µmol to 8.3 nmol/µmol hydroxiproline and the corresponding values of deoxypyridinoline from 0.26 nmol/µmol to 1.02 nmol/µmol hydroxiproline, giving an average PYD/DPD ratio of 18.4. Our findings of averaged PYD and DPD contents of (4766 ± 3125) nmol/l and (233 ± 191) nmol/l, respectively can not be compared directly since they result from measurements on intact membranes. Nevertheless, our present average PYD/DPD ratio of (23.8 ± 5.7) is in good agreement with Stuart et al. (2005).

Pyrodinoline cross-links are a mature form of cross-links associated with collagen I, but not with collagen type IV, (Avery and Bailey, 2008; Eyre and Wu, 2005). These cross-links occur between tropocollagens to form fibrils and also between fibrils for higher level organization. Type IV collagen contains keto-amine (immature) and di-sulphide cross-links, not quantified in this study. Tissues with high collagen turnover, and in particular fetal tissues, contain larger amounts of immature cross-links (also for fibrillar collagen). Immature cross-links are thus expected to be relevant in this tissue, as part of type IV collagen as well as in developing fibrillar collagen. A full rationalization of mechanical behavior would thus require a description of cross-linking maturity.

3.11.6 Correlation between mechanical and microstructural data

The high stretch stiffness as well as the critical membrane tension were found to be proportional to collagen content and the concentrations of the PYD and DPD cross-links. Multiple linear regression analysis shows that the cross-links and collagen content do not correlate independently with mechanical parameters. Since mature cross-links (PYD, DPD) are associated only with fibrillar collagen in its mature form, this finding might indicate that total collagen is predominantly made of fibrillar collagen. The values reported in Table 3.12 show somewhat larger correlations between membrane mechanical parameters (high strain stiffness, $K_2$, and critical tension, $T_{crit}$) and pyridinoline cross-links. A lower correlation for the cross-links would have indicated an important contribution of non-fibrillar collagen to mechanical parameters. Thus, the present observations do not support previous hypotheses (Bachmaier and Graf, 1999; Moore et al., 2006) which attributed to collagen type IV a significant role in mechanical strength of fetal membranes. Collagen type IV contributes to fetal membranes microfibrils complex (Malak et al., 1993;
Bachmaier and Graf, 1999) which might influence small strain deformation behavior. The absence of correlation between $K_1$ and collagen content might be due to the lower amount of type IV in total collagen as compared to fibrillar collagen. Quantitative determination of each collagen type, as well as divalent and keto-amine cross-links would enable identifying the contribution of fibrillar and non-fibrillar collagen to the mechanical behavior of fetal membranes.

The organization of collagen fibrils in loose bunches, not forming higher-order bundles, determines the deformability of fetal membranes. In this sense it is interesting to compare mechanical and microstructural characteristics of amnion and liver capsule, see Hollenstein (2011). Although the amnion contains a similar amount of collagen (per dry weight) compared to liver capsule, and it is of the same thickness, it is much softer than the capsule. The difference is related to the fact that, in contrast to amnion, collagen in liver capsules form thicker (4 µm to 6 µm) fiber bundles.

In contrast to our previous observations from uniaxial stress tests (Jabareen et al., 2009), no correlation could be observed between the elastin content and low stretch resistance to deformation within equibiaxial tension. Moreover, an attempt to reproduce the data of Jabareen et al. (2009) illustrates the difficulties involved in the determination of the initial response of soft biological tissues and its microstructural constituents. Analysis of the data from uniaxial tension tests (section 3.7.1) and the corresponding biochemical data (Table 3.9) lead only to very weak correlations between the initial stiffness and the elastin content (see appendix A). This discrepancy might be interpreted with respect to the role of microstructural constituents in the deformation behavior as well as by the general difficulties and uncertainties related to the determination of the elastin content. We hypothesize that in the case of uniaxial stress, collagen fibers are reoriented at low strains, such that resistance to deformation is provided by the elastin matrix. On the other hand, in case of a biaxial state of stress collagen fibers contribute to low deformation resistance, in that the initially crimped collagen fibers are straightened in all directions of the membrane plane, without major global reorientation. The mechanisms of progressive fiber recruitment and un-crimping and its relationship with the highly nonlinear tissue response (leading to the transition from low to higher stiffness) are well described in the literature (Lanir, 1983; Sacks and Wei, 2003; Hill et al., 2012). We propose that, for the uniaxial stress state, low strain compliance is enhanced by global fiber reorientation. An additional argument supporting this interpretation is the observed increase in the value of low stretch stiffness $K_1$ in the biaxial state of stress compared to a uniaxial stress state. A recent study from our group (Mauri et al., 2013) shows that collagen fiber reorientation already happens at moderate deformations. On the other hand, it should be noted that
the low stretch stiffness value strongly depends on the definition of a reference configuration (the normalization of the pressure-displacement curves at a preload of 1 mbar in the present case, or the threshold force value used in uniaxial tensile stresses). Furthermore, low stretch stiffness values are also influenced by the history dependence of the FM mechanical response.

3.11.7 Rupture sequence in inflation experiments

Concerning the rupture sequence of FM it was observed in this study that the amnion ruptures first in 73% of the cases in inflation testing. A more recent study from our group confirms this finding. Perrini et al. (2013) found a frequency of amnion rupture first of 80% and reported a more detailed rupture sequence, i.e. amnion ruptures at the periphery of the sample within a pure-shear state rather than in the center, chorion on the contrary ruptures in the middle of the sample. The results in terms of rupture sequence in mechanical testing depend on the experimental procedures. Artal et al. (1976), Lavery and Miller (1977), and Arikat et al. (2006) found that chorion ruptures first. In contrast, Schober et al. (1994b), Helmig et al. (1993), and Oxlund et al. (1990) observed that amnion ruptures first. Differences in the rupture sequence are likely to be due to the loading configuration (uniaxial tension, puncture test, inflation) and probably also to artifacts related to sample fixation. In that regard we consider the present experimental setup more reliable in that biaxial loading is induced by fluid pressure. A detailed analysis of the rupture sequence in puncture tests can be found in chapter 5.

Perrini et al. (2013) reported that the amnion and chorion layers were separated after inflation testing. Separation of amnion and chorion has as well been observed in puncture testing by Arikat et al. (2006) who stated that the process of separation accounts for a significant fraction of the work required to rupture the sample. Consistently, in this work it was also observed that amnion and chorion separate prior to membrane failure in tensile tests. The separation of the two constitutive layers is caused by a difference in the layer specific Poisson’s effect. All these results give evidence that the intermediate or “spongy” layer of the FM fails first before the other membrane layers rupture. In agreement to this hypothesis, Mauri et al. (2013) have shown that the intermediate layer possesses an altered collagen structure after cyclic loading.
3.12 Conclusions

There are several studies covering the biochemical characteristics of premature ruptured membranes (PROM). Skinner et al. (1981) reported a significantly lower collagen content in the amnion from PROM membranes compared to membranes from patients without PROM. In contrast, Mac Dermott and Landon (2000) found no association between a reduced collagen content of amnion and PROM, and hypothesize that PROM is caused by a localized membrane weakness. Apart from that, both studies reported a decrease of the collagen content, in the amnion layer, with progressing gestational age. More recently, Stuart et al. (2005) confirmed the reduced collagen content in prelabour membranes, but found no difference in the collagen content between samples from the rupture site and the non-rupture site. Furthermore, there was no difference in the collagen cross-link profile between the PROM and control group, but there was a regional variation in the cross-link ratio (PYD/DPD) for samples from the rupture site. Stuart et al. (2005) concluded that collagen cross-linking is not involved in the etiology of PROM, but that the formation of rupture initiation is a function of the regional variation in the cross-link ratio. Our results clearly indicate that a weakening of the membrane is expected if the collagen content is reduced or less cross-links are present. This supports the hypothesis that PROM might be associated with a (local) decrease of resistance of the collagen fiber network.

This work aimed at the mechanical and biochemical characterization of the fetal membrane tissue as well as to quantitatively analyze the relationship between microstructure and nonlinear deformation behavior. Biochemical assays were used to quantify the content of elastin and total collagen and concentrations of pyridinoline and deoxypyridinoline cross-links were determined by HPLC measurement. Low and high stretch tangent moduli ($K_1$ and $K_2$), critical membrane tension ($T_{crit}$), and failure stretch ($\lambda_{crit}$) at rupture were determined for the intact FM as well as for the separate amnion and chorion layers, based on different experimental configurations. Inflation experiments were analyzed by the use of a finite element based procedure. The influence of the constitutive model formulation on the results of this analysis was investigated. FM tissue was shown to behave as an $I_2$ material, that is, a material with higher resistance to area change as compared to elongation with free lateral contraction. Experimental results show that there is a pronounced difference between the uniaxial and equibiaxial mechanical response of the FM as well as between the contributions of the two constitutive layers amnion and chorion. Correlations were found between microstructural data and mechanical parameters: there is a distinct relationship between the total collagen content and the concentrations of PYD as well as DPD cross-links, and the me-
Mechanical parameters of high stretch stiffness as well as critical tension. No correlation was found between the elastin content and the low stretch stiffness, in contrast to a previous uniaxial study.

Immature (keto-amine and di-sulphide) cross-links are expected to be relevant in this fetal tissue, as part of type IV collagen as well as in developing fibrillar collagen. Quantitative assessment of cross-linking maturity is expected to provide relevant contributions toward rationalization of the mechanical behavior of fetal membranes. The most important improvement for future inflation experiments concerns the introduction of a robust system for image based strain measurement at the apex of the inflated membrane. In this way, uncertainties related to constitutive model formulation will be eliminated for the quantitative determination of mechanical parameters from inflation test measurements.
Development of a constitutive model for the mechanical response of fetal membranes

4.1 Introduction

The development of methods to avoid FM preterm rupture requires a better understanding of their mechanical behavior. Modeling the deformation behavior of FM is a required input for mechanical analyses aiming at understanding the physiology of FM rupture, optimizing medical procedures in order to reduce the risk of iPPROM, developing FM repair strategies, and analyzing the results of corresponding biomechanical testing procedures. Only few attempts were reported for the determination of a constitutive model of FM tissue (Miller et al., 1979; Prevost, 2004; Jabareen et al., 2009; Joyce, 2009). Most previous studies focused on the extraction of parameters to quantitatively characterize FM rupture and on the effects leading to PPROM (Artal et al., 1976; Lavery and Miller, 1979; Al-Zaid et al., 1980b). Chorioamniotic membrane separation was observed to lead to a premature rupture of the membranes in 63% of the cases after surgery (Sydorak et al., 2002). The separation of the FM layers has been observed in puncture experiments (Arikat et al., 2006). Separation of amnion and chorion in uniaxial tension tests on intact FM samples was also consistently observed in this work, see Figure 3.14. In this case, separation is caused by differences in the contraction behavior (Poisson’s effect) of the two layers, with amnion displaying a much greater lateral contraction.

The microstructure of the fetal membrane and the compositions of the different sublayers is described in section 2.2. Figure 4.1 shows scanning electron micrograph (SEM) images of a FM sample. It illustrates the crimped collagen fibers, forming a random network in the membrane plane, with no distinct directionality. The thin collagen fibers are expected to determine the deformation behavior and strength of amnion. Moreover, the previous
results in chapter 3 show a strong difference between the uniaxial and equibiaxial mechanical response of FM tissue. This difference in the mechanical response depending on the state of loading and the observed large Poisson’s effect are indication of a mechanical behavior that is determined by a network-like microstructure.

![Scanning electron micrograph of a human fetal membrane sample. Cross-sectional view (a) and top view (b) show an irregular network of single collagen fibers with no distinct directionality. With permission from Hollenstein (2011).](image)

**Figure 4.1:** Scanning electron micrograph of a human fetal membrane sample. Cross-sectional view (a) and top view (b) show an irregular network of single collagen fibers with no distinct directionality. With permission from Hollenstein (2011).

Large contraction of amnion in tensile tests had never been reported in previous works. This motivated a series of tensile tests on amnion and chorion layers with quantitative determination of lateral contraction using image correlation techniques. Observations in these tests along with considerations on mechanisms of rotation, stretching and buckling of fibers in a random network (Stylianopoulos and Barocas, 2007; Kabla and Mahadevan, 2007; Picu, 2011) led to the selection of a constitutive model formulation. Model parameters were determined to simultaneously match tension-stretch and contraction-elongation curves of tensile tests. Model response to equibiaxial tension was finally compared with corresponding data from previous measurements, section 3.7.3, in order to evaluate the predictive capabilities of the proposed equations. Finally, the constitutive model is implemented in a commercial FE code in order to use it for numerical simulations of physiological loading conditions or membranes containing lesions.
4.2 Experimental methods

4.2.1 Tensile testing of separate layers

In order to test the FM samples in a uniaxial stress configuration, the experimental setup as introduced in section 3.3.1 was used. The setup was modified with a light source underneath the water bath, which is helpful to achieve a better quality of the recorded image series, and adjusted to allow the testing of larger samples. Samples with a larger aspect ratio (80 \times 15 \text{ mm}) were used to reduce possible boundary effects from the clamping which might limit the lateral contraction in the central region of the sample. The tensile tests were performed as monotonic tension to failure with an elongation rate of 0.5\% nominal strain per second, starting from an initial free length of 60\text{ mm}. No preconditioning was applied. All tests were performed at room temperature in a bath filled with saline solution to avoid drying or swelling of the sample. The raw force and displacement data were normalized according to section 3.3.3 but with a lower force threshold of 5 \text{ mN} which accounts for the separate layers.

Data on the response of amnion and chorion in a biaxial state of stress were obtained using the inflation setup. The inflation setup, the experimental protocol (monotonic inflation to failure), and the procedures for extraction of biaxial tension-stretch curves are described in section 3.4.

Figure 4.2: Sample preparation for the tension tests on separate layers. Samples are created by the use of a die cutter (a) and markers of India ink are applied on the sample surface (b).

Fetal membranes were collected according to the protocol described in section 3.2. Amnion and chorion from fresh membranes were separated manually by gently pulling the layers apart. The separate layers were spread on a lubricated plastic mat and a die cutter (see
Chapter 4. Development of a constitutive model for the mechanical response of FM

Figure 4.3: Illustration of a quadrilateral element utilized for the evaluation of the in-plane kinematic response. A grid of $3 \times 3$ measurement points is used for the determination of the averaged nodal displacement trajectory $\hat{\mathbf{u}}$ based on digital image correlation.

Figure 4.2(a)) was used to create rectangular samples with the dimension of $80 \times 15$ mm. Small markers of India ink (Ausziehtusche Sepia, Rohrer & Klingner, Zella-Mehlis, Germany) were applied on the surface of the sample with the aid of a brush, which allows better optical tracking of the deformation field.

4.2.2 Analysis of the deformation field in uniaxial experiments

The recorded image series serves as basis for the determination of the kinematic response of the amnion and chorion layer. The digital image correlation (DIC) software VEDDAC 4.0 (Chemnitzer Werkstoffmechanik GmbH, Chemnitz, Germany) is used to trace a set of user defined measurement points corresponding to the markers in the central region of the sample. A pattern of $3 \times 4$ nodes is defined for the image analysis, where each node consists of a grid of $3 \times 3$ measurement points. Averaging of the displacement trajectories within each subgroup of measurement points provides the discrete nodal displacements $\hat{\mathbf{u}}^{(a)}$ in the global coordinate system. The resulting discrete displacement field is used for the approximation of the in-plane stretches by the use of element based interpolation functions, as applied in the finite element (FE) method. An approach based on linear quadrilateral elements was chosen to discretize the displacement field, since quadrilateral elements can be spanned adequately over the free (approximately rectangular) membrane region.

A material point originally located at $\mathbf{X}$ is mapped to its current position $\mathbf{x}$ by a displacement $\mathbf{u}$ ($\mathbf{x} = \mathbf{X} + \mathbf{u}$). This causes a deformation gradient in the form of

$$\mathbf{F}_{2d} = \frac{\partial \mathbf{x}}{\partial \mathbf{X}} = \mathbf{I}_{2d} + \frac{\partial \mathbf{u}}{\partial \mathbf{X}}$$ (4.1)
for a planar system, where $I_{2d}$ denotes the second-order identity tensor in two dimensions. The displacement field $\mathbf{u}$ can be approximated by consideration of the discrete nodal displacements $\hat{\mathbf{u}}^{(a)}$ and linear shape functions $N^{(a)} (\xi, \eta)$ as a function of the local coordinate system $(\xi, \eta)$, see Figure 4.3.

$$
\mathbf{u} = \sum_{a=1}^{4} N^{(a)} (\xi, \eta) \hat{\mathbf{u}}^{(a)}
$$

(4.2)

The superscript $(a)$ refers to the corresponding node number, i.e. $a \in \{1, 2, 3, 4\}$. This approach allows calculating the in-plane components of the element specific deformation gradient $F_{el}$ as a function of the associated nodal displacements $\hat{\mathbf{u}}^{(a)}$ and the linear shape functions $N^{(a)}$.

$$
F_{el} = I_{2d} + \sum_{a=1}^{4} \hat{\mathbf{u}}^{(a)} \otimes \frac{\partial N^{(a)}}{\partial \mathbf{X}}.
$$

(4.3)

The derivatives of the shape functions $N_{(\xi, \eta)}$ with respect to $\mathbf{X}$ can be obtained by consideration of the chain rule of differentiation.

$$
\frac{\partial N^{(a)}}{\partial \mathbf{X}_1} = \frac{\partial N^{(a)}}{\partial \xi} \frac{\partial \xi}{\partial \mathbf{X}_1} + \frac{\partial N^{(a)}}{\partial \eta} \frac{\partial \eta}{\partial \mathbf{X}_1}
$$

(4.4)

$$
\frac{\partial N^{(a)}}{\partial \mathbf{X}_2} = \frac{\partial N^{(a)}}{\partial \xi} \frac{\partial \xi}{\partial \mathbf{X}_2} + \frac{\partial N^{(a)}}{\partial \eta} \frac{\partial \eta}{\partial \mathbf{X}_2}
$$

(4.5)

Formulation in matrix form

$$
\begin{bmatrix}
\frac{\partial N^{(a)}}{\partial \mathbf{X}_1} \\
\frac{\partial N^{(a)}}{\partial \mathbf{X}_2}
\end{bmatrix}
= 
\begin{bmatrix}
\frac{\partial \xi}{\partial X_1} & \frac{\partial \eta}{\partial X_1} \\
\frac{\partial \xi}{\partial X_2} & \frac{\partial \eta}{\partial X_2}
\end{bmatrix}
J^{-T}
\begin{bmatrix}
\frac{\partial N^{(a)}}{\partial \xi} \\
\frac{\partial N^{(a)}}{\partial \eta}
\end{bmatrix}
$$

(4.6)

shows that the inverse transpose of the Jacobian $J$ relates the partial derivatives of the local to the global coordinate system. Isoparametric elements use the same set of shape functions for the approximation of the displacement field as well as the geometry in the global reference coordinates.

$$
\mathbf{X} = \sum_{a=1}^{4} N^{(a)} (\xi, \eta) \hat{\mathbf{X}}^{(a)}
$$

(4.7)

Thus, the Jacobian of each element can be obtained from the nodal positions in the reference configuration and partial derivatives of the shape functions.

$$
J_{el} = \frac{\partial (X_1, X_2)}{\partial (\xi, \eta)} = 
\begin{bmatrix}
\hat{\mathbf{X}}_1^T & \hat{\mathbf{X}}_2^T \\
\mathbf{X}_1^T & \mathbf{X}_2^T
\end{bmatrix}
\begin{bmatrix}
N_{\xi} & N_{\eta} \\
\hat{N}_{\xi} & \hat{N}_{\eta}
\end{bmatrix}
$$

(4.8)
\( \mathbf{X}_i^T \) denotes a vector of the corresponding nodal positions in the reference state in direction \( i, i \in \{1, 2\} \) and \( \mathbf{N}_{ij} \) denotes a vector composed of the derivatives of the shape function with respect to \( j \), where \( j \in \{\xi, \eta\} \). The element specific Jacobian is a function of the local coordinate system \((\xi, \eta)\). For the present study the Jacobian is evaluated in the element center, i.e. \((\xi, \eta) = (0, 0)\).

All common measures of deformation can be calculated based on the deformation gradient. For the load cases considered in this work, only the two in-plane principal stretches \( \lambda_1 \) and \( \lambda_2 \), which coincide with the longitudinal \( a_{01} \) and lateral \( a_{02} \) direction of the sample, are determined based on the 2d representation \( \mathbf{C}_{2d} \) of the right Cauchy-Green deformation tensor.

\[
\mathbf{C}_{2d} = \mathbf{F}_{2d}^T \mathbf{F}_{2d} \tag{4.9}
\]

\[
\lambda_1^2 = a_{01} \cdot \mathbf{C}_{2d} a_{01} \tag{4.10}
\]

\[
\lambda_2^2 = a_{02} \cdot \mathbf{C}_{2d} a_{02} \tag{4.11}
\]

Averaging over the entire region, in this case a \( 2 \times 3 \) element scheme, provides the kinematic response of the amnion layer, see Figure 4.4. The typical variability of elongation values in different elements was in the range of 5%.

The Poisson’s ratio is defined as the ratio of lateral strain (contraction, in direction 2) to longitudinal strain (elongation, in direction 1). It is strictly defined only for infinitesimal deformations. For large strain behavior, an incremental Poisson’s ratio or Poisson’s function, according to the definitions of Alderson et al. (1997) and Smith et al. (1999) can be used, which is based on the ratio of derivatives of logarithmic strains in the lateral and axial directions.

\[
\nu_{12}^{incr} = \frac{\lambda_1}{\lambda_2} \frac{\partial \lambda_2}{\partial \lambda_1} \tag{4.12}
\]

This incremental Poisson’s ratio might be regarded as akin to the definition of a tangent modulus for stiffness.

### 4.3 Anisotropic continuum model for FM

The notation used in the current chapter follows mainly the one used in Holzapfel et al. (2000). Further information and a general introduction into nonlinear continuum mechanics can be found in Holzapfel et al. (2000) and many other classical textbooks.
4.3. Anisotropic continuum model for FM

Figure 4.4: Illustration of the image analysis procedure. Image shows the transparent amnion layer with markers of India ink which are used to trace a set of user defined measurement points (red squares) by DIC method. A finite element scheme is used for the determination of the principal stretches.

4.3.1 Model formulation

Figure 4.1 shows the collagen microstructure of amnion. Thin, randomly oriented collagen fibers characterize this tissue and are expected to determine the mechanical response of amnion. A corresponding phenomenological model has to include terms representative of the mechanisms of stretching, rotation and buckling of the fibers. In this work the constitutive model formulation developed by Rubin and Bodner (2002) was chosen to model the response of the amnion layer. The model allows considering (families of) fibers embedded in an isotropic matrix, which model the anisotropic response of the tissue. It was shown in previous investigations to provide useful representation of the uniaxial (Jabareen et al., 2009) stress response of intact FM but underestimated the biaxial stress response (Buerzle et al., 2013) if formulated without fibers.

The Rubin-Bodner model includes terms to also describe dissipative deformation behavior. Our experimental data do not provide information on time and history dependence of amnion’s response. Thus, only the elastic part of the equations was considered in this work. The corresponding strain energy function, $\psi$, is a composition of different contributions, describing the dilatational ($g_1 (J)$, not included in the present formulation) and pure distortional, $g_2 (\beta_1)$, parts as well as the stretch of the single families of fibers,
Chapter 4. Development of a constitutive model for the mechanical response of FM

g_3 (\lambda_I), and determines the strain energy per unit reference volume (note that the original Rubin-Bodner model is formulated in terms of energy per unit mass).

\[
\psi = \frac{\mu_0}{2q} [e_{gg} - 1] \tag{4.13}
\]

\[
g = g_2 (\beta_1) + g_3 (\lambda_I) \tag{4.14}
\]

\[
g_2 (\beta_1) = m_2 (\beta_1 - 3) \tag{4.15}
\]

\[
g_3 (\lambda_I) = \sum_I m_3 m_4 (\lambda_I - 1)^{2m_4} \tag{4.16}
\]

\[
\beta_1 = tr (B) \text{ is the first invariant of the left Cauchy-Green deformation tensor } B = FF^T,
\]

and \( \lambda_I = |m_I| \) is the stretch of the I-th family of fibers, with \( m_I = FM_I \). The directions of the fibers in the reference configuration are defined by the unit vector \( M_I \). Very important for the present application, the fibers are considered not to bear compression loads, as indicated by the use of the Macaulay brackets \( \langle x \rangle = 1/2 (x + |x|) \). \( m_2, m_3, m_4, \mu_0, \) and \( q \) are model parameters which have to be determined from an inverse analysis of the experimental data. Due to the high water content (up to 90\% (Halaburt et al., 1989)), volume preservation \( (J = 1) \) was assumed in this case. Note that at present this assumption cannot be verified experimentally, since the thickness or the water content of the test pieces cannot be monitored during the tests.

4.3.2 Model parameters determination

The Cauchy stress tensor \( \sigma \) is obtained from derivatives of the strain energy function with respect to the corresponding deformation tensor. The membrane is in the 1-2 plane and the deformation gradient is (for all cases considered here) of the form:

\[
F = \begin{bmatrix}
\lambda_1 & 0 & 0 \\
0 & \lambda_2 & 0 \\
0 & 0 & \lambda_3
\end{bmatrix} \tag{4.17}
\]

with

\[
\lambda_3 = \frac{1}{\lambda_1 \lambda_2}. \tag{4.18}
\]

Reliable measurement of the sample thickness prior to mechanical testing was not possible. Therefore, experimental results as well as model predictions are presented as membrane tension values. The Cauchy stress tensor \( \sigma \) is transformed to a membrane tension tensor \( t = \sigma h = \sigma h_0 \lambda_3 \) by multiplication with the present thickness \( h \). The averaged initial
4.4. Results

thickness $h_0$ was determined in the separate layer tests in section 3.8. Representative average values used in the present work are: 100$\mu$m for amnion and 400$\mu$m for chorion. The membrane tension tensor $t$ (in the current configuration) reads

$$
t = \frac{m_2\mu}{\lambda_1\lambda_2} \left[ B - \frac{1}{3}(B \cdot I)I \right] + \sum_{l=1}^{8} \frac{m_3\mu}{\lambda_1\lambda_2} (\lambda_l - 1)^{2m_4-1} \frac{1}{\lambda_l} (M_l \otimes m_l) - \frac{p h_0}{\lambda_1\lambda_2} I 
$$

(4.19)

$$
\mu = \mu_0 h_0 e^{\gamma g}.
$$

(4.20)

The membrane tension tensor $t$ must fulfill two boundary conditions in a state of uniaxial tension. First, the stress vector in the thickness direction must vanish, which can be achieved by the corresponding determination of the hydrostatic pressure $p$, which is a general function of position and time due to the incompressibility constraint. Second, also the stress vector in the lateral (in-plane) direction must vanish. This condition for the 22 component of $t$:

$$
t_{22} = 0 = \frac{m_3}{m_2} \sum_{l=1}^{8} (\lambda_l - 1)^{2m_4-1} \frac{1}{\lambda_l} (M_l \otimes m_l)_{22} + \lambda_2^2 - \frac{1}{\lambda_1^2\lambda_2^2}
$$

(4.21)

determines simultaneously the kinematic response of the constitutive model, since it provides the stretch $\lambda_2$ as a function of $\lambda_1$ and $\lambda_l$. The kinematic response in the uniaxial stress state depends only on two model parameters: the ratio $m_3/m_2$ and the parameter $m_4$. The other model parameters do not affect the kinematic response. This circumstance can be used to split up the inverse analysis in two steps. First, only the parameter-ratio $m_3/m_2$ and the parameter $m_4$ are determined in order to match the experimental kinematic response (contraction-elongation curve). Next, the remaining parameters $q$, $\mu_0$, and $m_2$ (or $m_3$) are determined to match the tension-stretch response, while keeping $m_3/m_2$ and $m_4$ unchanged. The best fit model parameters are determined by the use of the Nelder-Mead simplex method, `fminsearch`, in MATLAB 2010a (The MathWorks, Inc., Natick, MA, USA).

4.4 Results

4.4.1 Uniaxial tests

Uniaxial tension tests have been performed on samples from six different membranes. Out of a total of 66 measurements, 12 amnion and 11 chorion samples allowed the optical
analysis of the displacement field. The remaining samples had to be discarded for reasons like wrapped borders, large wrinkles in the sample, diffuse markers and so on, which made the optical evaluation of the displacement field impossible. Since the intact membrane was separated prior to the sample preparation, there is no unique assignment between one amnion and one chorion sample and an original area of intact FM. The clamped tissue sample is initially slack. A low force threshold (5 mN in this case, accounting for the separate layers) was applied to define the initial configuration.

Figure 4.5(a) shows the experimental results for the amnion samples and 4.5(b) for the chorion samples. Both figures illustrate the tension-stretch response above and the corresponding in-plane kinematic response below, i.e. $\lambda_2$ versus $\lambda_1$. Note that the membrane tension is given as Cauchy tension which is defined as force per current sample width.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.5}
\caption{Experimental results for amnion (a) and chorion (b). Both figures illustrate on top the tension-stretch response and below the corresponding contraction-elongation curves for all tested samples.}
\end{figure}
It can be seen in Figure 4.5 that the tension-stretch curves demonstrate a typical nonlinear behavior with low initial stiffness followed by a transition into a region of higher stiffness. Moreover, the tension-stretch data show a pronounced (but common) scatter. On the other hand, the kinematic response of amnion in Figure 4.5(a) is highly reproducible and shows a very large lateral contraction. Table 4.1 reports corresponding values of amnion’s membrane tension and lateral contractions at $\lambda_1 = 1.1$, and maximum values of incremental Poisson’s ratio. The corresponding standard deviations reported in the table highlight the reproducibility of contraction data, as opposed to the large variability of tension values. The maximum values of the incremental Poisson’s ratio are up to 8.2 for amnion. The mechanical response of chorion is characterized by smaller values of membrane tension, larger failure stretches, and a much larger scatter in the corresponding in-plane kinematic response as illustrated in Figure 4.5(b).

<table>
<thead>
<tr>
<th>Amnion sample</th>
<th>$t^* \cdot 10^{-2}$ N/mm</th>
<th>$\lambda_1^*$ [-]</th>
<th>$\nu_{incr}^{max}$ [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.3</td>
<td>0.66</td>
<td>6.1</td>
</tr>
<tr>
<td>2</td>
<td>6.1</td>
<td>0.68</td>
<td>5.4</td>
</tr>
<tr>
<td>3</td>
<td>6.7</td>
<td>0.60</td>
<td>7.9</td>
</tr>
<tr>
<td>4</td>
<td>1.6</td>
<td>0.63</td>
<td>6.4</td>
</tr>
<tr>
<td>5</td>
<td>3.2</td>
<td>0.68</td>
<td>6.4</td>
</tr>
<tr>
<td>6</td>
<td>6.9</td>
<td>0.66</td>
<td>5.7</td>
</tr>
<tr>
<td>7</td>
<td>6.3</td>
<td>0.67</td>
<td>5.6</td>
</tr>
<tr>
<td>8</td>
<td>6.5</td>
<td>0.63</td>
<td>6.8</td>
</tr>
<tr>
<td>9</td>
<td>1.4</td>
<td>0.69</td>
<td>6.4</td>
</tr>
<tr>
<td>10</td>
<td>2.7</td>
<td>0.65</td>
<td>8.2</td>
</tr>
<tr>
<td>11</td>
<td>1.1</td>
<td>0.57</td>
<td>7.3</td>
</tr>
<tr>
<td>12</td>
<td>5.0</td>
<td>0.55</td>
<td>7.7</td>
</tr>
</tbody>
</table>

For completeness, also stiffness parameters $K_1$ and $K_2$ were evaluated as defined in section 3.3.3. Averaged values of low and high stretch stiffness as well as membrane tension and longitudinal strain at rupture of amnion and chorion are reported in Table 4.2. However, values of maximum membrane tension and strain at rupture cannot be considered as material properties, since rupture often occurs at the clamping site.
Table 4.2: Mechanical parameters extracted from the Cauchy tension-stretch curves of the samples used for uniaxial tension testing. Parameters are given as overall average ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>$K_1 \cdot 10^{-2} \text{N/mm}$</th>
<th>$K_2 \text{N/mm}$</th>
<th>$T_{\text{crit}} \text{N/mm}$</th>
<th>$\lambda_{\text{crit}} [-]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amnion</td>
<td>3.4 ± 2.3</td>
<td>5.17 ± 2.01</td>
<td>0.42 ± 0.19</td>
<td>1.23 ± 0.04</td>
</tr>
<tr>
<td>Chorion</td>
<td>0.9 ± 0.4</td>
<td>1.02 ± 0.32</td>
<td>0.12 ± 0.04</td>
<td>1.38 ± 0.06</td>
</tr>
</tbody>
</table>

4.4.2 Constitutive model parameters

It can be seen in Figure 4.5 that chorion shows smaller values of membrane tension, larger failure strains, and a lower Poisson’s effect than amnion. Although the determination of the constitutive model parameters is performed for both tissue layers, the results are only shown on the example of the amnion data, since these data make higher demands on the capabilities of the model formulation.

The model response should be (i) isotropic in the membrane plane. In fact, no preferential directions have been reported in any previous study. It should display the typical (ii) j-shaped tension-stretch curve, and, in particular, reproduce the observed (iii) very large lateral contraction. Simultaneous fulfillment of all these conditions is a challenging task.

The Rubin-Bodner model (Rubin and Bodner, 2002) has been implemented considering eight identical families of fibers in the initial directions of $\{0, \pm 22.5, \pm 45, \pm 67.5, 90\}^\circ$, thus providing a quasi-isotropic behavior in the membrane plane. Recent work on a discrete fiber model based on fibers oriented in the directions of opposing vertices of a regular icosahedron shows that this model is not exactly isotropic (Flynn et al., 2010; Flynn and Rubin, 2012). Figure 4.6 shows the results of the two step optimization procedure (explained in section 4.3.2) applied to the averaged uniaxial response of amnion. The model very well matches both, the tension-stretch response as well as the contraction-elongation curves. Since comparison between model and measurements is performed for the membrane tension (and not for stress), there is no need to determine a representative initial thickness of the “average” amnion sample. The “best fit” model parameters for the averaged amnion and chorion response are reported in Table 4.3.

The key for representing the observed kinematic response is the fact that the fibers do not resist compressions (no stress for $\lambda_i < 1$). Figure 4.7 illustrates the expected rotation of two fibers for a prescribed elongation in direction $e_1$. Fiber A has an initial orientation (dashed line) with a small angle with respect to $e_1$, whereas fiber B has an initial large angle deviation from $e_1$. Macroscopic deformation in direction $e_1$ leads to fiber stretching and rotation. Fiber A requires large lateral contraction in order to minimize its stretch...
Figure 4.6: Optimization results for averaged amnion response. (a) tension-stretch and (b) kinematic response.

Table 4.3: Best fit model parameters of the Rubin-Bodner model, optimized for the averaged uniaxial response of amnion and chorion. The model includes eight identical families of fibers in the directions of $\{0, \pm22.5, \pm45, \pm67.5, 90\}^\circ$ in the reference configuration.

<table>
<thead>
<tr>
<th></th>
<th>$m_2$ [-]</th>
<th>$m_3$ [-]</th>
<th>$m_4$ [-]</th>
<th>$\mu_0$ [MPa]</th>
<th>$q$ [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amnion</td>
<td>6.55E-4</td>
<td>3.15</td>
<td>1.45</td>
<td>4.47</td>
<td>1.36</td>
</tr>
<tr>
<td>Chorion</td>
<td>2.63E-2</td>
<td>6.08</td>
<td>1.59</td>
<td>0.07</td>
<td>1.39</td>
</tr>
</tbody>
</table>

by rotation, whereas fiber B minimizes its deformation (shortening) in case of low lateral contraction. This opposite requirement would lead to a “balanced” contraction behavior when the resistance to lengthening (tension) and shortening (compression) would be comparable. In case fibers are unable to resist compression, fiber A dominates and induces a large lateral contraction, leading to minimized lengthening of fiber A but significant shortening of fiber B.

Figure 4.8 illustrates the comparison of the Rubin-Bodner model response for two cases: fibers with and without compressive stiffness. There is only a minor difference in the tension-stretch response between the two cases but the kinematic response changes completely, leading to a behavior close to the one expected for an isotropic incompressible material ($\nu = 0.5$) for the case with compressive stiffness. Note that the original form of the Rubin-Bodner model already includes the assumption of zero fiber stiffness under compression. The obtained descriptive capabilities are thus related to the explicit representation in this model of the mechanisms of stretching, rotation, and buckling of a random fiber network.
4.4.3 Constitutive model verification

Averaged biaxial tension-stretch data obtained from inflation experiments on separate layers (section 3.7.3) are used to evaluate the predictive capabilities of the proposed model equations, i.e. their performance is evaluated without changing any of the five model parameters reported in Table 4.3. The biaxial tension-stretch data are normalized with respect to the same level of initial membrane tension as the uniaxial data in order to obtain a comparable reference configuration. Figure 4.9(a) compares the model with experimental results and illustrates the remarkable quality of the prediction of amnion’s biaxial response. Figure 4.9(b) shows correspondingly the prediction of the biaxial response of chorion. Even if the reproduction of the uniaxial behavior of chorion (data not shown) is as good as the one of amnion, the prediction of chorion’s biaxial response differs evidently from the measurements.

4.5 Implementation in ABAQUS/Standard

4.5.1 The UMAT subroutine

The purpose of the constitutive model development is to use the model for different finite element simulations. In order to do that the model needs to be implemented in a (commercial) FE code. The FE software ABAQUS (Abaqus Inc. RI, USA) allows to implement user defined materials by the use of the subroutine UMAT. This subroutine is called at all material calculation points of the elements which are assigned to have a user-defined material. The routine has to be programmed to provide the Cauchy stress
tensor and the corresponding tangent stiffness. The implementation of a purely elastic material is quite simple, but complexity and effort increase dramatically if time or rate dependency is included. Further details to several aspects involved in the use of the subroutine UMAT and the implementation of the Rubin-Bodner model in its full capacity can be found in the work of Papes (2012). In ABAQUS versions 6.8 and newer, anisotropic hyperelastic materials can be implemented in terms of the invariants of the deformation tensor or components of the Green strain tensor by the subroutines UANISOHYPER_INV or UANISOHYPER_STRAIN. However, these routines are limited to three families of fibers for which reason they cannot be used for the implementation of the current model.

The implementation of the constitutive model is based on the work of Papes (2012) and adapted to the current formulation with eight identical families of fibers and without time dependency. The model is implemented in a slightly compressible formulation due to its benefits for convergence. Compressibility has no particular influence on the deformation behavior if material parameters related to dilatation are much larger than parameters related to distortion so that only very small volume changes are possible.

The implementation is done for continuum elements, for which reason the strain energy has to be formulated in terms of energy per unit volume. Compressibility requires including the factor $g_1(J)$ accounting for the energy related to volume change as a function of the
Chapter 4. Development of a constitutive model for the mechanical response of FM

Figure 4.9: Comparison of uniaxial (UA) and equibiaxial (EB) experimental data with the corresponding model response for amnion (a) and chorion (b). Equibiaxial model prediction has been calculated without changing any of the model parameters determined based on the averaged uniaxial data.

Determinant of the deformation gradient \((J = \text{det}(F))\). Thus, the implemented equations are similar to the original ones.

\[
\psi = \frac{\mu_0}{2q} [e^{gq} - 1] \quad (4.22)
\]
\[
g = g_1 (J) + g_2 (\beta_1) + g_3 (\lambda_I) \quad (4.23)
\]
\[
g_1 (J) = 2m_1 [(J - 1) - \ln (J)] \quad (4.24)
\]
\[
g_2 (\beta_1) = m_2 (\beta_1 - 3) \quad (4.25)
\]
\[
g_3 (\lambda_I) = \sum_1^8 \frac{m_3}{m_4} (\lambda_I - 1)^{2m_4} \quad (4.26)
\]

The factor \(m_1 \mu_0 = \kappa\) determines the small strain bulk modulus. Because of the high water content, the parameter \(m_1\) is estimated from the compressibility of water \(\kappa_{H_2O}\) of 2200 MPa.

The definition of the six material parameters has to be done in the input file of ABAQUS by the command \texttt{*User Material, constants=6}. Furthermore, the number of (solution dependent) state variables (STATEV) has to be defined by the command \texttt{*DEPVAR, 24}. The initial fiber directions are defined by unit vectors. Each \(X, Y, Z\)-component of each fiber vector is assigned to a STATEV, so that a total of 24 STATEVs is required. The state variables are called separately at each timestep in each integration point so that the
4.5. Implementation in ABAQUS/Standard

dehomed configurations of each fiber can be calculated. The initialization of the state
variables has to be done before the actual simulation. This is done by the subroutine
SDVINI, which is called by the command *INITIAL CONDITIONS, TYPE=SOLUTION, USER
in the input file.

4.5.2 Validation

The implementation of the user material has to be checked for reliability. A first check
is related to the stress-strain and kinematic response which has to match analytical so-
lutions. This check was performed on single element (C3D8R) benchmark problems of
homogeneous uniaxial and equibiaxial deformations. A second check is related to the
convergence behavior. Convergence was checked with a linear brick element (C3D8R),
fixed on three sides and loaded by a nodal force applied in one corner. The analysis of
all benchmark problems showed a very good agreement between stress-strain curves from
analytical solutions and FE simulations. The analysis of the convergence behavior shows
that the implementation achieves quadratic convergence, which means that the number
of correct digits is doubled in each iteration.

Further validation of the implementation and the constitutive model in general is per-
formed by the simulation of inflation tests, as known from section 3.4.1. The present
implementation of the constitutive model for continuum elements allows also the use of
axisymmetric elements. The FM is modeled as a realistic bilayer structure with an am-
nion layer of 100 µm thickness and chorion of 400 µm. Since the interaction of amnion
and chorion is unknown, the two extreme cases of free relative sliding and tied interaction
(no relative movement) are investigated. 1920 axisymmetric elements (CAX4R) are used
to model the membrane with an outer diameter of 50 mm. The clamping cover is mod-
eled as rigid body and the pressure is applied by a pressure load on the inner surface of
the membrane. The resulting pressure-displacement curves are normalized by a pressure
threshold as the experiments and compared to the experimental data of section 3.7.2.

Figure 4.10(b) shows the resulting pressure-displacement curves from the inflation sim-
ulation. It can be seen that the bilayer model, with the parameters obtained in section
4.4.2, matches very well the experimental data in an average sense. Furthermore, it can
also be seen that the interaction of amnion and chorion does not contribute to the overall
pressure-displacement response. This circumstance might be attributed to the in general
small possibilities of relative movement between the layers within inflation testing. The
fact that the equibiaxial inflation response can accurately be predicted from uniaxial data
is a further confirmation of the validity of the assumptions of the model.
Figure 4.10: FE simulation (axisymmetric) of inflation tests with the user defined material model. (a) shows the axisymmetric bilayer FE model in a deflected configuration and (b) compares the model prediction with experimental data from section 3.7.2.

4.6 On the reference configuration of soft biological tissues

When does a material start to bear load? This is a fairly simple question but rather hard to answer when it is about soft biological tissues.

Determination of a reliable reference configuration is an essential step for the characterization of materials. The highly nonlinear mechanical response of soft tissues, with a very low initial stiffness and a subsequent stiffening, makes this particularly difficult. Most of the studies in the literature about the mechanical characterization of biological tissues do not even mention a reference configuration. Instead their reference configuration corresponds usually to a zero position of the experimental setup. This results typically in stress-strain curves with no initial stiffness (Prevost, 2004; Joyce, 2009). The consequence is that the horizontal position of the stress-strain curve is uncertain and therewith causes a large variability in all parameters depending on the definition of strain.

The definition of a reference configuration requires the introduction of a criterion to normalize the raw (force-displacement) data. Most often a force threshold (Wilshaw et al., 2006; Pandey et al., 2007; Jabareen et al., 2009) is introduced. But the value of this threshold typically depends on the test type, resolution of the force signal, sample geometry and so on. For a suitable choice of the threshold value a compromise has to be made in order to on one hand group the raw data and on the other hand avoid loosing to
many data from the initial response. A reliable definition of the reference configuration is not only important to normalize experimental data but also to compare data obtained in different experimental configurations.

This section addresses different aspects of the reference configuration of soft biological tissues with respect to different experimental configurations and their influence on extracted scalar parameters will be shown.

4.6.1 Normalization of inflation tests

In order to obtain accurate mechanical measurements it is essential to reliably define a reference configuration. The inflation data obtained in section 3.7.2 were normalized with the pressure criterion as described in section 3.4.4. The clamped membrane sample is initially slack and has wrinkles. According to section 3.4.4, we define the start of the measurement as the point when the measured total pressure starts to deviate from the hydrostatic pressure. Due to noise in the pressure signal and its limited resolution, a pressure threshold value of 1 mbar was used.

However, there is a variability in the absolute pressure and apex displacement of the membrane at which the pressure criterion is fulfilled caused by the in general large scatter in the mechanical properties and possibly also due to slight differences in the sample preparation and handling. Since the membrane tension is a function of pressure and curvature, also the initial membrane tension changes. According to this pressure preload the average initial membrane tension is $(2.1 \pm 0.4) \cdot 10^{-3} \text{N/mm}$, see section 3.7.4. This value is less than 1% of the critical membrane tension. Considering the overall averaged initial stiffness $K_1$ of $(0.105 \pm 0.030) \text{N/mm}$ shows that the corresponding strain at normalization is in the range of 1% to 3%. This is on the one hand a small value of initial strain but on the other already approximately 10% of the strain at rupture.

If this normalization criterion was not applied (in fact, this step is usually omitted in the literature) the reference configuration would be assumed to be that of a flat membrane. Resulting from this assumption the membrane deformations would be clearly higher. The critical strain at membrane rupture would change from $(20 \pm 3)\%$ to $(43 \pm 8)\%$ and the high stretch stiffness would reduce from $(2.64 \pm 0.49) \text{N/mm}$ to $(1.88 \pm 0.41) \text{N/mm}$. In addition, as illustrated in Table 4.4, all coefficients of correlations from section 3.9 would be reduced and the demonstrated relationship between mechanical and microstructural data could not be obtained any more. The reason for this lies in the higher variability of the mechanical data, caused by uncertainties in the initial configuration. The handling of soft biological tissues prior to mechanical testing is user subjective and even if done with
great care, small wrinkles in the tissue or clamping the tissue under slight tension can never be excluded. In that sense the use of the pressure criterion is a major improvement in the analysis of inflation tests. Nevertheless, it would be beneficial to reliably apply the normalization on the level of the membrane tension. However, this would require the raw tension-displacement data to be available. The membrane sample is almost flat at the beginning of the inflation test, so that the image analysis is difficult to perform and causes large variations in the determination of the curvature. Moreover, the signal to noise ratio of the pressure measurement is low at the beginning of the test. Those two effects cause large variations in the tension data, which makes the application of a criterion on the tension level rather difficult.

4.6.2 Comparison of data from different experimental configurations

Obtaining a reliable reference configuration is not only important to group the data within one study. It is also much more important to enable comparisons of data obtained in different experimental configurations. Obviously, using a system parameter like a force threshold, which depends on the sample size and test configuration, is not a suitable choice. Definition of the threshold value on the level of membrane tension or stress (for a homogeneous material) is beneficial, because it is independent from the experimental configuration. When comparing data obtained from different stress states it is worth mentioning that the initial stiffness of an isotropic and linear elastic material changes depending on the state of stress. It can be shown by the use of the linear elastic constitutive equations and implementation of the corresponding static or kinematic boundary conditions that the initial slope of the stress-strain curve scales by a factor of 1 : 1.3 : 2 depending on the corresponding loading state, i.e. uniaxial : pure-shear : equibiaxial.

In order to illustrate this approach, the uniaxial and equibiaxial data from section 3.7 as well as the data from the pure-shear tests (see appendix C) are normalized by the same level of initial membrane tension. Due to the limited resolution of the pressure signal in

<table>
<thead>
<tr>
<th></th>
<th>Collagen</th>
<th>Elastin</th>
<th>PYD</th>
<th>DPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_2$</td>
<td>0.394</td>
<td>0.134</td>
<td>0.679</td>
<td>0.590</td>
</tr>
<tr>
<td>$\lambda_{\text{crit}}$</td>
<td>0.337</td>
<td>0.138</td>
<td>0.138</td>
<td>0.223</td>
</tr>
</tbody>
</table>

*Table 4.4: Coefficients of correlation $R$ between mechanical and biochemical parameters. The mechanical parameters reported in this table have been obtained without the use of the normalization criterion. Values in bold are statistically significant ($p < 0.05$).*
4.6. On the reference configuration of soft biological tissues

Table 4.5: Comparison of the initial stiffness values obtained in different experimental configurations. The experimental have the same reference configuration which is characterized by the initial membrane tension of $2.1 \cdot 10^{-3}$ N/mm.

<table>
<thead>
<tr>
<th></th>
<th>Initial stiffness $K_1$ [N/mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>uniaxial stress</td>
<td>$(44.4 \pm 18.3) \cdot 10^{-3}$</td>
</tr>
<tr>
<td>pure-shear</td>
<td>$(60.6 \pm 12.3) \cdot 10^{-3}$</td>
</tr>
<tr>
<td>equibiaxial stress</td>
<td>$(105 \pm 31) \cdot 10^{-3}$</td>
</tr>
</tbody>
</table>

the inflation tests, the initial membrane tension of $2.1 \cdot 10^{-3}$ N/mm was taken as reference. The initial stiffness of the uniaxial and pure-shear data is determined on the first loading ramp of the preconditioning (virgin response).

Table 4.5 shows the overall averaged values of the initial stiffness for the different experimental configurations normalized with the same initial membrane tension. The different initial stiffnesses $K_{UA}^1 : K_{PS}^1 : K_{EB}^1$ scale with the values of $1 : 1.36 : 2.36$, which is in the same range as expected from linear elasticity. However, it has to be mentioned that the experimental basis of pure-shear tests is, with only five samples, rather low. Furthermore, the initial membrane tension from the inflation tests is variable, and was not considered for the determination of the corresponding stiffnesses of the other test methods.

4.6.3 Alternative criterion based on the kinematic response

The foregoing discussed criteria to define the reference configuration were all relating to the mechanical loading. If optical analysis of the deformation field is possible, the question about the start of the material response can also be approached by analysis of the kinematic response. Elongation of a sample causes not only forces or stresses in the direction of loading but also a contraction in the lateral direction (within uniaxial loading). The clamped uniaxial sample is initially slack and the borders are wrapped. If stretched in longitudinal direction the sample unfolds in a first step and gets wider. At a certain level of longitudinal deformation the lateral contraction starts. This indicates that the material starts to bear load, since this lateral contraction is caused by the reorientation of the collagen fibers. Figure 4.11 shows the raw data of the kinematic response of amnion. It can be seen that the stretch in the lateral direction $\lambda_2$ increases at the beginning of the test and starts to decrease afterward.

The raw data were normalized with respect to the state at which the slope of the lateral contraction becomes negative. Figure 4.12(a) shows the tension-stretch curves and 4.12(b) the corresponding curves for the in-plane kinematic response. It can be seen in Figure
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4.12 that the variability of the data increases compared to the data in section 4.4.1. However, the main finding of a much smaller scatter in the kinematic response compared to the scatter in the tension data is preserved. Figure 4.12(a) also shows that most of the samples start with zero initial stiffness. In consequence, even if the kinematic response indicates that the material starts to respond to the loading, the corresponding forces cannot be measured with the present setup. This makes an accurate analysis of the onset of material response difficult or it demands for better resolution of the force measurement.

4.7 Discussion

4.7.1 Experiments

For the first time, image analysis based determination of longitudinal and lateral strains allowed reliable determination of the state of deformation of separate amnion and chorion layers in uniaxial stress experiments. Local deformation analysis is very important in these experiments. In fact, differences of up to 25% were observed between nominal and actual elongations, which points to experimental difficulties to firmly clamp the soft tissue samples. Due to the fact that the sample is clamped at the two extremities, reliable analysis of the lateral contraction requires a large aspect ratio in order to obtain a uniaxial state of stress in the central region of the sample. The major advantage of the deformation analysis is the availability of quantitative data on lateral contraction, which was essential in this case for constitutive model development.
4.7. Discussion

Reliable identification of the “zero stress state” is required for the mechanical characterization of materials. This state is difficult to attain for soft biological tissues because of their low initial stiffness, which results in an associated uncertainty in the horizontal position of the tension-stretch curves (Miller, 2001; Gao and Desai, 2010). A low force threshold (less than two orders of magnitude lower than the maximum force) was introduced in the current study in order to normalize the curves. This approach allowed the comparison of uniaxial and equibiaxial tension data with respect to the same level of initial membrane tension.

The observed values and the scatter in membrane tension of amnion and chorion is in line with previously reported experimental results (Oxlund et al., 1990; Helmig et al., 1993; Prevost, 2004). The observed variability in tension data is common for biological tissues, and contrasts with the high reproducibility of the kinematic response of amnion, see Figure 4.5(a) and values in Table 4.1. This indicates that the amount and distribution of collagens type I, III, IV, V, and VI in each amnion sample varies to a significant extent (Malak et al., 1993), whereas the mechanism of deformation of the fiber network is an intrinsic characteristic of this type of tissue.

The mechanical behavior of chorion is significantly different to that of amnion, i.e. the critical membrane tensions are smaller and the failure stretches are larger. Moreover, the kinematic response of chorion shows much smaller lateral contractions and the scatter in the tension data is comparable to the one in the in-plane kinematic response. The main cause for this difference can be attributed to the role of collagen in determining the me-
chanical behavior, since it is known that chorion contains only about half of the amount of collagen of amnion. On the other hand, it is more difficult to obtain a clean chorion layer from an experimental point of view. The fetal membrane is separated manually into amnion and chorion layers during sample preparation. The separation of the membrane happens in the intermediate or “spongy” layer (Fawthrop and Ockleford, 1994) which is composed of collagens (Malak et al., 1993) and allows a relative movement of amnion and chorion (Bourne, 1962). When the membrane is separated, the collagens from the spongy layer are divided by an unknown ratio, thus causing a high variability of the mechanical behavior of chorion.

The resistance of fibers to elongation leads to rotation and to buckling of the fibers initially oriented at a larger angle with respect to the direction of tension. This causes remarkable lateral contraction. This interpretation is in line with the findings of Kabla and Mahadevan (2007) for soft fibrous networks and with results obtained using random fibers network models (Picu, 2011). The maximum observed incremental Poisson’s ratio of amnion is 8.2, which is, to the author’s knowledge, larger than any previously reported values for biological or synthetic materials. There are two recent studies (Vader et al., 2009; Lake and Barocas, 2011) in which the Poisson’s ratio has been determined for collagen gels, reporting values of up to 5. Other studies measuring the Poisson’s ratio of anisotropic biological tissues such as human annulus fibrosus, human hip joint capsule ligaments, and sheep flexural tendon (Elliott and Setton, 2001; Hewitt et al., 2001; Lynch et al., 2003) report values in the range of 0.6-1.8, of 1.4, and of 3, respectively.

No preconditioning was applied in the present tests. The data from section 3.7.1 show that the initial stiffness increases with increasing cycle number during preconditioning. This can be explained by pronounced fiber reorientation during preconditioning, which also leads to a shift of the reference configuration. However, additional tests should be performed to investigate if these changes are irreversible or if these mechanisms are subjected to recovery.

The initial stiffness of amnion and chorion was determined in the present work to be $(3.4 \pm 2.3) \cdot 10^{-2} \text{N/mm}$ and $(0.9 \pm 0.4) \cdot 10^{-2} \text{N/mm}$, respectively. The corresponding averaged value of the virgin response of intact FM was determined as $(1.0 \pm 5.0) \cdot 10^{-2} \text{N/mm}$ in section 3.7.1. Comparison of these values shows that the initial stiffness of the intact FM might be determined by the chorion layer only. The same behavior was already observed in the inflation tests on separate amnion and chorion layers in section 3.7.4.
4.7.2 Constitutive modeling

Key for the rationalization of amnion’s behavior is the formulation of a (i) transversely isotropic model (quasi-isotropic in-plane fiber distribution) with (ii) fibers active in tension only (no stiffness for shortening, finite stiffness for lengthening). Consideration of distributed families of fibers in a homogeneous matrix is common to several proposed models for soft biological tissue (Holzapfel et al., 2000; Sacks, 2003; Chen et al., 2011). The particular aspect highlighted in this work was not investigated in previous publications, mainly for the two following reasons: (1) little attention has been paid so far to the kinematic response of uniaxial tension tests, focusing on the stress-strain behavior; (2) when fibers are introduced often pronounced anisotropy is described, so that the contraction perpendicular to the preferred fiber direction is dominated by the matrix (typically $\nu \approx 0.5$).

Physically motivated selection of the model formulation made it possible to achieve excellent predictive capabilities of amnion’s biaxial response with only five model parameters. Note that this is the same number of material constants required to describe the behavior of a transversely isotropic material in linear elasticity. Furthermore, determination of model parameters was particularly efficient, using the split between parameters related to the kinematic response and those determining the tension-stretch behavior. The determination of the parameters related to the kinematic response ($m_3/m_2$ and $m_4$) was shown to lead to the same results for a broad range of initial values. Note, on the contrary, that the solution for the parameters related to the tension-stretch response is not unique.

The transversely isotropic model formulation determined in the present work is able to predict large incremental Poisson’s ratios. Within the realm of isotropic linear elasticity, the Poisson’s ratio is restricted to values between -1 and 0.5. However, Ting and Chen (2005) showed in a recent study that under the prerequisite of positive definiteness of the strain energy density the Poisson’s ratio of anisotropic elastic materials can have no bounds. Although the current model formulation is able to predict large Poisson’s ratios, the linearized value for small deformations is 0.5, thus being within the given bounds. Even if the averaged uniaxial response of chorion can be reproduced in the same quality as that of amnion, the prediction of the biaxial response of chorion differs substantially from the measurement. The reason for this discrepancy might be related to experimental difficulties in obtaining a clean chorion layer, as discussed in the previous section. It seems that especially the biaxial data of chorion are dominated by an additional amount of collagen originating from the spongy layer. Further indication for this interpretation is the good agreement between the resulting pressure-displacement curve from the FE infla-
tion simulation (with uniaxial parameters) and the corresponding experimental data for intact FM. If the model were to underestimate the contribution of chorion, as illustrated in Figure 4.9(b), then there should also be a larger deviation toward a more compliant FE response.

The present study shows that the key for the rationalization of the kinematic response is the fact that the fibers do not contribute under compression. It is obvious that the thin and initially crimped collagen fibers will buckle under minor compression. However, they will possess a certain (very low) compressive stiffness. Thus, the present results are limited to the descriptive capabilities of the model formulation, but are valid as long as the compressive stiffness of the fibers is much lower as the corresponding tensile stiffness. The current model predicts an increase of the thickness for the uniaxial experiments due to the assumption of incompressibility. This behavior cannot be verified in the experimental setup utilized for the present work. Current efforts in our laboratory are focused toward in-situ measurement in a multi photon microscope in order to measure thickness changes for different states of in-plane deformation. On the other hand, the assumption of incompressibility can also be discussed. Biological tissues are usually assumed to be incompressible because of the high water content. But the unphysical prediction of an increase of the sample thickness by a factor of 2 to 3 leads to the question as to what is happening to the water inside of the sample. Preliminary results from the measurements in the multi photon microscope indicate that the thickness of amnion is decreasing. This causes a reduction of the sample volume and leads to the interpretation that the water content is reduced during uniaxial stretching.

4.7.3 Implications of large lateral contractions

Our findings show that human amnion possesses an exceptionally high Poisson’s effect. This might be a key property for fulfillment of its function. In fact, large lateral contraction in a state of uniaxial stress (such as at hole- or crack-like defects in a membrane) cause stress redistribution and local stiffening, thus providing higher toughness and making amnion a defect-tolerant membrane. This interpretation is in line with the findings of Koh and Oyen (2012) who observed in numerical simulations of fibrous networks corresponding fiber reorientations at the crack tip and crack blunting, representing an important toughening mechanism. In addition, differences in the contraction behavior of amnion with respect to chorion and maternal decidua facilitate mechanisms of local relative sliding of the layers. This relative movement is considered a prominent mechanism to avoid amniotic fluid leakage in case of localized FM defects (Gratacós et al., 2006).
4.7.4 FE implementation

The implementation of the constitutive model in ABAQUS works well and has passed all verification benchmark problems. However, stability problems appeared during FE simulations of different experimental configurations that are related to the nearly incompressible formulation. The model parameter $m_1 = \kappa/\mu_0$ that determines the strain energy contribution due to dilatation is estimated from the small strain bulk modulus. Parameter $\mu_0$ is also related to the small strain shear modulus by $m_2\mu_0$, which is in general a small number for soft biological tissues. In consequence, parameter $m_1$ is about six orders of magnitude larger than parameter $m_2$, which is related to the distortion of the matrix component. Since parameters $m_1$ to $m_4$ are in the exponent of an exponential function, a small change in the volume during an iteration step can lead to numerical singularity which aborts the calculation. The simplest solution to this problem is to reduce the assumed bulk modulus for the determination of the parameter value of $m_1$. However, the value should still be large enough to preserve a nearly incompressible behavior. In any case, these problems related to the (nearly) incompressible formulation of the model might be considered as an indication that the assumption of constant water content might not reflect the real behavior, as already discussed in the previous section.

4.8 Conclusion

This study contributes significantly toward the determination of a constitutive model for simulation of the mechanical behavior of human FM tissue. Uniaxial tensile tests were performed on single amnion and chorion layers and the response analyzed with particular focus on the lateral contraction behavior.

The results confirmed established knowledge that amnion is stiffer, stronger, and less extensible than chorion and can therefore be considered as the mechanically dominant layer of the FM. Results showed that the amnion layer possesses unique and highly repeatable contraction properties, despite a pronounced but typical scatter in the tension-stretch curves. The kinematic response of chorion is less reproducible, which can be attributed to its lower concentration of collagen. Maximum incremental Poisson’s ratios in the range of 5.4 - 8.2 were calculated for amnion, which is larger than any previously published value for biological or synthetic materials. This structural behavior can be rationalized through mechanisms of rotation, stretching, and buckling of collagen fibers. A corresponding phenomenological constitutive model formulation was identified allowing a perfect match of
the averaged tension-stretch and contraction-elongation curves. Reproducing the kinematic behavior is very important in order to explain the large biaxial stiffness of amnion. In fact, model prediction of the biaxial tension response of amnion was shown to be very good.

The successful implementation of the constitutive model in a FE code enables future investigations of different experimental configurations in more detail. Reliable definition of the reference configuration was shown to be important for mechanical analysis and comparison of data from different studies or originating from different experimental configurations. However, the very low initial stiffness complicates the detection of the onset of material response. A compromise is to introduce a material specific threshold value (initial membrane tension), which is independent from the experimental configuration and sample dimensions.

The present findings enable the development of numerical models aiming at (i) identifying fetal membranes at higher risk of rupture, (ii) analyzing the mechanical response of FM in different experimental configurations, (iii) modeling and simulating medical procedures in order to minimize the risk of iatrogenic rupture, and (iv) developing synthetic materials with comparable performance in terms of deformability and toughness.
Puncture testing applied to fetal membrane tissue

5.1 Introduction

Understanding the mechanical behavior of fetal membrane tissue is necessary for the development of methods to prevent preterm rupture or repair of the fetoscopic entry site after minimally invasive fetal surgery. The composition of the two constitutive layers amnion and chorion and their sublayers were extensively analyzed and described in the literature (Malak et al., 1993; Ilancheran et al., 2009). The thin amnion layer, which is mainly composed of collagen types I, III, IV, V, VI is attached to the thicker and more cellular chorion layer. The connection between amnion and chorion is referred to as the intermediate or “spongy” layer, which is usually attributed to amnion and originates from the fusion of amnion and chorion around 17 to 20 weeks of gestation (Ilancheran et al., 2009). Although the biochemical composition and characteristics have been studied in great detail, a consistent mechanical characterization of these layers is still missing.

Mechanical data of FM were mainly obtained by the use of three types of mechanical testing: (i) uniaxial tension, (ii) inflation or burst, and (iii) puncture testing. The latter is characterized by simple sample handling and preparation and allows many samples to be tested from one membrane. In a first report McGregor et al. (1987) applied puncture testing for the evaluation of the strength of FM exposed to bacterial protease. Oyen et al. (2004b) assessed the puncture failure of intact FM, chorion, and amnion and evaluated the effect of labor and preterm delivery on the puncture force. Later, Oyen et al. (2006) evaluated the relative contributions of amnion and chorion to the strength of intact fetal membrane by puncture testing and correlated the findings with gestational age. Pressman et al. (2002) performed a similar study on intact FM. Several studies reporting results
Chapter 5. Puncture testing applied to fetal membrane tissue

from puncture tests on FM originate from one group, whose results are summarized in the review paper of Moore et al. (2006). El Khwad et al. (2005) provided evidence of the mechanical changes in the zone of altered morphology (ZAM) which overlays the cervix and is presumed to be the natural FM rupture site. Arikat et al. (2006) also studied the biophysical properties of individual components (amnion and choriodedicidua) and compared them with intact FM. Arikat et al. found that the separation of amnion from choriodedicidua, which happens prior to rupture, constitutes a significant component of the work required to rupture the sample. Finally, Pandey et al. (2007) used puncture testing to investigate the effect of cyclic stretching on the rupture strength of FM and observed that rupture strength increased after preceding cyclic loading.

At present, there is no final conclusion in the literature about the rupture sequence of FM components (amnion or chorion to rupture first) and how this might depend on the experimental configuration used for testing the membranes. For example, Helmig et al. (1993) (uniaxial) and Schober et al. (1994b) (puncture) found that amnion ruptures first, whereas Artal et al. (1976) (uniaxial), Lavery and Miller (1979) (inflation), and Oyen et al. (2004b) (puncture) stated that chorion ruptures first. The work by Arikat et al. (2006) provided evidence of the rupture sequence of FM by video documentation of puncture tests, showing that chorion ruptures first. This is in contrast to observations from our studies (Buerzle et al., 2013; Buerzle and Mazza, 2013; Perrini et al., 2013) using inflation and uniaxial tension experiments on intact FM as well as on separate amnion and chorion layers.

The method of puncture testing originates from the textile industry and became more popular for applications to FM tissue since the work of Schober et al. (1994b), who showed that results from puncture testing can be extrapolated and compared to results from inflation testing. There are several theoretical studies that cover the topic of deflections of a circular membrane. Bhatia and Nachbar (1968) proposed a nonlinear membrane solution for the stresses and deflections of the finite indentation of an elastic membrane. Begley and Mackin (2004) also treated the spherical indentation of circular elastic films in the membrane regime and proposed closed-form analytic solutions, which allow the determination of elastic parameters from experiments. Scott et al. (2004) and Komaragiri et al. (2005) used a behavior map which allows to evaluate if the circular film behaves as plate, linear membrane or nonlinear membrane depending on two dimensionless parameters. A computational estimation of the load-deflection response based on uniaxial experiments as well as an analysis of the effect of friction can be found in Selvadurai (2006). Two recent studies applied puncture testing for the characterization of extracellular matrix
materials, where Freytes et al. (2005) implemented a rather simple and Cloonan et al. (2012) a more sophisticated analytical model for the analysis of the test data. Despite the large amount of literature available, detailed understanding of the method of puncture testing under consideration of aspects related to biological tissues is still missing. This study aims at puncture characterization of fetal membrane tissue and at a general analysis of the test method. Puncture experiments are performed and analyzed with focus on the rupture sequence. Corresponding finite element simulations are carried out to investigate particular phenomena observed in the experiments. Careful analysis of the experiments and insights from the numerical simulation allow the evaluation of puncture testing as a method to characterize FM tissue.

5.2 Methods

5.2.1 Experimental setup and protocol

In order to perform puncture tests on fetal membrane samples, a dedicated setup was built on the model of the one used in Moore et al. (2006). The setup consists of a vertical tension test machine (Stentor II, Andilog Industries, France) with a vertical travel distance of 200 mm. Attached to the traverse is a force gauge with 50 N capacity, see Figure 5.1(a). The tension test machine has a position accuracy of 0.01 mm and the force sensor a resolution of 5 mN. The device is controlled by the software CALIFORT (Andilog Industries, France) which allows a sequential programming of the test protocol. The software allows only tests performed under a displacement controlled (constant velocity) regime. Two different clampings were designed to investigate their influence on the resulting puncture test data as well as on the rupture sequence, see Figure 5.1. The “long clamping” (LC) was designed to allow a gentle clamping of the FM sample. The “short clamping” (SC) was designed to be similar to the one used for the inflation tests (see section 3.4.2) to provide a tight sample fixation. Figure 5.2 illustrates schematic drawings including geometrical dimensions of the two clampings utilized in the present study. A spherical plunger with diameter 10 mm was used for all tests in this work. The LC was designed to be similar to the one used in El Khwad et al. (2005) and Moore et al. (2006). Relevant but not specified clamping dimensions were read out of the images in the corresponding publications (e.g. Figure 1 in Arikat et al. (2006)). In detail, the inner diameter of the clamping is 25 mm and a $\varnothing 5$ mm sealing ring of inner diameter 40 mm is inserted in the lower part of the clamping, see Figure 5.2(a). In addition, a digital camera (Microsoft,
Chapter 5. Puncture testing applied to fetal membrane tissue

LifeCam Cinema, 720p) is placed below the clamping to monitor the rupture sequence. The tests are performed with a constant plunger speed of 25 mm/min. The experimental data consist of the force and displacement data and the recorded movie from the bottom view of the sample. A low force threshold of 25 mN is used to normalize the experimental data unless otherwise stated, thus a deflected reference configuration is considered for the data analysis.

Figure 5.1: The puncture test setup. A vertical tension test machine (a) is equipped with a dedicated clamping and plunger to perform puncture tests. A digital camera is placed underneath the clamping to record the rupture sequence. Two different clampings are utilized for the investigation of the dependency of the mechanical characteristics on the boundary conditions. The two clampings are denoted as long clamping (b) and short clamping (c). The notation long and short refers to the length of the membrane segment fixed in the clamping.

5.2.2 Sample collection and preparation

Collection of fetal membranes follows the procedure reported in section 3.2. The sample preparation is slightly different for samples tested on the long or short clamping. The fetal membrane is spread with the chorion side downward on a lubricated mat. For samples tested on the LC, a piece of plastic-coated paper with the dimensions of approximately $50 \times 50$ mm is placed on the amnion side and the sample cut along the borders of the paper. The paper is used to support the membrane for the transport into the clamping.
Figure 5.2: Schematic drawings including geometric dimensions of the two clampings utilized for the present study, i.e. (a) long clamping (LC), and (b) short clamping (SC).

The preparation of samples tested on the SC is identical to the preparation of samples used for inflation testing by gluing sandpaper rings (grade P400, inner diameter 27 mm, outer diameter 45 mm) on both sides of the sample, see section 3.4.2. For all tests the membrane sample is placed on the fixture with the chorion side downward and is clamped by the cover plate. Saline solution is frequently sprayed on the sample to avoid dehydration and to reduce possible friction effects.

5.2.3 Analysis of puncture test data

Material characterization by puncture testing is usually done by determination of maximum values of plunger force and displacement as well as calculation of the membrane tension in the apex region. More information can be obtained if the second derivative of the force-displacement curve is considered, since this provides information about slippage in the clamping or weakening of the material prior to failure. Monotonic stiffening during elongation is expected for an intact material. This implies that the first derivative of the force-elongation curves is monotonically increasing. An indication of a beginning material damage or slippage is therefore when the second derivative at a certain time $t^*$ becomes negative:

$$\frac{\partial^2 F(t^*)}{\partial u^2} < 0.$$ (5.1)

At $t^*$ the first derivative will still be positive, giving the impression that the material is intact. Calculation of derivatives of experimental data is challenging due to noise in the signal. Therefore, a fifth order polynomial is fitted to the experimental data first, and derivation is performed on the polynomial fit. Subsequently, the time point at which the second derivative becomes negative is evaluated.
5.2.4 Finite element model

The finite element software package ABAQUS 6.9-1 is used for numerical simulations of puncture tests. The fetal membrane tissue is assumed to be homogeneous in thickness and in its material properties. Therefore, an axisymmetric model of the puncture test is set up and nonlinearities are considered. The FM is modeled as bilayer structure composed of amnion and chorion, and the transversely isotropic constitutive model presented in section 4.3 is used to represent their individual mechanical behavior. Corresponding model parameters can be found in Table 4.2. Modeled layer thicknesses are 100 µm for amnion and 400 µm for chorion, according to the results in section 3.8.3. The interaction of amnion and chorion in terms of a constitutive relation is unknown. Amnion and chorion are connected through the “spongy” layer which possesses a certain adherence (Strohl et al., 2010) but on the other hand allows relative movement between the main layers (Bourne, 1962). Moreover, separation of amnion and chorion was observed to occur in uniaxial tension tests of intact FM caused by differences in the layer specific Poisson’s effects (Buerzle and Mazza, 2013). The two extreme cases of free relative sliding and tied contact are implemented in the FE model to capture this behavior.

Figure 5.3 shows the axisymmetric finite element model for the short and long clamping. Both models are in principle identical, only the length of the membrane segment inside the clamping varies depending on the clamping type. The membrane is fixed in the radial direction at the outer extremity. The vertical movement of the membrane is constrained by the clamping. The top and bottom parts of the clamping are modeled as analytic rigid surfaces, both being fully constrained at their reference point. The membrane is deflected by a spherical plunger which is also modeled as a rigid body. The lateral and rotational degrees of freedom of the plunger are fixed while a displacement ramp in the axial direction is applied. The resulting puncture force is assessed from the reaction force at the reference point of the plunger. Values of membrane tension in the finite element simulations refer to the values of the radial Cauchy stress component and the current thickness at the axis of symmetry.

Definition of the interaction between master surface (rigid body) and slave surface (deformable membrane) is required for contact analysis. Surface-to-surface contact with finite sliding formulation was used. For the contact behavior in the normal direction the default (penalty method) constraint enforcement method with hard pressure overclosure was used and separation after contact was allowed. The tangential behavior of the two contact pairs either includes Coulomb friction or was modeled frictionless depending on the study type, see Table 5.1. Linear quadrilateral elements (CAX4R) with reduced in-
5.2. Methods

Figure 5.3: Illustration of the axisymmetric finite element model (with representative mesh size) utilized for the analysis of the puncture test method. The figure shows the SC printed in black and the extension added for modeling of the LC in gray. Clamping and plunger are modeled as analytical rigid surfaces and the deformable membrane as bilayer structure. The transversely isotropic constitutive model derived in section 4.3.1 is used to describe the mechanical behavior of the FM.

Integration and enhanced hourglass control are used for the deformable membrane. The appropriate mesh size was determined within a convergence study on the example of a circular plate under concentric point load because of its known analytical solution. Element size, shape function, and integration order were varied in order to find the best compromise between accuracy and calculation time for the current situation. The UMAT implementation was verified in section 4.5 with brick and axisymmetric elements. An extensive overview about the use of the UMAT subroutine in combination with different element types and formulations and their implications on precision and calculation time can be found in Papes (2012).

The finite element models for SC and LC, as illustrated in Figure 5.3, are used to investigate different aspects of puncture testing, which are considered to be important for the testing of FM tissue. Table 5.1 provides an overview about the different study types performed and summarizes the parameters characterizing the corresponding FE models. The aspects analyzed in the single studies are: the interaction of amnion and chorion (C1), the effect of friction within the clamping (C2) as well as at the plunger interface (C3), evaluation of the quality of the estimation of the membrane tension by analytical models (C4), analysis of the stress and stretch distributions across the thickness (C5), and further aspects of data analysis (C6).
Table 5.1: Summary of the FE model characteristics utilized for different numerical simulations.

<table>
<thead>
<tr>
<th>Study type</th>
<th>Amnion-Chorion interaction</th>
<th>Clamping type</th>
<th>Tangential behavior</th>
<th>Reference configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>tied &amp; free</td>
<td>LC &amp; SC</td>
<td>frictionless</td>
<td>frictionless flat</td>
</tr>
<tr>
<td>C2</td>
<td>tied &amp; free</td>
<td>LC</td>
<td>variable</td>
<td>frictionless flat</td>
</tr>
<tr>
<td>C3</td>
<td>tied</td>
<td>SC</td>
<td>frictionless</td>
<td>variable flat</td>
</tr>
<tr>
<td>C4</td>
<td>tied</td>
<td>LC</td>
<td>frictionless</td>
<td>frictionless flat</td>
</tr>
<tr>
<td>C5</td>
<td>tied</td>
<td>LC</td>
<td>frictionless</td>
<td>variable flat</td>
</tr>
<tr>
<td>C6</td>
<td>tied</td>
<td>LC</td>
<td>frictionless</td>
<td>variable flat</td>
</tr>
</tbody>
</table>

5.2.5 Analytical models to estimate contact angle and membrane tension

Puncture testing induces an inhomogeneous deformation field in the membrane sample. The deformation field is nearly equibiaxial in the center and transforms to a pure-shear state at the clamping similar as within inflation testing. No direct extraction of scalar material parameters from the measured force-displacement curves is possible due to the inhomogeneity of the displacement field. The membrane tension in the center of the plunger can be estimated from simple analytical models.

The plunger induces a spherical deformation in the center of the membrane. Axial equilibrium of forces inside the contact region (Laplace’s law)

\[ T = \frac{F}{2\pi R \sin^2(\beta_C)} \]  

provides a relation between the puncture force \( F \) and the membrane tension \( T \), based on the assumption of a constant equibiaxial stress state. The only unknown that remains is the contact angle \( \beta_C \), which can be estimated from analytical models. Two different models are introduced in the following and used for the estimation of the contact angle. The quality of the estimation will be evaluated by comparison with results from numerical simulations.

Conical model

The simplest analytical model can be obtained by assuming the deformed membrane to be composed of a conical segment and a spherical cap, see Figure 5.4. The contact angle \( \beta_C \)
5.2. Methods

**Figure 5.4:** Analytical models used for the analysis of the contact angle $\beta_C$. Left side shows the conical model and the right side the model by Begley and Mackin (2004).

can be assessed directly from geometric relations, since the conical segment is tangential to the plunger at the contact point. The length of the conical segment $f$

$$f = \sqrt{a^2 + (u - R)^2 - R^2}$$  \hfill (5.3)

as well as the contact angle $\beta_C$

$$\beta_C = \begin{cases} 
\tan^{-1}\left(\frac{a}{u-R}\right) - \tan^{-1}\left(\frac{f}{R}\right), & u < R \\
\pi - \tan^{-1}\left(\frac{a}{u-R}\right) - \tan^{-1}\left(\frac{f}{R}\right), & u \geq R \end{cases}$$  \hfill (5.4)

can be expressed solely as a function of the plunger displacement $u$, radius of the plunger $R$, and the half diameter of the clamping $a$. The plunger displacement refers to a flat reference configuration and it is assumed that the membrane thickness is much smaller than the planar dimensions.

**Model by Begley and Mackin**

The work of Begley and Mackin (2004) provides an extensive study of the mechanics of membrane deflections, and provides relationships between mechanical properties of the membrane sample, probe dimensions, and the load-deflection response. Their work uses the theory of thin shells and is based on the work of Bhatia and Nachbar (1968). The model by Begley and Mackin considers finite deflections and rotations but assumes small strains of the membrane material. The variables used to describe the deformed membrane
are shown in Figure 5.4. The radial and circumferential stretches can be obtained from the corresponding displacements \( u_r \) and \( u_z \) in the radial and axial directions

\[
\lambda_r = \sqrt{\left(\frac{d\rho}{dr}\right)^2 + \left(\frac{du_z}{dr}\right)^2} \tag{5.5}
\]

\[
\lambda_\theta = 1 + \frac{u_r}{r} \tag{5.6}
\]

with \( \rho = r + u_r \) being the deformed radial position of a material point. Expansion of these equations leads to the corresponding kinematic relations:

\[
\varepsilon_r \approx \frac{du_r}{dr} + \frac{1}{2} \left(\frac{du_z}{dr}\right)^2 \tag{5.7}
\]

\[
\varepsilon_\theta = \frac{u_r}{r} \tag{5.8}
\]

A (incompressible) neo-Hookean material description (elastic constant \( C_1 = E/6 \)) is used to express the membrane tensions (stress resultants) depending on the corresponding strains, the membrane thickness \( h \), and the Young’s modulus \( E \).

\[
T_r = \frac{2}{3} Eh (2\varepsilon_r + \varepsilon_\theta) \tag{5.9}
\]

\[
T_\theta = \frac{2}{3} Eh (2\varepsilon_\theta + \varepsilon_r) \tag{5.10}
\]

These general relations are used to obtain separate closed-form approximations for the contact and the freestanding region. For the calculation of the contact angle only the behavior inside the contact region is of interest. Assuming that the strains in the contact region in both directions are small and that the axial displacement \( u_z (r) \) of the membrane is dictated by the plunger displacement \( u \)

\[
u_z = u - R + \sqrt{R^2 - r^2} \tag{5.11}
\]

leads to an estimation of the averaged stretch \( \lambda_0 \) in the contact region

\[
\lambda_0 = 1 + \frac{1}{6} \left(\frac{\rho C}{R}\right)^2 \tag{5.12}
\]
as a function of the radius of contact $\rho_C$. Combination of the averaged stretch in the contact region with the stress resultants and considering axial force equilibrium leads to the expression of the contact angle

$$\sin(\beta_C) = \sqrt{\frac{3F}{2\pi EhR}}$$

(5.13)

for the case of zero prestress. This result defines simultaneously a limit of the validity of the approximation, since $\sin(\beta_C) \leq 1$ has to be fulfilled.

5.3 Experimental results

5.3.1 Puncture tests on fetal membrane samples

Puncture tests were performed on a total of 36 samples from seven different membranes, i.e. 26 samples on the long clamping and 10 samples on the short clamping. Figure 5.5(a) shows the force-displacement curves from each tested FM sample, normalized by a force threshold of 0.025 N. This force threshold corresponds to an overall averaged initial membrane tension (estimated by the conical model) of $(3.5 \pm 1.8) \cdot 10^{-3}$ N/mm which is comparable to the corresponding average of $(2.1 \pm 0.4) \cdot 10^{-3}$ N/mm used for the inflation tests in section 3.7.2. The force-displacement curves illustrated in Figure 5.5 show the typical bilinear behavior with low initial stiffness and subsequent transition into a region of higher stiffness. The sample is initially slack due to squeezing within the clamping. The reference configuration, defined by a force threshold, is characterized by an initial apex displacement $D_0$ similar as within inflation testing, see section 3.4.4. Thus a deflected reference configuration is considered for the further analysis.

The curves in Figure 5.5(a) illustrate the scatter in the data obtained from measurements on different membranes. The overall averaged force-displacement curves for the two clippings utilized, can be seen in Figure 5.5(b). The results obtained by the use of the SC are characterized by a smaller extensibility and much higher stiffness for large deformations. Table 5.2 summarizes the data of maximum force $F_{max}$, maximum apex displacement $u_{max}$, and critical membrane tension $T_{crit}$ for both clamping types and averaged for each membrane tested. The initial apex displacement $D_0$ which defines the reference configuration is characterized by an averaged value of $(6.9 \pm 1.8)$ mm for the LC and $(6.3 \pm 1.5)$ mm for the SC. Overall averaged data for the parameters specified in Table 5.2 are $(10.9 \pm 5.5)$ N for the maximum force, $(0.46 \pm 0.23)$ N/mm for the critical
Figure 5.5: Resulting force-displacement curves obtained by puncture testing on fetal membrane samples. Force-displacement curves of each single sample (a) and averaged curves for both clamping types utilized (b). LC: long clamping, SC: short clamping.

Table 5.2: Mechanical parameters extracted from the force-displacement curves obtained by puncture testing. Between four and seven samples were tested from each membrane.

<table>
<thead>
<tr>
<th>Clamping type</th>
<th>Membrane</th>
<th>$F_{\text{max}}$ [N]</th>
<th>$T_{\text{crit}}$ [N/mm]</th>
<th>$u_{\text{max}}$ [mm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long clamping</td>
<td>P1</td>
<td>6.7 ± 2.8</td>
<td>0.28 ± 0.1</td>
<td>9.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>7.7 ± 1.4</td>
<td>0.32 ± 0.04</td>
<td>9.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>9.5 ± 1.2</td>
<td>0.39 ± 0.04</td>
<td>11.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>17.9 ± 5.1</td>
<td>0.75 ± 0.22</td>
<td>11.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>10.2 ± 3.6</td>
<td>0.47 ± 0.16</td>
<td>8.2 ± 0.6</td>
</tr>
<tr>
<td>Short clamping</td>
<td>P6</td>
<td>8.2 ± 1.3</td>
<td>0.39 ± 0.04</td>
<td>6.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>P7</td>
<td>14.7 ± 4.1</td>
<td>0.79 ± 0.17</td>
<td>7.2 ± 0.4</td>
</tr>
</tbody>
</table>

membrane tension, and (10.0 ± 1.6) mm for the maximum displacement obtained by the use of the LC. Corresponding data for the SC are (11.5 ± 4.5) N, (0.59 ± 0.24) N/mm, and (6.9 ± 0.5) mm, respectively. Differences in the mechanical parameters obtained by the use of the two clamping methods are only significant ($p < 0.05$) for the maximum displacement. The values of membrane tension provided in Table 5.2 were calculated by the law of Laplace and the estimation of the contact angle by the conical model as introduced in section 5.2.5.
5.3.2 FM rupture in puncture tests

The rupture sequence of each FM sample was investigated by video recording from the lower side of the clamping. The rupture sequence differs for the two clamping types utilized, but follows a characteristic pattern for each clamping method. Characteristic force-time curves for the two clampings utilized are illustrated in Figure 5.6. For the long clamping, the rupture sequence was as follows: The plunger contacts the membrane and deflects it by simultaneous stretching of both tissue layers. At a certain level of deformation the puncture force reaches a first maximum $F_1$ and an audible “pop” sound can be recognized which is associated with a subsequent decrease of the puncture force. After this first event the puncture force remains constant or increases again. Somewhat later a second failure process reduces the puncture force from $F_2$ to zero. It can be seen in the recorded movie sequences that chorion ruptures first in the center of the sample and then slides radially over the plunger, as illustrated in Figure 5.7. Amnion, still intact and attached to the plunger, penetrates through chorion and ruptures later. Out of the 26 samples, 17 are classified as chorion rupture first by the above mentioned rupture sequence. Both tissue layers ruptured together for seven samples, and for two samples a possible rupture of the amnion layer happened first. This is assumed because a sudden decrease of the puncture force was observed but no rupture of chorion could be observed. The rupture sequence was highly consistent for the tests performed with the short clamping. Also these tests started with simultaneous stretching of both layers up to the deformation and force level $F_1$ where an audible “pop” sound could be recognized. However, in these tests the “pop” appeared along with visible shaking of the membrane sample and a sudden decrease of the force signal, while chorion was still intact. After this traumatic event there was only a moderate increase of the force up to the level $F_2$ where also chorion failed. This sequence was observed in all tests with the short clamping, thus amnion ruptured first in all tests. Failure in amnion happened most often in the center or at the periphery of the plunger. Only two samples ruptured close to the clamping site.

5.3.3 Damage or slippage before rupture?

In addition to maximum values of force and displacement, also the second derivative of the force-displacement curves was analyzed. Analysis of the curves from the FM measurements with the LC shows that the second derivative becomes negative prior to failure in 18 out of 26 samples (69% of cases), see Figure 5.8. The criterion is fulfilled in 14 out of 17 samples (82%) which were classified as chorion rupture first. The correspond-
Figure 5.6: Illustration of characteristic force-time curves obtained by the use of the two clampings LC (a) and SC (b). For tests performed with the LC, chorion ruptures in most cases at the first force peak $F_1$. The subsequent force trace is not consistent, since an almost stable force level (as illustrated), decrease or increase of the force was observed. Somewhat later, rupture of amnion at $F_2$ reduces the force to zero. For tests performed with the SC an audible “pop” can be recognized in combination with visible shaking of the membrane at the first peak $F_1$, while chorion is still intact. After moderate force increase to $F_2$ rupture of chorion can be observed.

Figure 5.7: Characteristic sequence of FM rupture during puncture testing with the long clamping. The dashed line indicates the border of chorion. (a) Chorion ruptures first in the center and glides radially over the plunger, while amnion (illustrated by the crosshatched region) is still intact. (b) Amnion still intact and attached to the plunger penetrates through chorion, and finally rupture of amnion (most often at the periphery of the plunger) which also moves radially over the plunger (c).
5.3. Experimental results

![Graph A](a)  ![Graph B](b)

**Figure 5.8:** Illustration of the analysis of the second derivative (b) of the original force-displacement data (a) obtained by the use of the LC. Circular marker indicates the time point $t^*$ at which the second derivative becomes negative.

In summary, there is strong indication for damage or slippage in the puncture tests performed with the LC. Similar, but much less pronounced mechanisms can also be expected to be present in tests performed with the SC.

5.3.4 Interrupted puncture tests

Additional interrupted puncture tests utilizing the LC were performed on 10 fetal membrane samples. For five samples markers of India ink were applied on the amnion layer close to the clamping site to monitor possible sliding inside the clamping. The samples underwent common puncture testing until the force reached the critical range (reduction of second derivative), then the plunger movement was stopped and the direction reversed. Maximum force measured in these tests was in the range of 4.9 N to 7.5 N. The samples were subjected to careful visual inspection after testing to investigate indications of dam-
Figure 5.9: Illustration of the original force-displacement data (a) and the corresponding second derivative (b) for one test performed with the SC. Although, the second derivative does not become negative for any test performed with the SC, the curves show for some samples a distinct maximum with subsequent decrease.

age. Apart from a marginal imprint of the plunger no damage could be observed in either of the two constitutive layers. The criterion of negative second derivative was fulfilled in four of these samples.

Figure 5.10 shows two images of a FM sample at the beginning and end of the interrupted test. The movement of the markers clearly displays the slippage of amnion inside the clamping. The radial movement is not related to irreversible tissue deformation, since amnion recovers to its initial shape (the markers move back to the border of the clamping) after loosening of the clamping. Sliding of chorion was only observed for few samples by careful visual inspection of the recorded movies. Sliding of chorion is less pronounced and depends on the thickness and inhomogeneity of the layer. Homogeneous and thick chorion layers show a lower tendency for slippage. No such slippage of either amnion or chorion was observed for tests performed with the SC.

The results from the current section show that pronounced slippage of the amnion layer in the tests performed with the LC is the main cause for the reduction of the second derivative of the corresponding force-displacement curves. No such slippage was observed by visual inspection of the tests performed with the SC. Moreover, microscopic tissue damage, which might also contribute to the reduction of the second derivative, cannot be excluded for tests performed with both clamping types.
5.4 Results from numerical simulations

The finite element model as introduced in section 5.2.4 is used to get a better understanding of the mechanical response of biological tissues when subjected to puncture testing and in particular to investigate effects leading to the unusual rupture sequence of fetal membrane tissue. All FE models used for the subsequent analysis refer to the one introduced in section 5.2.4, while possible model modifications are explained in the corresponding sections.

5.4.1 Interaction of amnion and chorion

The interaction of amnion and chorion in terms of a constitutive relation is unknown. Two simulations of the limiting cases of free relative sliding (frictionless) and tied contact interaction are performed for each clamping type to investigate the influence of this unknown parameter.

The simulation results in Figure 5.11 show that there is no contribution from the interaction of amnion and chorion on the resulting force-displacement curves for LC and SC. This result is due to the limited possibilities of relative sliding between amnion and chorion in the numerical simulations when the outer extremity of the membrane is fixed. Chorion undergoes additional shear deformations if the interaction restricts the relative movement. However, the associated strain energy contributions are small due to chorion’s compliance. For example, at a plunger displacement of 10 mm the puncture force differs only by 1% for LC and by 3% for SC.
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Figure 5.11: Results from puncture test simulations (case C1) with different interactions of amnion and chorion, i.e. free relative sliding and tied contact. The results show that there is no contribution from the interaction of amnion and chorion on the puncture force for both clamping types LC and SC. The numerical results were normalized by a force threshold of 0.025 N.

5.4.2 Effect of friction

Numerical simulations are performed with variation of the coefficient of friction (Coulomb friction) in order to get more insight in the dependency of the puncture test results on frictional effects. Influence of the friction at the interfaces of membrane-plunger and membrane-clamping was analyzed separately (simulation cases C2 and C3 in Table 5.1). Modification of the FE model from section 5.2.4 was necessary to apply friction in the clamping. In a first step of the simulation the membrane is compressed by moving the upper part of the clamping 0.1 mm downward, while the membrane is free to move in radial direction, i.e. the boundary condition at the outer extremity is removed for both simulation steps. In a second step, the common puncture test is performed by vertical movement of the plunger. Analysis of the frictional effects in the clamping is performed for both tied and free interaction of amnion and chorion. The initial compression of the membrane in the clamping generates contact pressure which, in combination with the introduced coefficient of friction, limits the slippage inside the clamping. The FE model with the short clamping was used for the analysis of the effect of friction at the plunger interface.

The friction at the membrane-clamping interface has a large impact on the force-displacement characteristics, as illustrated in Figure 5.12(a). There is a shift in horizontal direction as well as a change in the slopes of the force-displacement curves at small and large displacements. The change of the initial stiffness originates from the preceding com-
5.4. Results from numerical simulations

Figure 5.12: Analysis of the effect of friction at the interface of membrane-clamping (a) and membrane-plunger (b). Simulations were performed by variation of the coefficient of friction (Coulomb friction) at the corresponding interface. Data in (a) are shown as raw values (not normalized), pronounced differences in initial stiffness lead to unphysical representation after normalization.

pression step required to create contact pressure. Compression of the membrane leads to large radial expansions mainly of chorion, which, for the tied interaction, leads to a tensile prestress of amnion. The results in Figure 5.12(a) indicate that low coefficients of friction ($\mu < 0.1$) lead to sample slippage in the clamping, which significantly reduces the puncture force. It is interesting to note that even a frictionless simulation without boundary condition at the outer border reaches a puncture force of approximately 12 N at 12 mm displacement. This is caused by the limited possibilities of radial displacement within the axisymmetric configuration. Radial displacements of the outer border of the membrane are in the range of 2.6 mm for $\mu = 0$, and 0.6 mm for $\mu = 0.2$ at a plunger displacement of 15 mm. Only one simulation, allowing free relative movement (frictionless) between amnion and chorion, could be performed due to pronounced convergence problems involved in these simulations. The corresponding result with $\mu = 1$ at the clamping agrees with the simulations of the tied interaction, except from the initial stiffness, i.e. free sliding between amnion and chorion prevents radial prestress. However, large influence of the amnion-chorion interaction for cases with $\mu < 1$ cannot be excluded. The change of the slopes at larger deformations for different coefficients of friction originates from the restricted stretching of the membrane in the clamping. The results from simulations with the SC agree qualitatively well with the one obtained by introducing friction in the clamping when the radial movement in the clamping is restricted by friction effects.
Figure 5.12(b) shows the resulting force-displacement curves with varying coefficients of friction at the plunger interface. The different curves are close to each other, but there are still deviations due to the exponential type of curves. For illustration, the force at a plunger displacement of 8 mm differs about 14% between the frictionless simulation and the simulation with $\mu = 1.0$.

5.4.3 Estimation of membrane tension by analytical models

The membrane tension in the apex region can be approximated by Laplace’s law, once the contact angle is known. The accuracy and validity of the analytical models, allowing an estimation of the contact angle, is evaluated by comparison with numerical results (simulation case C4). The reference value of the contact angle is determined in numerical simulations by analysis of the contact status (closed or open).

Figure 5.13 shows the progress of the contact angle as a function of plunger displacement for different models. The rough progress of the FE result is caused by the discrete nature of the FE mesh. As can be seen in Figure 5.13, the simple conical model provides a reasonable approximation of the FE result. The Begley-Mackin model requires the input of a Young’s modulus, which was determined as $8.6 \cdot 10^{-2}$ MPa by the separate layer tests in section 4.4.1, and corresponds to the initial stiffness of the model used for the FE simulation. The prediction of the contact angle from the Begley-Mackin model differs clearly from the FE result and the conical model, as can be seen in Figure 5.13. This large deviation can be explained by general differences between the (incompressible) neo-Hookean model and the transversely isotropic formulation of the Rubin-Bodner model. Moreover, the results of the Begley-Mackin model depend strongly on the value of the Young’s modulus, which also influences the range of validity of the model. Model predictions of membrane tension are calculated from the corresponding force and contact angle data, and are compared to two different FE results. First, the membrane tension within the FE simulations is obtained by analyzing the elements on the axis of symmetry due to the equibiaxial stress state in the center. Second, an approximation of the membrane tension is obtained by application of Laplace’s law (equation 5.2) to the FE results of force and contact angle. Figure 5.14 illustrates the results of membrane tension as a function of the puncture force obtained from different models. The Begley-Mackin model is only valid up to a force of approximately 0.4 N or a membrane tension of 0.01 N/mm, for which reason it is not included in the figure. The simple conical model provides a useful approximation of the membrane tension. The deviations between the FE tension and the one obtained by the conical model are 19% at a force of 6 N and 24% at 12 N. Differences between
5.4. Results from numerical simulations

![Graph](image)

**Figure 5.13**: Illustration of the progress of the maximum contact angle $\beta_C$ between membrane and plunger as a function of the plunger displacement. Values of contact angle were obtained by the use of different models. The displacement value refers to a flat membrane.

...the true FE tension and the application of Laplace’s law on the FE data are related to the inhomogeneous stress field in the apex region. In fact, the ratio of circumferential to radial stresses at the border of the contact region is in the range of 0.5. Thus, the assumption of constant equibiaxial stress is not fulfilled.

5.4.4 Stress and stretch distribution in the center

Analysis of the stress and stretch distribution is based on a FE simulation (case C5) according to section 5.2.4. The corresponding distributions are determined by evaluation of the element specific stress and strain values across the thickness. The FE simulations are performed with different values of friction at the membrane-plunger interface to investigate the influence of a local support of the amnion layer.

Figure 5.15 illustrates the distributions of radial Cauchy stress and stretch across the nominal sample thickness (y coordinate), whereby the distributions are evaluated at the average experimental rupture force of 11 N, representative for both clamping types. The rough progress of the distribution is caused by the FE discretization. As can be seen in the two graphics, there are four elements across the thickness of the chorion layer and two elements across the amnion layer. It can be seen in Figure 5.15 that the stretch distribution, for a frictionless contact, is almost constant. In consequence, the corresponding stresses in amnion are much larger than the ones in chorion. On the contrary, the top layer of amnion is supported by the plunger interface if a certain amount of friction is present at the plunger. However, comparison of the values in Figure 5.15 with the corre-
Figure 5.14: Results of the predictions of membrane tension obtained by the use of different models, plotted as tension-force curves. The Begley-Mackin model is only valid up to a force of approximately 0.4 N or a tension of 0.01 N/mm, respectively, for which reason the corresponding curve is not included in the figure. All model predictions refer to the reference configuration of a flat membrane.

In general, evaluation of the stress and strain distributions at an averaged experimental rupture force leads to values of stress and strain that highly overestimate the corresponding critical experimental values. The reason for this overestimation might be found in a too stiff modeling of the sample fixation. Therefore, an additional simulation was performed with the ambition to reproduce the experimental observation of amnion slippage in the clamping while chorion is fixed. The FE model had to be modified slightly in order to allow slippage of the amnion layer. The displacement constraint in the radial direction at the outer side of the membrane was removed, while the lower side of chorion was constrained in radial direction. The remaining parameters (LC, frictionless at upper side of the clamping, tied interaction) of the simulation remained unchanged.

Figure 5.16(a) illustrates the force-displacement curves of the simulations which include slippage of amnion, where a pronounced difference in the force data can be observed. A comparison of the model prediction with experimental data can be found in Figure 5.20. The stretch distributions in Figure 5.16(b) refer to the same level of plunger displacement of 10 mm. It can be seen that the possibility of slippage of amnion slightly reduces the
5.4. Results from numerical simulations

![Stress and Stretch Distributions](image)

Figure 5.15: Illustration of the Cauchy stress (a) and stretch (b) distributions (in radial direction) across the thickness in the center of the sample. The distributions correspond to a puncture force of 11 N which is representative of the averaged experimental rupture force.

The corresponding strains. Pronounced support of the amnion layer can be observed if friction at the plunger interface is included. The gray shaded area in Figure 5.16 illustrates the expected region of membrane failure, i.e. it represents the stretch at rupture within the range of $1.20 \pm 0.03$, as determined in section 3.7.2 by inflation testing. The results in Figure 5.16 illustrate the possibility of chorion rupturing first if amnion glides in the clamping and if friction at the plunger is present.

5.4.5 Normalization of puncture test data

Aspects related to the analysis of puncture test data are investigated by a numerical benchmark problem. The deflected reference configurations are generated by displacing the outer diameter of the membrane toward the inner side, mimicking a rigid body motion as as caused by squeezing in the clamping. In addition, a small pressure load ($1 \cdot 10^{-6} \text{ MPa}$) is applied on the top surface of the sample to ensure that the membrane is deflected toward the lower side. Variation of the boundary displacements leads to different (approximately stress free) reference configurations.

Figure 5.17(a) shows the raw force and displacement data obtained in puncture test simulations with different reference configurations, which are characterized by a horizontal shift of the curves. Figure 5.17(b) illustrates the same curves after a normalization with a force threshold of $0.025 \text{ N}$. The deflected reference configurations are characterized by an initial apex displacement $D_0$. Corresponding values are: $D_0 = 3.0 \text{ mm}$, $D_{01} = 4.6 \text{ mm}$,
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Figure 5.16: Illustration of force-displacement (a) and strain distributions (b) across the thickness for the models which consider amnion slippage in the clamping. Continuous line: amnion fixed, dashed line: amnion slippage, frictionless, and dash-dotted line: amnion slippage, $\mu = 0.6$ at the plunger. The force-displacement data were normalized by a force threshold of 0.025 N. The gray shaded area illustrates the expected region of membrane failure from the inflation data in section 3.7.2.

$D_{02} = 5.8$ mm, $D_{03} = 6.8$ mm. It can be seen in Figure 5.17(b) that the order of the curves invert, meaning that the curve with the largest initial displacement shows the stiffest response after normalization. Since all simulations were performed with the same material description, the characteristic of a successful normalization is the superposition of all curves on one line. The geometric nonlinearities involved in the membrane deflection cause differences in the initial tension at the level of the threshold force. Therefore, normalization with respect to the same level of membrane tension is more meaningful, as already discussed in section 4.6. Figure 5.18(a) shows the progress of the membrane tension versus raw plunger displacement. The corresponding curves after normalization with an initial membrane tension (0.01 N/mm) are illustrated in Figure 5.18(b).

It can be seen that even the normalization on the same level of membrane tension does not provide a successful method to regroup the curves. The consequences that arise from this behavior can be seen in Figure 5.19(a). There is a dependency of the membrane tension not only on the force but also on the reference configuration. For example, at an average rupture force of 11 N the corresponding membrane tensions vary between 0.73 N/mm and 0.79 N/mm (8% difference) just due to the differences in the reference configuration. Successful normalization of the puncture test data is only possible if the corresponding stretch values are known, as shown in Figure 5.19(b).
5.4. Results from numerical simulations

Figure 5.17: Illustration of the raw force-displacement data (a) and the corresponding curves after normalization (b) by a force threshold of 0.025 N. The small force threshold does not allow to group the curves. Corresponding values of apex displacement at the reference configuration are: $D_0 = 3.0 \text{ mm}$, $D_{01} = 4.6 \text{ mm}$, $D_{02} = 5.8 \text{ mm}$, $D_{03} = 6.8 \text{ mm}$.

Figure 5.18: Illustration of the raw tension-displacement curves (a) as obtained by numerical simulations. Figure (b) shows the same curves after normalization by a threshold of the membrane tension of 0.01 N/mm. Corresponding values of apex displacement at the reference configuration are: $D_0 = 3.1 \text{ mm}$, $D_{01} = 4.8 \text{ mm}$, $D_{02} = 6.1 \text{ mm}$, $D_{03} = 7.1 \text{ mm}$.
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Figure 5.19: Illustration of the relation between membrane tension and plunger force (a). (b) shows tension-stretch curves after normalization with membrane tension threshold of 0.01 N/mm. Grouping of the curves with different reference configurations is only possible if the corresponding stretch data are known.

5.4.6 Comparison of model prediction with experimental data

A suitable numerical model is not only characterized by its ability to reproduce particular experimental effects but also by its capability to adequately reproduce the macroscopic behavior in terms of the force-displacement response. Figure 5.20 illustrates the comparison of the force-displacement curves obtained from numerical simulations (case C1, according to Table 5.1) and experiments for the two clampings utilized. The comparison of the data obtained for the LC is done on the example of both, the standard FE model from section 5.2.4 and the model that allows slippage of amnion in the clamping. Figure 5.20(a) reveals the difficulty of reproducing the experimental data obtained with the LC by numerical simulations. The standard model is too stiff for large displacements and the model including amnion slippage underestimates the initial response. This might indicate a transition between the two states in the course of a test. Reproduction of the data obtained with the SC works much better, although the numerical simulation slightly overestimates the response at large deformations.
5.5 Discussion

This study was dedicated to the analysis of the method of puncture testing and its application to fetal membrane tissue. Measurements were performed on fetal membrane samples and numerical simulations were utilized for the investigation of particular effects observed during the experiments. Variability of the data and uncertainties in the modeling are discussed and compared with data from the literature.

5.5.1 Experiments on fetal membrane samples

Mechanical behavior of FM samples

Puncture tests were performed on 36 samples from seven membranes. Two different clippings (denoted as long and short) were used to investigate the dependency of the mechanical characteristics on the boundary conditions. The long clamping (LC), enabling a smooth sample fixation, was designed to be similar to the one used in Moore et al. (2006), thus a comparison of corresponding data is feasible. Three publications from this group determined the rupture force of FM in puncture tests. El Khwad et al. (2005) and Pandey et al. (2007) determined the rupture force of fresh FM specimens from cesarean sections and measured forces from $(9.07 \pm 2.61)$ N to $(10.13 \pm 2.96)$ N. Arikat et al. (2006) determined a rupture force of samples from fresh vaginal deliver-

Figure 5.20: Comparison of the averaged experimental data and the results from the numerical simulations for the long clamping (a) and the short clamping (b). Experimental data and numerical results were normalized by a force threshold of 0.025 N.
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ies of (9.71 ± 2.42) N. The maximum forces of (10.9 ± 5.5) N measured in the present study agree well with the aforementioned data. Only the variability of the present data is considerably larger, which might be related to the fact that the zone of altered morphology (El Khwad et al., 2005) was not excluded in this work. On the other hand, remarkable differences can be observed when values of the displacement at rupture are compared. Arikat et al. (2006) and (El Khwad et al., 2005) determined maximum membrane deflections of (4.59 ± 1.54) mm and (6.7 ± 1.0) mm which is significantly smaller than the present value of (10.0 ± 1.6) mm. The large difference between these values is very likely related to a different definition of the reference configuration, which is not defined in the previous studies. However, in a later study from the same group (Pandey et al., 2007) a force threshold value of 0.44 N was specified, which is much larger than the herein used value of 0.025 N. The maximum membrane deflections of the current study reduce to (7.1 ± 1.6) mm when the larger threshold value is applied. Remaining (small) differences might be attributed to differences in the plunger speed, 84 mm/min in the specified references and 25 mm/min in the current study, and to differences in the sample preparation and handling.

Comparison of the data obtained in this study by the use of two different clampings shows pronounced differences depending on the fixation technique. Force-displacement curves obtained by using the SC are stiffer and less extensible. The averaged maximum displacement reduces to (6.9 ± 0.5) mm, while the maximum force of (11.5 ± 4.5) N is comparable to values obtained with the LC. If the larger force threshold of 0.44 N is applied to these data, the corresponding values reduce to (4.7 ± 0.5) mm and (11.0 ± 4.5) N, respectively. Differences to the data obtained with the LC can be related to significant slippage of the membrane sample in the long clamping, as will be discussed later in more detail.

Several other studies in the literature performed puncture test on human FM samples but with different dimensions of plunger and clamping which complicates a comparison. Schober et al. (1994b) and Pressman et al. (2002) provide data of maximum force and displacement for their experimental setup. The corresponding membrane tension can be estimated by the use of Laplace’s law and the conical model to assess the contact angle. Estimation of membrane tension leads to values from 0.53 N/mm to 0.60 N/mm for the data of Schober et al. (1994b) and 0.46 N/mm to 1.33 N/mm for the data of Pressman et al. (2002). Oyen et al. (2004b, 2006) reports values of maximum force of (4.26 ± 1.09) N and (4.10 ± 1.62) N for a setup with 3.2 mm plunger diameter and 20 mm diameter of the clamping (no displacement measurement). The maximum membrane tension of (0.46 ± 0.23) N/mm to (0.59 ± 0.24) N/mm as determined in the current study as well as the values approximated from data reported in the literature are within the range.
of values determined in other experimental configurations. Values of maximum uniaxial membrane tension range from 0.15 N/mm (Jabareen et al., 2009) to 0.98 N/mm (Artal et al., 1979), see section 3.11 for a summary of mechanical parameters of FM reported in the literature. Nevertheless, the present values of maximum membrane tension are much higher than the values determined in section 3.7.1 and 3.7.2, i.e. \((0.19 \pm 0.06)\) N/mm (uniaxial tension) and \((0.26 \pm 0.07)\) N/mm (inflation). Analysis of the second derivative of the force-displacement curves and additional interrupted tests have shown that significant slippage is involved in the tests performed with the LC. The membrane tension evaluated at the time point \(t^*\) (negative second derivative) reduces to \((0.39 \pm 0.24)\) N/mm. No visible slippage was observed in the tests performed with the SC, which is confirmed by positive values of the corresponding second derivatives. For the sake of completeness, evaluation of the membrane tension at the time point when the second derivative is maximum leads to values of \((0.19 \pm 0.14)\) N/mm for the tests performed with the LC and \((0.44 \pm 0.29)\) N/mm for the SC. These values illustrate the early onset of slippage in the LC and indicate that similar but less pronounced mechanisms might also be present in the tests performed with the SC. Minor uncertainties in these data remain, since the detection of maximum or negative values of the second derivative depends on the order of the polynomial function and the quality of the polynomial fit. Visual inspection of the sample after testing showed no signs of damage, except from the obvious tear line. However, microscopic damage in the tissue cannot be excluded and might also be related to the overestimation of membrane’s strength. Some portion (approximately 20%) of these differences can also be related to the estimation of the membrane tension by analytical models. Moreover, detection of failure of the tissue sample depends on the experimental configuration utilized. For example, already a very small lesion in the sample is sufficient to cause pressure loss in inflation tests and to provoke failure. On the other hand, in puncture tests rather a macroscopic failure of the sample is detected.

The experimental raw data from the present study were normalized by a force threshold of 0.025 N, which was mainly determined by consideration of the force signal resolution of 5 mN. Normalization by this threshold leads to an averaged initial membrane tension of \((3.5 \pm 1.8) \cdot 10^{-3}\) N/mm, which is in good agreement to the corresponding value of \((2.1 \pm 0.4) \cdot 10^{-3}\) N/mm used for the normalization of the inflation tests in section 3.7.2.

The data obtained in this study show that the variability of the maximum displacement \((10.0 \pm 1.6)\) mm is much smaller than the variability in the maximum force \((10.9 \pm 5.5)\) N. This indicates that failure of FM tissue is mainly determined by its deformation capacity and not by its strength. The same tendencies can also be observed in the data obtained by inflation testing in section 3.7.2.
Rupture behavior of FM samples

Installation of a video camera underneath the sample allowed the investigation of the rupture sequence. The sequence of events observed during rupture, with chorion rupturing before amnion in most of the cases for tests performed on the LC, agrees with the observations reported in the literature (Arikat et al., 2006). Also the shape of the holes in amnion and chorion after penetration of the plunger, where amnion shows a larger and circular hole and chorion a hole with elliptic shape, is consistent with the findings of Oyen et al. (2004b). However, additional interrupted puncture tests as well as analysis of the second derivative of the force-displacement curves showed that there is significant slippage of amnion inside the long clamping. These observations along with the ability to reproduce the data and findings reported in El Khwad et al. (2005) and Arikat et al. (2006) implies that slippage was present in these studies too. The rupture sequence with chorion rupturing first is in conflict with other data obtained in this work. All data obtained by tensile testing or inflation testing of intact FM samples or separate layers of the FM indicate that amnion is less extensible than chorion, see chapter 3 and 4, and is therefore expected to fail first if both layers adhere and thus experience the same deformation. On the contrary, using a clamping with more confined sample fixation leads to a reversed rupture sequence, with amnion rupturing first in all cases. Only one study in the literature applied puncture testing to FM tissue and found amnion rupturing first. Schober et al. (1994b) also used a sophisticated clamping where the FM sample was glued on the front face of a plastic tube and the overlapping tissue fastened additionally to the tube section. Furthermore, the plunger was wrapped with a polytetrafluoroethylene (PTFE) film to minimize friction. Thus, it seems that the rupture sequence of FM in puncture testing is mainly determined by the quality of the sample fixation.

Importantly, the rupture sequence of chorion rupturing first and amnion penetrating through chorion is to some extent representative of the in vivo situation at delivery. The uterus does not hold either of the membranes rigidly, therefore amnion and chorion can and likely do slide over each other (Bourne, 1962). Moreover, it is known that the cervical tissue undergoes dramatic changes in consistency with the onset of labor which lead to progressive softening and radial opening of the cervix (Badir et al., 2013). Funneling of the cervix might create a situation where the choriodecidua is attached to the uterine wall at the border of the funnel and amnion sliding over it. Deformation of the “free” membrane segment overlaying the funnel, due to fetal movement or amniotic fluid pressure, might lead to rupture of chorion and amnion protruding through chorion, in a similar way as observed in the puncture tests with the LC.
5.5.2 Numerical simulations

Interaction of amnion and chorion

Although the interaction of amnion and chorion is unknown in terms of a constitutive relation, the FE simulations for LC and SC show that the interface between the two layers contributes only marginally to the force-displacement relation. However, this observation is based on numerical results where the deformable membrane is fixed at the outer extremity. Mechanisms of sample slippage were not included in these models, which might not represent the real experimental situation. The predictions from the present numerical simulations contradict the experimental findings of Arikat et al. (2006) who concluded that the interaction of amnion and chorion contributes a significant amount of the work required to rupture the FM. On the other hand, the results from the numerical simulations are related to the in general limited possibilities of relative sliding between amnion and chorion in puncture testing when the outer extremity is constrained. Since the membrane is thin compared to the planar dimension, deflection of the membrane induces only a small difference in the layer specific stretches. Chorion undergoes additional shear deformations if the interaction restricts the relative movement. However, the associated strain energy contributions are small due to chorion’s compliance.

The study of Arikat et al. (2006) observed by puncture testing that the separation of amnion and chorion constitutes a significant amount of the work required to rupture the sample. The authors observed no difference in the strength of intact FM, the sum of amnion and chorion, and the reapproximated (separated and recombined) samples. However, there was a significant difference in the corresponding work to rupture between the sample groups, whereby the reapproximated samples showed a smaller work to rupture as the intact FM samples. The results from the present study show that the interaction of amnion and chorion does only marginally contribute to the force-displacement behavior. This is in line with the fact that the measured maximum puncture force in Arikat et al. (2006) is not influenced by the separation of amnion and chorion. The work to rupture, on the other hand, was probably mainly influenced by the force-displacement progress in the late phase of the test and after the first rupture event, where the reapproximated samples are expected to have a larger tendency of slippage, thus the apparent puncture stiffness being lower and thereby leading to smaller values of work to rupture. However, potentially clarifying force-displacement curves are missing in the publication. Separation of amnion and chorion was also observed in the tensile experiments in section 3.10. These results indicate that the interlaminar strength of the FM has to be smaller than the
bending stiffness of the soft and compliant chorion. Otherwise bending and wrapping of chorion should have been observed in these tests. In a recent report, Strohl et al. (2010) provide values of the adherence of amnion and chorion, measured in a peeling test (see Kumar et al. (2009) for a description of the method). Although the loading situation of the peeling test differs from the one investigated in the present study, the reported values in the range of $5 \cdot 10^{-3}$ N/mm illustrate the weak adherence of amnion and chorion. Moreover, slippage in combination with changes in the interface layer might have also significantly contributed to the findings of El Khwad et al. (2005) about the mechanical changes in the ZAM, see appendix D for more details.

**Effect of friction**

The numerical results show that a moderate compression of the sample in the clamping and a small amount of friction would be sufficient to prevent relative movement in the LC. On the contrary, experimental observations show that there is slippage of the amnion layer in the clamping even for firmly fixed samples. These discrepancies might be related to the present implementation of friction in the LC models, i.e. friction is assumed to act over the whole clamping surface in contact with the membrane. In the experiments with the LC, the contact between membrane and clamping might be limited to the contact point with the sealing ring, thus facilitating sample slippage. The experimental observation of significant slippage in the LC is related to a transition between initial adherence and subsequent gliding. This effect is difficult to implement in FE simulations with the bilayer FM model, for which reason more investigations are required to achieve accurate modeling of the LC. Moreover, experimental sample fixation is related to particular compression of the chorion layer and might cause water outflow of the sample which could serve as a lubricating film and thus reduce friction. Results from the analysis of the effect of friction at the plunger or clamping interface are limited to the descriptive capabilities of the present FE model. Friction between tissue sample and interface is in reality an unknown parameter and might even change depending on the state of hydration and thus change from sample to sample.

**Estimation of contact angle and membrane tension**

Puncture testing does not provide intrinsic material parameters. However, simple analytical models allow estimating the membrane tension in the apex region. Two different models were implemented for the evaluation of their predictive capabilities. The simple
conical model provides a suitable estimation of the contact angle, while the more complex Begley-Mackin model (Begley and Mackin, 2004) only provides accurate values for small plunger displacements and small plunger forces. Similarly, the conical model provides an acceptable estimation of the membrane tension with deviations in the range of 20% with respect to the FE solution. The Begley-Mackin model fails to predict the membrane tensions at large strains due to its limited range of validity. This model was originally formulated for materials with a neo-Hookean behavior. Corresponding predictions of contact angle and tension depend mainly on the Young’s modulus, which is difficult to quantify for a highly nonlinear material like FM tissue. However, it has to be mentioned that the Begley-Mackin model does provide accurate approximations for elastomeric materials like Ecoflex (Ecoflex0030, Smooth-On, Inc, USA), i.e. if modeled as neo-Hookean material. Freytes et al. (2005) used a similar conical model for the analysis of multilaminated extracellular matrix devices. The main differences to the current work are that they considered the surface area of the spherical plunger in contact with the membrane for the calculation of the contact pressure and that they used a modified description of a Mooney-Rivlin material for the corresponding FE simulations. Freytes et al. (2005) also observed a deviation of 50% to 55% between FE stresses and analytical results. The main cause for these large differences can be referred to the use of Laplace’s law for the estimation of the membrane tension. The analytical solution assumes a constant equibiaxial state of stress, which does not reflect the real situation of a finite sized indenter. The stress distribution in the apex region can be affected by the choice of suitable dimensions of plunger and clamping. Schober et al. (1994b) suggest the use of a probe diameter to clamping diameter ratio of 1 : 5 ($R/a = 0.2$). In fact, ratios of circumferential to radial stress at the border of the contact region are in the range of 0.94 for a situation with dimensions of $R/a = 5/25$. Thus, the present dimension of plunger and clamping ($R/a = 5/12.5$) cannot be considered suitable for reliable determination of membrane tension.

**Stress and stretch distribution across thickness**

Analyzing the stress and stretch distribution in the center of the sample is important for a better understanding of the mechanisms leading to the unique rupture sequence of FM in puncture tests. Two numerical models were used for the analysis of the stress and stretch distribution in the central region of the sample. The results show that the outermost layer of amnion is supported by the plunger if friction at the membrane-plunger interface is present. However, the phenomenon of chorion rupturing before amnion can only be explained by numerical simulations if slippage of the amnion layer inside the clamping
Chapter 5. Puncture testing applied to fetal membrane tissue

is allowed similar to experimental observations. The present analyses are restricted to the tied interaction of amnion and chorion due to numerical difficulties involved if free sliding between the layers is included. It can be expected that the supporting effect from the plunger on amnion gets larger if amnion is allowed to slide on chorion. Note that the material point with the largest stretch shifts to the periphery of the plunger for large plunger displacement due to the tied interaction and frictional effects. Finer discretization of the FE mesh or use of higher order elements would provide a smoother resolution of the of the stress and strain values across the thickness. In any case, it seems that the observation of chorion rupturing before amnion is a consequence of the characteristics of sample fixation. Correspondingly, chorion rupturing first has rarely been observed in inflation tests (see section 3.10.1) where the deformation field is comparable to puncture testing, except for the contact with the plunger.

Normalization of puncture test data

Reliable determination of a reference configuration is an essential step for material characterization, as already discussed in section 4.6. The deflection of the membrane in puncture tests starts with almost zero stiffness (even at $D_0 \neq 0$) due to the low initial stiffness of the material and the vanishing bending stiffness of the membrane. Accurate measurement of the initial response requires theoretically an infinite resolution of the force signal. In reality, the force signal has a limited resolution and there is also noise superposed on the signal. For these reasons normalization of the data with respect to a reference configuration is important. So far, a deflected reference configuration in puncture tests has only been mentioned in Pandey et al. (2007) where a force threshold was introduced. The results in section 5.4.5 illustrate the difficulty of normalizing puncture test data on the force-displacement level by the use of a threshold value of force or tension. In consequence, there is a remaining uncertainty in the relation between membrane tension and plunger force, originating from the reference configuration, see Figure 5.19. Adequate normalization is only possible if the corresponding stretch data are known, which implies an experimental assessment of the deformation field. The use of a large force threshold, as 0.44 N in Pandey et al. (2007), reduces the scatter in the corresponding displacement data, but leads to significant loss of data of the initial response. On the other hand, it has to be mentioned that the variability of the reference configuration in the experiments is small compared to the cases investigated in the numerical simulations, i.e. average $D_0$ is $(6.9 \pm 1.8)$ mm. The corresponding uncertainty in the determination of the membrane tension is in the range of 3 %, which is negligible compared to the large scatter involved.
in testing FM tissue. On the other hand, all results from the analysis of the reference configuration depend on the numerical model utilized for the analysis. For the specific procedure adopted in the present study, creation of a deflected reference configuration requires the displacement of the outer boundary of the membrane and the application of a small pressure load. The effect of the pressure load on the surface is approximately equivalent to gravitational loading but has the advantage that it can be applied as a ramp function over the time increments of the calculation, thus being beneficial for convergence. However, the reference configuration in the numerical simulations is not exactly stress free, because the pressure load as well as the boundary displacement cause minor pre-stresses (approximately $10^{-3}$ MPa). ABAQUS would allow to reset stress and strain measures after each step of the calculation but with the disadvantage that also the deformed fiber configurations of the Rubin-Bodner model implementation would be lost. However, the residual stresses in the numerical simulations are small compared to their maximum values and appear locally at the outer boundary of the model. Moreover, calculations with different values of the initial pressure load did not lead to significant different results. For these reasons the present findings are not affected by residual stresses in the numerical models.

**Comparison with experiments**

Measurements obtained by use of the short clamping can be well reproduced, although the model somewhat overestimates the stiffness at large deformations. Even if the implemented transversely isotropic constitutive model fits the uniaxial data very well and is able to represent inflation data without any change in the parameters, reproduction of data from puncture testing is much more difficult. The main difficulties can be attributed to the complex contact situations and the unknown coefficients of friction. Moreover, the type and quality of the sample fixation plays an essential role. The averaged initial response of the measurements performed with the long clamping can be reproduced very well. However, the model prediction starts to deviate dramatically at the onset of slippage in the experiments. The FE model which includes amnion slippage allows explaining the rupture sequence, but is too compliant in terms of the force-displacement response due to an overestimation of amnion’s movement within the clamping. The difference is related to the complex transition between initial adherence in the clamping and later sliding of the amnion layer. These effects are only partially included in the present models and are in general difficult to reproduce in a model.
5.6 Conclusions

Conclusions of the present study mainly concern the question as to whether puncture testing is a suitable method for the characterization of human fetal membrane tissue. Puncture testing does not provide intrinsic material parameters but allows approximate evaluation of the membrane tension (or stress) values from experiments. Inadequate sample fixation enables slippage of amnion in the clamping and leads to a rupture sequence with chorion failing before amnion. However, the use of a more reliable clamping method leads to a rupture sequence (amnion rupture first) that is in agreement with findings from other experimental configurations. Numerical simulations were performed to bring insight into several mechanisms involved in puncture testing. FE results showed that for firmly fixed samples (SC) no contribution from the interaction of amnion and chorion can be observed on the resulting force-displacement curves. Friction in the clamping or at the plunger has a large impact on the force-displacement data, but is an unknown parameter and might even change from sample to sample, this being the main disadvantage in the analysis of test data for determining the deformation behavior.

Analytical models allow the approximate evaluation of critical tension values. The experimental values of maximum membrane tension from puncture tests are about twice the values determined by inflation or tensile testing. These differences are probably caused by slippage and microscopic damage during puncture testing as well as differences in the detection of failure between the different experimental configurations. Also, estimation of membrane tension by analytical models causes deviations in the range of 20%, which can be reduced if a more suitable ratio of plunger to clamping diameter (approximately 1 : 5) is used. The unique observation of chorion rupturing first can be explained by numerical simulations. Chorion ruptures first if a certain amount of friction is present at the plunger interface and amnion has the possibility to slide in the clamping, and this is the observed behavior in the experiments. Moreover, numerical simulations also reveal the difficulty of normalization of force-displacement data by a threshold value of force or tension. In consequence, there is a minor uncertainty involved in the relation between plunger force and membrane tension if deflected reference configurations are considered. However, this uncertainty is small compared to the in general large scatter in the data of soft biological tissues.

Puncture testing induces a deformation field comparable to the one of inflation testing, i.e. the deformation field is equibiaxial in the center and transforms into a pure-shear state at the border. Comparison of both methods reveals two main differences. First, the contact between membrane and plunger, including an unknown coefficient of friction,
leads to uncertainties related to the stress distribution in the central region, which causes deviations in the estimation of the membrane tension. The determination of the membrane tension in the apex region is more accurate for inflation tests when images from the side view profile are available. Friction at the plunger interface also leads to protection of the outermost layer of amnion, which contributes to differences in the rupture sequence.

Second, there is a pronounced difference in the detection of tissue failure between the two methods. Inflation testing is sensitive to even small lesions in the sample, which cause pressure loss and provoke the detection of failure, while in puncture testing rather a macroscopic tissue rupture is observed. Thus, puncture testing overestimates tissue’s strength compared to other test methods. No difference regarding the experimental effort required to perform accurate measurements between the two methods can be observed if a suitable procedure of sample preparation is followed.

The main conclusion of the present study is that puncture testing can be applied to characterize FM tissue if some prerequisites are fulfilled. Puncture testing requires a suitable clamping method and sample preparation as well as an adequate ratio of plunger to clamping diameter. The method in general allows simple and fast testing of tissue samples.
Chapter 6

Toward prophylactic plugging for prevention of iPPROM

6.1 Introduction

Operative fetoscopy on fetus and placenta has become a therapeutic option. Treatment of the twin-to-twin transfusion syndrome or the congenital diaphragmatic hernia are widely accepted fetoscopic interventions (Senat et al., 2004; Jani et al., 2009). However, needle and fetoscopic punctures of fetal membranes for diagnostic or surgical interventions lead to localized defects that carry a significant risk for subsequent iatrogenic preterm prelabor rupture of fetal membranes (iPPROM). The potentially beneficial fetoscopic interventions are limited by the high occurrence of iPPROM which is in the range of 4% to 100% depending on the type of intervention and the size of the instruments (Deprest et al., 2010; Beck et al., 2011). Healing of the FM tissue has been shown to be very limited or even absent (Devlieger et al., 2006). Closure of the fetoscopic entry site was discovered to be caused by relative sliding of amnion and chorion preventing leakage of the amniotic fluid (Gratacós et al., 2006). On the contrary, it has also been observed (Sydorak et al., 2002) that chorioamniotic membrane separation leads to a premature rupture of the membranes in 63% of the cases after surgery. To date, no method for artificial sealing or repair of the FM after invasive intervention has made it into clinical practice, although several methods such as plugging, stimulation of biological repair, or sealing by surgical glues have been proposed and evaluated (Mallik et al., 2007; Ochsenbein-Köible et al., 2007; Bilic et al., 2010).

The synthetic formation of a new hydrogel glue that mimics the ability of marine blue mussels to firmly adhere to a wide variety of materials under wet and saline conditions, herein referred to as “mussel glue”, has recently been described in Lee et al. (2002, 2007).
In the meantime, it has been shown that mussel glue is a non-cytotoxic sealant that adheres to FM tissue (Bilic et al., 2010) and the long term in vivo stability of mussel glue has been demonstrated as well (Brubaker et al., 2010). Recently, mussel glue was successfully applied for in vivo sealing of FM defects in the rabbit model (Kivelio et al., 2013). All these results show great promise for the use of mussel glue for the sealing of FM defects. From a mechanical point of view, the problem is obvious: defects in the tissue lead to local stress concentrations that provoke failure. The linear elastic solution of the stress concentrations around a circular hole in a semi-infinite plate has been known for a long time and is part of many classical textbooks such as Timoshenko and Goodier (1951). Extensions of the problem to solutions of hyperelastic materials under large deformations were made (Rivlin and Thomas, 1951; Verma and Rana, 1978) and show an increase of the stress concentrations for large deformations. Similar studies were performed in the field of composite materials for the investigation of the effect of orthotropy on the stress concentrations. The results in Konish and Whitney (1975); Hwu and Yen (1991) show that very large stress concentrations can occur, depending on the laminate structure, even for small deformations. There is only one study investigating the stress concentrations around circular defects at large deformations of anisotropic hyperelastic materials (Fung-type) with particular consideration of aspects related to biological tissues. David and Humphrey (2004) concluded that a circumferentially stiffer material can reduce the stress concentrations. Recent developments go in the direction of the application of the methods of fracture mechanics to soft elastic materials at large deformations (Krishnan et al., 2008). Also, effects of crack propagation and crack tip opening were recently investigated in fibrous networks (Stachewicz et al., 2011; Koh and Oyen, 2012; Koh et al., 2013). All three studies observed interesting mechanisms of toughening due to a region of higher compliance near the crack tip. There exists plenty of literature about the fracture toughness and crack-growth resistance of bone (Norman et al., 1995; Vashishth, 2004). However, application of the methods of fracture mechanics to soft tissues are in their infancy. Taylor et al. (2012) reported data of fracture toughness of porcine muscle and a detailed analysis of all previous work. Although there is to date less literature available, Taylor et al. (2012) concluded that soft tissues are highly tolerant of defects.

The current work aims for improvements to the repair of defects in FM tissue after minimally invasive interventions. Experiments were performed for the qualification of mussel glue as a possible sealant and mechanical analyses were performed for the investigation of the stress concentrations around defects in FM tissue under consideration of its unique mechanical behavior. This study offers guidelines for future developments to recreate the mechanical integrity of punctured FM by application of a mussel glue patch.
6.2 Qualification of mussel glue as possible sealant

The studies performed for the qualification of mussel glue as possible sealant for FM defects were conducted in collaboration with the Department of Obstetrics from the University Hospital of Zurich, Switzerland and the group of Dr. P.B. Messersmith from the Biomedical Engineering Department of the Northwestern University in Evanston, USA. The results presented in this section are a brief summary of the ones already published in Haller et al. (2011) and Haller et al. (2012) but with a focus on mechanical aspects.

6.2.1 Methods

**Standardized inflation tests on elastomeric membranes**

Very high bonding (VHB 4905 and 4910, 3M AG, Rüschlikon, Switzerland) elastomeric membranes with thicknesses of 0.5 mm and 1.0 mm, were used as substrate membrane for the standardized evaluation of the sealing capability of mussel glue. To obtain membranes of 2.0 mm thickness, two 1.0 mm membranes were bonded together by the use of their inherent bonding properties. The membrane was attached to the cover ring of the clamping and punctured in the center using a biopsy punch device with 3 mm diameter (BP-30F, kai medical, Solingen, Germany). A lubricated mold was placed above the defect and 150 µl of mussel glue were applied to create a cylindrical patch with a thickness of approximately 1.4 mm and diameter of 12.9 mm. The “repaired” membrane was then clamped on the fluid filled cylinder and the inflation test was performed with a constant flow rate of 12 ml/min. The deformed dimensions of the defect and the diameter of the glue patch were evaluated in addition to the measured fluid pressure inside the deformed membrane.

**Tests on fetal membrane samples**

The sealing performance of mussel glue was also tested on fetal membrane samples. To this end, membranes were collected according to section 3.2 and the samples prepared by following the procedure explained in section 3.4.2. Defects were created in the center of the mounted FM samples using a 16-gauge needle (1.6 mm diameter, Somatex, Teltow, Germany) or a 11-French three-side pointed trocar of 3.7 mm diameter (Richard Wolf GmbH, Knittlingen, Germany). The resulting lesions were directly sealed with approximately 125 µl mussel mimetic sealant. The glue was applied in terms of drops out of a
Chapter 6. Toward prophylactic plugging for prevention of iPPROM

static mixture device (blending and mixing applicator SA-3678, Nordson Micromedics, St. Paul, USA) that was attached to two syringes, where each syringe contained one component of the glue. The applied glue was allowed to cure for about 5 min at room temperature before mechanical testing.

Inverse analysis for estimation of mussel glue properties

An inverse finite element (FE) procedure was applied to estimate the mechanical parameters of mussel glue based on the tests of mussel glue on VHB membranes. The commercial FE software package ABAQUS 6.9-1 was used to set up the corresponding axisymmetric model consisting of the punctured membrane and the glue patch. The FE model corresponds in principle to the one described in section 3.4.6 but with a central hole of 2.9 mm diameter and a glue patch on the top surface with 12.9 mm diameter and a thickness of 1.36 mm. Perfect adhesion of the glue onto the membrane surface is assumed in the numerical simulations. Pressure was applied on the whole internal surface of the VHB membrane and the glue patch. Geometric and material nonlinearities were included. The elastomeric membrane had already been mechanically characterized by tensile testing in Wissler (2007) and a suitable representation was achieved by the use of a Yeoh model formulation. The mussel glue is assumed to behave as a hyperelastic neo-Hookean material. First, the Yeoh model parameters of the intact VHB response (1 mm membrane) are obtained by the solution of the inverse problem. A separate characterization of the VHB response was necessary, since the mechanical response of VHB changes with time due to storage of the material and because the parameters published in Wissler (2007) were determined for very large stretches (\( \lambda \) up to 5). Next, the inflation experiment of the punctured and repaired VHB membranes was simulated by FE with iterative modification of the material parameters for the glue model to achieve a good agreement between simulated \( d_{Simu} \) and measured \( d_{Exp} \) time histories of hole diameter. The optimization problem is automatically solved by the use of the function \texttt{fminsearch} in MATLAB 2010a (The MathWorks, Inc., Natick, MA, USA).

6.2.2 Results

Tests on elastomeric membranes

The mechanical response of VHB membranes differs from the one of fetal membranes, as can be seen in Figure 6.2. The response of VHB is characterized by a stiff initial response,
6.2. Qualification of mussel glue as possible sealant

Figure 6.1: Axisymmetric finite element model in reference configuration (a) and in the deformed state (b) as well as corresponding experimental pictures in reference (c) and deformed (d) state. The hole diameter $d_{\text{Simu}}$ is analyzed in the FE simulation and compared to the corresponding measurement $d_{\text{Exp}}$. The material parameter of the mussel glue is iteratively updated to achieve a good agreement between experiment and simulation.
where the pressure increases with the apex displacement. After the pressure has reached a first maximum it decreases again. If the experiments were performed under a pressure controlled regime the membrane would “snap through” from the first pressure peak to an other stable equilibrium at larger deformations. This effect is well known from inflation of circular elastomeric membranes (Adkins and Rivlin, 1952). Since the experiments were performed with a constant flow rate, the “snap through” phenomenon is not critical and becomes only noticeable in a decrease of the pressure after a first local maximum. In consequence, the maximum pressure achieved within the tests of the sealed membranes depends mostly on the thickness of the VHB membrane. The inflation tests were performed with a constant flow rate of 12 ml/min until rupture of the glue patch. Rupture was characterized by a sudden decrease of the pressure and a visible fluid leakage through the defect. Rupture happened typically in the glue patch at the site of the defect. For some samples, the glue patch did not adhere to the membrane surface and detached from the membrane at the beginning of the measurement, for which reason these samples were excluded from the analysis.

The formation of the membrane defects and the glue application were standardized in order to accurately determine the sealing performance of mussel glue. The defects created with the biopsy punch had a diameter of \((2.9 \pm 0.2)\, \text{mm}\). Defects were sealed by applying 125 µl mussel glue in a lubricated mold, resulting in glue patches with dimensions of \((12.9 \pm 0.1)\, \text{mm}\) diameter and \((1.36 \pm 0.05)\, \text{mm}\) thickness, thus illustrating the well defined glue application. By changing the membrane thickness from 0.5 mm to 1.0 mm and 2.0 mm also the burst pressure changes from \((12.6 \pm 1.6)\, \text{mbar}\) to \((22.4 \pm 1.7)\, \text{mbar}\) and \((45.1 \pm 5.5)\, \text{mbar}\), respectively. To assess the deformability of mussel glue, changes of the outer diameter of the glue patch \((P_0, p_c)\) as well as the opening of the defect \((D_0, d_c)\) were analyzed, see Figure 6.3. Comparison of the initial dimensions with corresponding dimensions just before rupture showed that the diameter of the patch increased at most by \((49 \pm 34)\%\) and the hole by \((94 \pm 55)\%\). The values characterizing the deformability did not change significantly for the different membrane thicknesses. Table 6.1 summarizes the data of maximum pressure and deformability for the different membrane thicknesses.

**Tests on fetal membrane samples**

In order to quantify the sealing properties of mussel glue, fetal membrane samples were punched with a small diameter needle \((1.6\, \text{mm})\) or a 11-French three-side pointed trocar \((3.7\, \text{mm})\) as would be used during minimally invasive fetal surgery. The achieved mean inflation pressures of \((42.3 \pm 17.8)\, \text{mbar}\) for small defects and \((48.6 \pm 18.4)\, \text{mbar}\) for the
6.2. Qualification of mussel glue as possible sealant

![Graph showing averaged pressure-displacement data for VHB membranes of different thicknesses.](image)

**Figure 6.2:** Averaged pressure-displacement data obtained by inflation tests of intact VHB membranes of different thicknesses.

![Images of inflation test on sealed 2 mm VHB membrane.](image)

**Figure 6.3:** Top view images of an inflation test on a sealed 2 mm VHB membrane. Images show the reference configuration (a) and the deformed membrane just before rupture at 50.3 mbar (b) as well as the geometric quantities evaluated to assess mussel glue’s deformability. The insert on the top right corner of both images shows the corresponding side view profile and illustrates the remarkable deformability of mussel glue.

**Table 6.1:** Mechanical parameters characterizing the sealing performance of mussel glue on VHB membranes of different thicknesses. The table reports values of the maximum pressure $p_{\text{max}}$ achieved in the inflation tests and quantifies the deformability of the mussel glue. Maximum engineering strains were evaluated by analysis of the change of the outer diameter of the glue patch $\varepsilon_{\text{max}}^{\text{Patch}}$ as well as the opening of the defect $\varepsilon_{\text{max}}^{\text{Defect}}$.

<table>
<thead>
<tr>
<th>VHB membrane thickness</th>
<th>0.5 mm</th>
<th>1.0 mm</th>
<th>2.0 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p_{\text{max}}$ [mbar]</td>
<td>12.6 ± 1.6</td>
<td>22.4 ± 1.7</td>
<td>45.1 ± 5.5</td>
</tr>
<tr>
<td>$\varepsilon_{\text{max}}^{\text{Patch}}$ [%]</td>
<td>40 ± 24</td>
<td>43 ± 25</td>
<td>49 ± 34</td>
</tr>
<tr>
<td>$\varepsilon_{\text{max}}^{\text{Defect}}$ [%]</td>
<td>81 ± 31</td>
<td>87 ± 42</td>
<td>94 ± 55</td>
</tr>
</tbody>
</table>
large defects demonstrate the sealing performance of mussel glue. However, the present values are much lower than the maximum pressures of \((153 \pm 51)\) mbar for intact FM, measured in the inflation tests in section 3.7.2. Mussel glue is able to seal both defects sizes under wet conditions, which in this case means that the glue was applied on a water layer on the membrane surface, originating from membrane puncturing.

In contrast to the tests with the elastomeric membranes, the opening of the defect and the distention of the glue patch could not be evaluated due to the poor color contrast between fetal membrane and mussel glue. The tests were ended by rupture of the glue patch which happened typically at the site of the defect and leakage through a small hole in the glue could be observed.

**Mechanical behavior of mussel glue**

Figure 6.2 illustrates the pressure-displacement response of intact VHB membranes of different thicknesses. The “snap through” phenomenon in combination with the compliance of VHB leads to very large apex displacements and volumes of the deformed membrane even at moderate pressures. Thus the measurements on intact VHB samples were stopped after the first peak of the pressure was achieved to ensure a reasonable size of the inflated membrane. Inverse analysis of the average pressure-displacement response of 1 mm VHB membranes provides the coefficients of the Yeoh model formulation for VHB, i.e. \(C_1 = 1.5 \cdot 10^{-2} \text{MPa}, C_2 = 2.6 \cdot 10^{-5} \text{MPa},\) and \(C_3 = -8.3 \cdot 10^{-5} \text{MPa}\.\) The value of the first elastic constant \(C_1\) is in good agreement to corresponding parameters determined in indentation tests by Farine (2013), i.e. \(C_1 = 1.2 \cdot 10^{-2} \text{MPa}, C_2 = -8.7 \cdot 10^{-8} \text{MPa},\) and \(C_3 = 1.2 \cdot 10^{-7} \text{MPa}\.\) The corresponding uniaxial stress-strain responses differ by about 25% at 20% longitudinal strain.

The results of the optimization problem using sealed VHB membranes show that the mechanical response of the mussel glue can be represented by a neo-Hookean material model with an elastic coefficient \(C_1 = 9.63 \text{kPa}\). The linearized uniaxial response of this model corresponds to a Young's modulus of 57.8 kPa.

**6.3 Stress concentrations around circular defects in FM tissue**

Puncture of the fetal membrane is related to a high risk of preterm membrane rupture (Deprest et al., 2010). In general, defects in a material, such as holes or cracks, cause
local stress concentrations and redistributions. The linear elastic solution of the stress concentrations around a circular hole in a semi-infinite plate has been known for long time (Timoshenko and Goodier, 1951) (original solution was obtained by G. Kirsch in 1898), but less is known about stress concentrations for defects in nonlinear anisotropic materials at large deformations. In this section, stress concentrations around a circular hole in FM tissue are analyzed under consideration of its unique mechanical behavior. First, an analytical solution is obtained for the single layers which is followed by the investigation of the effect of the interaction of amnion and chorion by numerical simulations. Finally, different methods of FM repair are analyzed by numerical simulations and their sealing performance is evaluated.

6.3.1 Methods

Analytical model

The effect of puncturing the fetal membrane for medical interventions is investigated by the analysis of the stress redistributions around a circular hole in an otherwise equibiaxially stretched membrane. A circular membrane of outer radius $R_o$ and central hole of radius $R_i$ subjected to a uniform radial stress at the outer boundary is considered in this section, see Figure 6.4. The derivation of the corresponding equations follows the procedure published in David and Humphrey (2004). A similar problem was also already analyzed by Rivlin and Thomas (1951).

Using cylindrical coordinates, a material point initially located at $(R, \Phi)$ is mapped to $(r, \varphi)$. The angular position of the material point remains constant ($\Phi = \varphi$) due to the
rotational symmetry of the problem. Thus, a motion of \( r = r(R) \) leads to a deformation gradient in the form of
\[
\mathbf{F} = \begin{bmatrix} \lambda_R & 0 \\ 0 & \lambda_\phi \end{bmatrix} = \begin{bmatrix} r' & 0 \\ 0 & \frac{r}{R} \end{bmatrix}
\]
(6.1)
where \( r' \) denotes derivation with respect to \( R \). Note that only the in-plane components are considered due to the plane stress situation. The only non vanishing equilibrium equation is
\[
\frac{\partial \sigma_{rr}}{\partial r} + \frac{1}{r} (\sigma_{rr} - \sigma_{\phi\phi}) = 0.
\]
(6.2)
Helpful relations can be obtained by application of the chain rule of differentiation
\[
\frac{d\sigma_{rr}}{dR} = \frac{\partial \sigma_{rr}}{\partial r} \frac{dr}{dR} + \frac{\partial \sigma_{rr}}{\partial \phi} \frac{d\phi}{dR} = \lambda_R \frac{\partial \sigma_{rr}}{\partial r}
\]
(6.3)
and consideration of the symmetry
\[
\frac{d\sigma_{rr}}{dR} = \frac{\partial \sigma_{rr}}{\partial \lambda_R} \frac{d\lambda_R}{dR} + \frac{\partial \sigma_{rr}}{\partial \lambda_\phi} \frac{d\lambda_\phi}{dR}.
\]
(6.4)
By combination of the previous equations, the radial equilibrium can be expressed as a second order ordinary differential equation for the function \( r(R) \):
\[
r'' = \left( \frac{\sigma_{\phi\phi} - \sigma_{rr}}{r} + \frac{\partial \sigma_{rr}}{\partial \lambda_R} \right) \frac{\lambda_R}{R}.
\]
(6.5)
The solution of the differential equation must fulfill two boundary conditions. First, either a static or a kinematic constraint can be applied by specifying a radial traction \( \sigma_0 \) or a radial stretch \( \lambda_0 \) at the outer boundary.
\[
\sigma_{rr}(R_o) = \sigma_0 \quad \lambda_R(R_o) = \lambda_0
\]
(6.6)
Second, the radial component of the stress must vanish at the hole site
\[
\sigma_{rr}(R_i) = 0.
\]
(6.7)
The mechanical response of the fetal membrane tissue is represented by the transversely isotropic model formulation derived in section 4.3. Thus, the radial and circumferential stress components can be obtained by derivation of the strain energy potential with respect to the corresponding deformation tensor.
The boundary value problem (BVP) of equation 6.5 can be transformed into two first order ordinary differential equations \( y_1 = r, y_2 = r' \) and solved in MATLAB 2010a by
6.3. Stress concentrations around circular defects in FM tissue

Figure 6.5: Illustration of the axisymmetric finite element model utilized for the analysis of the stress concentrations around circular holes in fetal membrane tissue. The transversely isotropic constitutive model derived in section 4.3.1 is used to describe the mechanical behavior of the FM.

The application of the intrinsic function \texttt{bvp4c}, which applies a finite difference code for the solution of the BVP by iterative solution of the corresponding initial value problem.

Numerical simulations of punctured membranes

The analytical solution is limited to the single layer behavior. Numerical simulations are performed for the investigation of the behavior of the intact FM and possible effects of local deformations in thickness direction. The finite element software ABAQUS 6.9-1 is used for the analysis of the stress concentrations and redistributions around a hole in the fetal membrane tissue. The situation is represented by a circular membrane sample of 10 mm outer radius with a central hole of 1 mm radius, subjected to a uniform radial stretch at the outer boundary, similar to the case described in the previous section. The membrane is assumed to be homogeneous in thickness and in its mechanical properties, therefore an axisymmetric model, as illustrated in Figure 6.5, is set up. The load is applied by a radial displacement of the outer boundary. Geometric and material nonlinearities are considered due to the large deformations. The transversely isotropic constitutive model from section 4.3.1 is used to represent the mechanical behavior of the fetal membrane. The corresponding model parameters of amnion and chorion are chosen according to Table 4.3. Second order axisymmetric elements CAX8R are used to achieve a fine resolution of the stress distributions. The fetal membrane is modeled as realistic bilayer structure composed of amnion and chorion, with corresponding layer thicknesses of 100 \( \mu \text{m} \) for amnion and 400 \( \mu \text{m} \) for chorion. Due to the lack of knowledge of the interaction of amnion and chorion, two types of contact behavior are investigated, i.e. free relative sliding and tied contact. The stress concentrations are evaluated as the Cauchy stress components in the radial and circumferential directions at the hole site with respect to those in the far field region.
Chapter 6. Toward prophylactic plugging for prevention of iPPROM

Numerical simulations of repaired membranes

The effectiveness of fetal membrane repair by application of a glue patch is investigated by numerical simulations. Four different configurations are simulated and evaluated: a rigid glue patch on either the amnion or chorion side as well as a compliant mussel glue patch on one of the surfaces of the fetal membrane. The FE model is similar to the one of the punctured FM (Figure 6.5), but extended by a glue patch and modified in its geometry for similarity to the mussel glue tests in section 6.2.2. The FE model represents the situation of a membrane sample of diameter 30 mm with a central hole of 2 mm diameter. The defect is repaired by a glue patch of 9 mm diameter and a thickness of 1 mm, see Figure 6.10 for the example of a glue patch on the amnion side (in the deformed state). The mechanical behavior of mussel glue is represented by a neo-Hookean material model with an elastic constant of $C_1 = 9.63$ kPa. Perfect adherence (no sliding) of amnion and chorion as well as between the glue patch and the membrane surface is assumed in the simulations. For the models representing a rigid patch, the glue patch in Figure 6.10 is replaced by a displacement constraint of the corresponding nodes on the membrane surface.

6.3.2 Results

Stress concentrations in amnion and chorion

Analytical solutions of the boundary value problem were obtained for a prescribed radial stretch at the outer boundary of the circular membrane ($R_o = 10$ mm, $R_i = 1$ mm). Figure 6.6 shows the progress of the corresponding stretch and stress components as a function of the radial position. The circumferential stretch reaches its peak at the site of the hole and declines toward the outer site, whereas the radial component of the stretch is smaller than one at the inner side and increases toward the outer site. The radial stretch matches the prescribed deformation at the outer boundary. The stress components show a similar progress over the radial distance. The circumferential stress is maximal at the inner side and declines toward the outer boundary, while the radial stress vanishes at the inner side and converges toward a constant value at the outer boundary. Both stress and stretch components converge to a constant value in the far field, which has to be fulfilled due to the equibiaxial loading.

Figure 6.6 illustrates that the stress concentration is a very localized effect, i.e. the circumferential stress declines by 75% within approximately 0.5 mm distance (5.5% of length). The intensity of the stress concentration can be assessed by evaluation of the
6.3. Stress concentrations around circular defects in FM tissue

stress concentration factor (SCF) which relates the maximum circumferential stress at the hole site to the circumferential stress in the far field \( \text{SCF} = \sigma_{\phi\phi}(R_i) / \sigma_{\phi\phi}(R_o) \). Figure 6.7 shows the progress of the stress concentration factor of amnion and chorion as a function of the prescribed radial stretch \( \lambda_0 \). In addition, different ratios of \( R_i/R_o \) were evaluated to investigate the dependency on the hole size. It can be seen in Figure 6.7 that both tissue layers (amnion and chorion) show a rapid increase of the stress concentration factor. In fact, values of SCF at 10\% radial strain are 3.4 for amnion and 3.0 for chorion, for the configuration of \( R_i/R_o = 0.1 \). Moreover, values of SCF for amnion tend toward 6.8 for 20\% radial strain. The data converge to a value of 2 for small deformations, which is in agreement with the well known solution of linear elasticity. However, the boundary value problem is difficult to solve for very small deformations due to numerical difficulties with the MATLAB code. The ratios \( R_i/R_o \) of 0.02 and 0.1 show approximately the same progress of the SCF, only the largest hole with 3 mm radius leads to a decrease of the SCF.

Different constitutive models were implemented to investigate if the unusual large values of SCF are related to large deformations, the exponential stiffening behavior, or the large lateral contraction of the material formulations. Figure 6.8 shows the SCF for three different constitutive models, i.e. a neo-Hookean model, an isotropic model with exponential stiffening, and the transversely isotropic model for the FM. The response of the isotropic model with exponential stiffening was obtained by using the amnion model, but setting the fiber parameter \( m_3 \) to a small value. It can be seen in Figure 6.8 that the fast increase of the SCF can be mainly related to the large lateral contractions of the fetal membrane tissue. On the other hand, large lateral contractions serve for a faster decay of the circumferential stresses as compared to other material formulations.

Stress concentrations in bilayer FM

The stress redistributions of amnion, chorion as well as intact FM in numerical simulations were also analyzed by means of the stress concentration factor (SCF). Resulting values of the stress concentration factor as a function of the applied radial stretch are illustrated in Figure 6.9(a) for all simulated cases. Single amnion and chorion layers show values of SCF around 2.7 at 10\% radial stretch. This value is much smaller than the corresponding values of 3.4 for amnion and 3.0 for chorion obtained by the analytical solution. The numerical results in Figure 6.9(a) reveal also that there is no pronounced difference in the stress concentrations between the intact FM and the separate layers. In fact, values of SCF at 10\% deformation are still 2.7 for amnion and 2.8 for chorion if amnion and
Figure 6.6: Solution of the boundary value problem for the amnion layer for 10% radial stretch. Graphics show the radial and circumferential components of the stretch (a) and stress (b) as a function of the radial position. Results are shown for the configuration of $R_i/R_o = 0.1$.

Figure 6.7: Stress concentration factors (SCF) for the amnion (a) and chorion (b) layer as a function of the applied radial stretch. The solutions were obtained for different ratios of hole radius $R_i$ and outer radius of the membrane $R_o$. 
6.3. Stress concentrations around circular defects in FM tissue

Figure 6.8: Stress concentrations factors (SCF) as a function of the applied radial stretch (a) for different constitutive models, i.e. transversely isotropic model for FM response, isotropic model with exponential stiffening, and neo-Hookean formulation. Figure (b) shows a normalized plot \( \frac{\sigma_{\phi\phi}(R)}{\sigma_{\phi\phi}(R_i)} \) of the decay of the circumferential stress depending on the radial position. Both figures refer to the configuration of \( R_i/R_o = 0.1 \).

chorion are firmly adhered. Differences between the analytical and numerical solutions might be attributed to the discretization of the FE mesh. The analytical solution shows that the circumferential stress at the hole decays around 50% within 0.2 mm distance, which corresponds to the length of nine finite elements in the present models. Despite the use of second order elements and a fine mesh discretization, the FE solution does not capture the pronounced stress decay within the very first element at the hole site, see Figure 6.6(b) for illustration. Moreover, the vanishing radial stress at the hole site creates a uniaxial state of stress which is related to large lateral contractions and causes a local thickening of the sample, see Figure 6.9(b). Results from numerical simulations of single layers show a local increase of the thickness of 71% for amnion and 21% for chorion, which is related to bulging of the membrane at the hole site. Such mechanisms are not included in the analytical models.

Stress concentrations in repaired membranes

Numerical simulations were performed on four configurations of repaired membranes, i.e. a rigid patch on amnion or chorion as well as a mussel glue patch on amnion or chorion. Figure 6.10 shows the deformed FE mesh for the situation with a mussel glue patch on the amnion side. Performance of the sealing methods is evaluated by the analysis of
Figure 6.9: Figure (a) shows the stress concentrations factors (SCF) obtained in FE simulations ($R_i/R_o = 0.1$) as a function of the applied radial stretch. Simulations were performed to investigate the single layer behavior “free” and the behavior of the intact FM by “tied” interaction of amnion and chorion. Figure (b) shows a close up view of the defect region of the deformed FE mesh at 10% radial deformation and the color bar illustrates the logarithmic strain component in the circumferential direction.

The ratio of circumferential to radial stretch ($\lambda_\phi/\lambda_R$) at the site of the defect and at the outer boundary of the glue patch. This ratio gives information about the local state of deformation and its deviation to the equibiaxial state in the far field. The analysis is limited to the amnion layer, since it is the mechanically dominant layer of the FM. The corresponding nodal values were determined in the middle of the amnion layer. The stretch ratios are in the rage of 0.99 - 1.69 at the hole site and between 0.03 and 1.01 at the outer side of the patch. Table 6.2 summarizes the stretch ratios at the hole and patch site for each configuration simulated. For comparison, the corresponding stretch ratio at the hole site of the punctured membrane is 1.92, see previous section. Values of the stretch ratio in amnion at the patch side around 1, for the cases with a glue patch on the chorion side, indicate that the stress concentrations decayed into an equibiaxial state of stress. Application of a rigid glue patch causes remarkable stress concentrations at the patch site in the particular layer, i.e. ratios of the radial stress at the patch site compared to the far field show values up to 18 for chorion and 7 for amnion.
6.4. Discussion

Different studies were performed for the qualification of mussel glue as possible sealant for FM defects and for the investigation of the effects of local defects in FM tissue. Difficulties and uncertainties in the experiments as well as assumptions made for the numerical analysis require the discussion of the results.

6.4.1 Mussel glue sealing

One strategy to reduce the risk of iPPROM is the application of a mussel glue patch for temporary closure of the defect site. Standardized conditions for defect formation and glue application were used for the evaluation of the mechanical stability of mussel glue. The use of elastomeric membranes enabled the determination of glue properties independent from the highly variable properties of FM. In fact, a relation between sealing performance and substrate material remains. The data show that the achieved burst pressures of $(12.6 \pm 1.6)\text{ mbar}$, $(22.4 \pm 1.7)\text{ mbar}$, and $(45.1 \pm 5.5)\text{ mbar}$ depend significantly
on the thickness of the VHB membrane. However, analysis of the dilatation of the glue patch showed almost identical average values for all membrane thicknesses tested. On average, the mussel glue could be stretched 87% in the defect and 44% in the patch area, thus illustrating a much larger extensibility than expected for FM tissue. For comparison, strains at rupture of FM tissue obtained by inflation tests are in the range of (20 ± 3)%. These results indicate that the deformation behavior of the sealed membrane is dictated by the base membrane, whereas failure occurs when a critical state of deformation of the glue is reached. In consequence, mussel glue seals effectively 3 mm defects in elastomeric membranes, but does not lead to significant local reinforcement that restricts membrane deformation.

In addition, mussel glue sealing properties were also determined on FM samples with small (1.6 mm) and large (3.7 mm) defects. Mussel glue effectively sealed both defect sizes even under wet conditions. Achieved burst pressures of (42.3 ± 17.8) mbar for small defects and (48.6 ± 18.4) mbar for the large defects are comparable to pressures measured during normal contractions (Maul et al., 2004), and thus might be sufficient to prevent preterm rupture. On the other hand, maximum pressures of the repaired FM samples are much lower than the corresponding values of (153 ± 51) mbar of the intact FM samples measured in section 3.7.2. Failure always happened through a defect in the glue patch, thus illustrating that the glue patch is still the weakest spot. Moreover, mussel glue was applied on the chorion in these experiments, which is known to play only a secondary role in terms of the mechanical response of FM. Despite the promising sealing performance of mussel glue in the inflation tests, in vivo glue application on chorion might be a challenging task, since the membrane had to be detached from the uterine wall prior to glue applications. Tests on FM samples were performed on membranes derived from the third trimester of pregnancy. Membranes from the second trimester, when fetoscopic interventions are performed, might have different mechanical properties. Thus, further data should be collected on second trimester membranes to confirm the present findings. Moreover, it has to be considered that there is subsequent membrane growth and stretching, when the glue is applied in the second trimester, which might also influence the sealing performance.

The result from the inverse optimization shows that the mechanical response of mussel glue can be represented by a neo-Hookean model formulation with an elastic constant of $C_1 = 9.63 \text{kPa}$. The corresponding linearized uniaxial initial stiffness of this model $K_1 = 0.029 \text{N/mm}$ is in good agreement to data of the initial stiffness of FM in the range of $(0.028 \pm 0.017) \text{N/mm}$, which points to the promising mechanical biocompatibility of mussel glue. This demonstrates the ability of mussel glue to match the deformation be-
behavior of FM and to avoid stress concentrations at the interface between membrane and glue at least for small deformations. On the other hand, the mechanical behavior at large deformations differs evidently between a neo-Hookean material and FM tissue. For an accurate mechanical description of mussel glue, more data should be obtained either by further inverse analysis of inflation tests or by conventional mechanical tests of homogeneous deformation. A mismatch between glue and fetal membrane properties will lead to additional shear stresses in the interface which can lead to delamination of the glue patch. Since the mechanical behavior of mussel glue, in terms of the tension-stretch as well as kinematic response, will never match that of FM tissue, shear stresses in the interface can not be avoided. For that reason, more research has to be conducted toward a modification of the glue properties and the optimization of the glue patch geometry under consideration of the shear stresses at the interface with respect to the adhesion strength of the glue.

6.4.2 Stress concentrations around circular defects in FM

Understanding the stress concentrations and redistributions around holes in the FM tissue is a prerequisite for later evaluation of sealing strategies. The solution of the boundary value problem reveals that both tissue layers, amnion and chorion, show a rapid increase of the stress concentration factor already at moderate strains. In fact, values of SCF at 10% radial strain are 3.4 for amnion and 3.0 for chorion. Moreover, SCF of amnion tend toward a value of 6.8 for 20% strain. Even though the numerical solution converges to a value of 2 for small deformations, which is in agreement with the well known solution for linear elasticity, the rapid increase for larger strains is surprising. Other studies on isotropic hyperelastic materials determined similar values of SCF but for much larger stretches. Oden (1972) analyzed a similar situation based on a Mooney-Rivlin material formulation and determined a value of 7 for a radial stretch of 2.5 (i.e. 150% strain). Similar results can be found in Rivlin and Thomas (1951) and Verma and Rana (1978) for the corresponding strain distributions. Studies on composite materials (Konish and Whitney, 1975; Hwu and Yen, 1991) found a particular influence of the anisotropy (composition of the laminate) on the stress concentrations around circular holes. They found values of SCF up to 7 for small deformations and uniaxial loading. Corresponding simulations of uniaxial loading of a punctured amnion sample (not reported here) show values of SCF up to 7.4 for approximately 5% strain. There is, to the best of the author’s knowledge, only one study that analyzes the stress concentrations for hyperelastic anisotropic materials at large deformations: David and Humphrey (2004) analyzed a similar situation as in this
study, but incorporating a Fung-type material and determined values of SCF around 3.5 for 25% strain and for an isotropic case. Moreover, David and Humphrey (2004) conclude that anisotropy plays a major role, since a radially stiffer behavior can increase the stress concentrations, whereas a circumferentially stiffer behavior can minimize it. According to the results in David and Humphrey (2004) the role of the anisotropy is greater for smaller holes. In the present study only the simulation with $R_i/R_o = 0.3$ shows an decrease of the SCF, which is caused by the fact that the remaining radial distance is too small for convergence of the stress components to the far field level.

The findings of the present study show that the very large SCF even at moderate strains are mainly caused by the large lateral contractions of the transversely isotropic model formulation. On the other hand, the large contractions related to the uniaxial state of stress at the hole site lead to very localized stress concentrations by a rapid decay of the stresses to the far field level. In fact, the stress decay of isotropic materials requires a larger spatial extent. Comparison of the stress concentration factors obtained by the analytical solution and the numerical simulations shows a deviation toward lower SCF in the FE simulations. Although the stress components in the far field show only a difference of 5% between the two solution methods, the FE solution seems not to be able to reproduce the very fast stress decay at the hole site despite the use of second order elements and a fine mesh discretization. Mechanisms of local thickening at the hole site in the FE simulations might also contribute to this deviation. The uniaxial stress state at the hole site is related to large lateral contractions which cause local thickening and bulging of the membrane at the hole site. These effects are related to stresses in the thickness direction (one order of magnitude lower than the stress magnitude in the far field), which are not considered in the plane stress analytical solution. A comparison between the single layer behavior and intact FM shows that there is almost no contribution from the interaction of amnion and chorion on the stress concentrations.

The analyses performed in this study focused on the determination of the stress concentrations around circular holes. On the other hand, the experimental results in chapters 3 and 4 have shown that FM failure is rather determined by strain limits. Despite the large stress concentrations, the uniaxial stress state at the hole site is related to a larger strain limit, due to the enabled lateral contractions. In fact, critical strains of intact FM are in the range of $(20 \pm 3)\%$ for equibiaxial loading and $(32 \pm 9)\%$ for uniaxial loading. Therefore, the FM tissue will be rather tolerant against circular defects. Similar observations were made in preliminary uniaxial tests of notched FM samples. Figure 6.11 illustrates a double notched FM sample (initial width 20 mm, initial crack length 4 mm) during a tension test. As can be seen, there is almost no crack growth and the pronounced
contraction of the sample leads to blunting of the initially sharp crack tips. This is in line with the findings of Stachewicz et al. (2011) and Koh and Oyen (2012) who have shown that fibrous networks tend to crack blunting instead of propagation due to a region of larger compliance near the crack-tip.

Figure 6.11: Double notched FM sample under uniaxial tension at the beginning (a) and just before rupture at around 1 N (b). Pronounced lateral contraction of the samples leads to crack blunting instead of propagation. Most of the samples tested did not even rupture at the crack site but rather at the clamping site.

6.4.3 Numerical simulations of repaired membranes

Four different simulations were performed for the preliminary evaluation of FM repair by application of a glue patch. The results show that out of the four cases the situation of a mussel glue patch on the amnion layer performs best if the evaluation is performed with focus on the local state of deformation of amnion. A stiff glue patch on amnion would also be helpful to reduce the stress concentrations at the defect site but creates additional stress concentrations at the outer side of the patch. Such a mismatch between glue properties and FM tissue can lead to delamination of the patch as seen in tests of fibrin glue on FM by Haller et al. (2012). Application of any glue patch on chorion seems not to be helpful for protection of the amnion layer even if tied interaction of amnion and chorion is assumed. These preliminary results depend strongly on the constitutive behavior of mussel glue, which is not well known yet, and the geometry of the glue patch. Moreover, the current study is based on the evaluation of circumferential and radial stretches at the defect site and at the outer boundary of the glue patch. An accurate analysis should also evaluate the shear stress distribution at the interface between glue and membrane and analyze maximum values of shear stress with respect to the adhesion of the glue. An
illustration of the optimization potential of the glue patch geometry by evaluation of the stress distribution in the bonding layer can be found on the example of the structural optimization of an onsert (Kress et al., 2004), a joining element used to achieve load introduction into lightweight structures.

6.4.4 Considerations for medical applications

Repair of the fetal membrane after invasive interventions has two particular aims. First, sealing of the defect to avoid amniotic fluid leakage, and second recreation of the mechanical integrity of the FM tissue. Leakage of amniotic fluid has not been considered a prominent problem. Gratacós et al. (2006) have observed that sealing is achieved by relative motion of amnion and chorion as well as reattachment of the FM to the decidua. However, preservation of the mechanical integrity of the FM is a more challenging task. Defects in the FM cause localized stress concentrations and redistributions as seen in the previous sections. Successful repair of the defect is characterized by recreation of the same local state of deformation at the defect site as in the far field. This requires an optimal match of the mechanical properties of the glue and the FM tissue as well as an optimized geometry of the glue patch for a smooth transition of the stresses from the membrane into the glue patch. Even though the mechanical properties of mussel glue can be adjusted by its chemical compositions and polymerization, it will not be possible to reproduce the unique mechanical behavior of amnion. It is known that amnion is stiffer and stronger than chorion and this leads to higher stress concentrations in amnion. For that reason, application of a glue patch is only meaningful if applied on the amnion side. A trade-off has to be made between compliance and toughness of the glue patch. On the one hand, the applied glue patch should be compliant enough to follow the general deformations of the membrane but on the other hand it should be tough enough to tolerate contact forces due to fetal movement. Also, the reliable application of the glue patch on the inner side of the FM is a challenging engineering problem that has to be solved in the future. The geometry of the glue patch as well as its adherence to the FM tissue is essential for the efficiency of the sealing and repair. However, these two features are difficult to control since the procedure of glue application has to be performed through the fetoscopic access site and the glue has to be applied in the wet environment of the amniotic fluid.
6.5 Conclusions

This study contributes toward the development of methods for repair of the fetal membrane after invasive interventions in the uterine cavity and to a reduction of the risk of iPPROM. Inflation experiments were performed on punctured elastomeric and fetal membranes that were sealed by application of a mussel glue patch. Stress concentrations around circular defects in FM tissue were analyzed by analytical and numerical methods. The results of standardized tests on elastomeric membranes show that mussel glue can be distended up to 87% above the defect area, which is much larger than critical strains of FM tissue. Mussel glue sealings on small and large defects in FM resisted pressures in the range of 45 mbar, comparable to pressures measured in normal contractions, but approximately 70% lower than maximum pressures of intact membranes. These findings confirm mussel glue’s suitability for membrane sealing.

For the first time, a mechanical model determined stress concentrations around circular holes in FM tissue. Corresponding results show large stress concentration factors even for moderate strains in amnion and chorion. Large lateral contraction at the hole site causes strong stress concentrations but leads to a very localized effect. Results from numerical simulations on repaired membranes show that the stress concentrations can effectively be reduced by the application of a mussel glue patch.

More research has to be done for the mechanical characterization of mussel glue as well as for a better understanding of the stress concentrations and their relation to tissue failure. The present results have to be considered as preliminary, but they suggest that repair methods might be developed to effectively reduce the risk of iPPROM.
Conclusions and outlook

A better understanding of the mechanical behavior of human fetal membrane tissue is essential for developments to prevent or treat (iatrogenic) PPROM. This thesis aimed to contribute to present research on FM tissue. The focus was on a comprehensive and detailed mechanical characterization, including measurements in various experimental configurations and the formulation of models for the analysis of the subfailure deformation behavior as well as the investigation of the failure behavior. Numerous measurements on FM samples were performed and analyzed with respect to well defined reference configurations. Thus, a large database of parameters characterizing the nonlinear mechanical response in different configurations and conditions could be obtained. Insight into the unique mechanical behavior of FM tissue was gained and many contributions could be made for the extension of the present knowledge.

7.1 Contributions of the present work

Mechanical characterization and constitutive model development  A comprehensive mechanical characterization was performed for intact FM tissue as well as separate amnion and chorion layers under uniaxial and equibiaxial loading. The experimental findings proved the established knowledge that amnion is stiffer and stronger than chorion and has to be considered as the mechanically dominant layer of the FM. Observations in these tests revealed large differences between the uniaxial and equibiaxial mechanical behavior of the FM, i.e. the biaxial response is characterized by a larger initial stiffness and a smaller deformability. Scalar parameters characterizing the stiffness for small and large deformations, the critical membrane tension, and the maximum stretch were determined to describe the nonlinear mechanical response. Characterization of the microstructural composition was done by biochemical assays for the determination of the
collagen and elastin content, and for one study the concentration of collagen cross-links was also measured. Corresponding membrane thicknesses were measured by histological sections. The results showed that amnion contains about twice the amount of collagen contained in chorion even though it is about four times thinner than chorion. Although the biochemical assays lead to repeatable values of collagen and elastin within different studies, skepticism about the real amount of elastin in the FM tissue remains. Analysis of the correlations between mechanical and histological parameters allowed for the first time detection of the relationship between microstructural composition and mechanical behavior under physiologic relevant loading conditions: there is a positive correlation between the high stretch stiffness as well as the critical membrane tension and the collagen content as well as the concentration of collagen cross-links.

Tension tests on separate amnion and chorion layers revealed the unique and highly reproducible in-plane contraction behavior of amnion. Maximum values of incremental Poisson’s ratio are in the range of 5 to 8, and such high values have not been measured before either for biological tissues or for synthetic materials. The reason for this behavior can be found in amnion’s microstructure, which is characterized by a dense network of randomly oriented collagen fibers. Mechanisms of fiber reorientation, stretching, and buckling lead to this unique contraction behavior, which is the only highly repeatable mechanical property of FM tissue observed within this work and might therefore be essential for in vivo functioning. These results allowed the formulation of a transversely isotropic hyperelastic constitutive model, where partial microstructural information is included by embedding of nonlinear reinforcement fibers in an isotropic matrix. Representation of the averaged uniaxial response as well as predictions of the mechanical response in other loading configurations were found to be very good, which makes it (probably) the first constitutive model for FM able to successfully describe the mechanical behavior in a wide range of loading situations. Implementation of this model in a commercial finite element code made it available for more complex numerical simulations.

**Failure behavior of fetal membrane tissue** Analysis of the failure behavior of FM tissue was performed in different studies and focused mainly on the determination of the rupture sequence. Mechanical characterization of the separate amnion and chorion layers under uniaxial and equibiaxial loading showed that amnion is in general less extensible than chorion. Therefore, it is expected that amnion ruptures first in tests performed with intact FM samples. Indeed, rupture of amnion first was observed in most of the inflation and uniaxial tension tests performed in this work. Moreover, uniaxial tension tests on intact FM samples revealed separation of the two constitutive layers during testing, caused
by a difference in the layer specific Poisson’s effect. Separation is expected to happen in the intermediate layer and was also observed to be present in puncture tests. Results from puncture tests documented in the literature lead to contradicting results in terms of the failure behavior of FM, which motivated the development of an in-house puncture setup. Tests performed with this setup and the use of two different clampings demonstrated that the rupture sequence of FM (amnion or chorion to rupture first) is a characteristic of the clamping method. Slippage of the sample inside the clamping as well as the possibility of relative movement between amnion and chorion has large influence on the failure behavior in this experimental configuration. In fact, tied sample fixation leads to amnion rupturing first while smooth sample fixation facilitates chorion rupturing first.

In general, the definition of failure and the sensitivity of its detection depend on the experimental configuration utilized. Inflation testing is sensitive to even small lesions in the sample, while puncture testing rather detects macroscopic failure of the tissue sample. However, there might be a discrepancy between the consistency of the failure behavior in well defined mechanical tests and its relevance for in vivo membrane rupture. During pregnancy the FM is supported by the uterus on the outer side, with chorion being firmly attached to the maternal decidua and amnion likely sliding over it, which illustrates the differences in the boundary conditions compared to the mechanical tests. Although a small lesion in the sample provokes failure in inflation tests, it might be not that critical in the in vivo situation as long as there is no dramatic loss of amniotic fluid. Moreover, changes in the consistency of the cervical tissue toward the end of pregnancy lead to funneling of the internal part of the cervix, which creates a situation of a free membrane region overlying the funnel, and chorion being attached to the decidua at the border of the funnel. Chorion rupturing first in combination with amnion gliding on chorion and protruding through the defect in chorion seems to be a realistic scenario in such a situation. However, the same rupture sequence observed in puncture testing was attributed to inadequate sample fixation and the corresponding clamping method considered not being beneficial for mechanical characterization.

Analysis of punctured membranes and development of repair methods  A new synthetic hydrogel glue, mimicking the ability of mussels to adhere to a wide variety of materials even under wet and saline conditions (herein referred to as “mussel glue”), was qualified in ex vivo tests as sealant for the repair of defects in FM tissue. Standardized tests on elastomeric membranes as well as tests on fetal membrane samples demonstrated mussel glue’s suitability for FM repair: mussel glue is highly distensible and seals physiologically relevant defect sizes in FM tissue even under wet conditions. Inverse optimization
led to the formulation of a constitutive model for the mechanical response of mussel glue. Analysis of the test data showed that the initial stiffness of mussel glue is comparable to FM tissue, which points to the promising mechanical biocompatibility of mussel glue. Analysis of the effect of membrane puncturing was performed for the first time by evaluation of the stress concentrations around circular defects in FM tissue. Analytical and numerical results of these investigations show a rapid increase in the stress concentration factors even for moderate strains. High values of stress concentration are caused by the large lateral contraction related to the uniaxial stress state at the defect site. On the other hand, the large stress concentrations are a very localized effect due to an increased rate of decay of the stress concentrations to the far field level compared to other material formulations. Moreover, failure of FM tissue is rather determined by its deformation capacity and not by its strength. The uniaxial stress state at the hole site is related to larger failure strains, as compared to the equibiaxial stress state in the far field, which serves as an additional toughening mechanism. In general, large lateral contraction in a state of uniaxial stress, such as at the front of a crack tip, is related to pronounced mechanisms of fiber reorientation and stress redistribution. This leads to local stiffening, thus providing higher toughness and making the FM a highly defect tolerant membrane. Methods to repair the fetoscopic entry site after minimally invasive surgery have the aim to recreate the mechanical integrity of the FM. Preliminary results show that a mussel glue patch on the amnion side of the membrane is helpful to effectively reduce the stress concentrations. However, more research is required for optimization of the shape and dimensions of the glue patch as well as for the solution of the challenging problem related to the in vivo application of a glue patch on the inner side of the fetal membrane.

7.2 General remarks

Different experimental configurations were used within the present work for the mechanical characterization of FM tissue. Comparison of the test methods (uniaxial tension, inflation, and puncture testing) shows that the effort required for sample preparation and execution of accurate tests is comparable among the methods. Limits of data acquisition or extractable information rather refer to the particular setup utilized than to general characteristics of the test method. For the choice of the test method and setup, a compromise has to be made between physiological relevance of the loading situation and the number of ascertainable mechanical parameters. There is no way around an optical evaluation of the displacement field for experimental configurations inducing an inhomogeneous deformation field if characterization of the deformation behavior is desired. On
the other hand, there is a tendency observable in the literature on mechanical behavior of FM tissue toward the choice of the test method or setup that allows simple and fast testing and the extraction of many samples from one membrane. However, with regard to accurate mechanical characterization the focus should rather be on the quality and not the quantity of measurements.

In the same way, normalization of the experimental raw data is essential for mechanical analysis. No reliable determination of tangent moduli, maximum strain or Cauchy stress values is possible without the definition of the reference state. However, this step is usually omitted in the literature. Precise definition of the reference configuration is not only important for data analysis but also for comparison of data obtained in different loading conditions or reported in the literature. Creation of a database collecting mechanical characteristics for the exchange within the scientific community is hindered if reported studies suffer from avoiding of essential steps or incomplete specifications. Therefore, general guidelines for testing and data analysis, as they exist for the characterization of engineering materials, should also be specified for the biomechanics community. Moreover, most studies in the field of biomechanics focus on the determination of the stress-strain behavior for the characterization of soft biological tissues. This traditional approach has been performed over decades for the characterization of engineering materials, which in general possess very repeatable properties. However, the stress-strain data of soft biological tissues typically include large scatter, for which reason this approach might not be appropriate for the characterization of these materials. Research should focus on identifying and characterizing the repeatable properties of biological tissues, since these properties might be more relevant for the rationalization of their in vivo mechanical behavior.

7.3 Outlook

The achievements of the present work contribute to a better understanding of the mechanical properties of human fetal membrane tissue. However, the topics of mechanics, failure, and repair of FM tissue still offer significant potential for future investigations.

Mechanical characterization Although the mechanical behavior of the separate amnion and chorion layers has been studied in great detail, their interaction through the intermediate layer is only poorly understood. A first study (Strohl et al., 2010) measured the adherence of amnion and chorion by the use of a “peeling test”, but more research should be conducted for the determination of the interlaminar shear strength. Such re-
results would be more relevant for the in vivo situation and could be used to explain the separation during tensile testing in more detail. These results could also be used for corresponding modifications of the present bilayer FE model toward an even more realistic model. Moreover, evaluation of the regional variability of the interlaminar strength would contribute to a better understanding of the mechanical changes in the zone of altered morphology and could explain the difficulties involved in the validation of these changes by the use of different experimental configurations. The present thesis focused on the quasi-static response of FM tissue. However, the natural environment of the FM is much more dynamic due to fetal and maternal movement. Therefore, future investigations should also focus on the time-dependent response. Relaxation and creep tests as well as cyclic loading could be investigated depending on different levels of strain rate and stress amplitudes. In this regard, also the behavior of the lateral contraction during cyclic loading or the relaxation phase should be investigated, since this would provide relevant information about the reversibility of the mechanisms of collagen fiber reorientation. Moreover, additional tests should be performed to clarify the thickness changes during uniaxial loading and to overcome potential limitations of the present formulation assuming incompressible behavior. All these findings could then be included in the constitutive model formulation. Furthermore, the present model should be formulated as a “membrane” formulation and implemented in a FE code for shell elements to reduce computational time and to avoid numerical problems caused by the nearly incompressible formulation.

**Failure of FM tissue** The analysis of the rupture sequence should be extended by tests of membranes containing initial defects for a better understanding of the failure behavior and the determination of fracture mechanics properties. The sensitivity to circular holes and other defect shapes should be evaluated in experiments. These results would allow to assess whether the large stress concentrations around defects provoke failure or if the stress concentrations can be compensated by the higher compliance of the uniaxial stress state at the defect site. According to personal communications with surgeons, the angle and speed during puncturing is essential to minimize the risk of iPPROM. For this reason mechanical analyses should be performed considering the defect shapes caused by syringes and trocars under varying parameters of instrument size, puncture angle, and speed to investigate the potential of subsequent iatrogenic rupture. Additional fracture mechanics tests could be performed for the evaluation of the sensitivity to defects under controlled conditions and for the determination of the crack resistance of FM tissue. These results could serve for future optimization of surgical tools and procedures to limit the risk of iPPROM.
7.3. Outlook

**Repair of punctured membranes** There is a need for a better mechanical characterization of mussel glue if it will be used in future for the repair of punctured membranes. Modification of the chemical composition allows tuning of the glue properties, which should be adjusted to match the FM behavior in the best possible way. If the membranes will be sealed and repaired by the application of a glue patch, more research has to be done on the optimization of its shape and dimensions. Since the mechanical properties of mussel glue will probably not perfectly match the ones of FM tissue, a compromise has to be reached between reliable mechanical sealing and minimization of stress concentrations at the border of the patch as well as in the interface. Also the application of the glue patch, on either side of the FM, through the fetoscopic entry site is a challenging engineering problem that has to be solved in the future.
Correlations between mechanical parameters and microstructural constituents under uniaxial loading

Motivation

The correlations presented in section 3.9 refer only to the equibiaxial mechanical response based on inflation testing, since the corresponding correlations under uniaxial loading are already known from a previous study (Jabareen et al., 2009). The mechanical and histological data of the intact FM samples used for uniaxial tension testing were obtained with the same protocol as used in Jabareen et al. (2009). Therefore, they enable an attempt to reproduce the findings of Jabareen et al. and an evaluation of the robustness of these correlations.

Methods

The mechanical parameters characterizing the nonlinear FM response under uniaxial loading are extracted as defined in section 3.3.3. For consistency with Jabareen et al. (2009) the stiffness parameters $E_1$ and $E_2$ are evaluated as the slopes of the stress-strain curves. The Rubin-Bodner model in its isotropic, elastic, and incompressible formulation was
Appendix A. Correlations between mechanical parameters and microstructural constituents under uniaxial loading

shown to provide a reasonable representation of the averaged uniaxial FM response. The corresponding nominal stress $\sigma_{xx}^{\text{nom}}$ in axial direction reads

$$
\sigma_{xx}^{\text{nom}} = m_2 \mu_0 e^{q m_2(I_1 - 3)} \left( \lambda_1 - \frac{1}{\lambda_1^2} \right),
$$

(A.1)

$$
I_1 = \lambda_1^2 + \frac{2}{\lambda_1}
$$

(A.2)

with $\lambda_1$ being the stretch in longitudinal direction and $I_1$ the first invariant of the left Cauchy-Green deformation tensor $B$. Best fit model parameters $m_2, \mu_0, q$ are determined by the method of least squares utilizing the MATLAB intrinsic function \texttt{fminsearch}. Optimization of the model parameters is performed with respect to the membrane specific averaged stress-strain curve.

A remarkable difference between the initial stiffness of the preconditioned state and the virgin response was observed in section 3.7.1. Therefore, optimization of the Rubin-Bodner model as well as evaluation of the initial stiffness $E_1$ were also performed on the virgin response.

Results

The mechanical characteristics of the 10 intact fetal membranes used for tensile testing are summarized in Table A.1. The parameters were evaluated for the preconditioned state as well as for the virgin response. For the virgin response only the initial stiffness $E_1$ was evaluated due to the limited strain amplitude within preconditioning (20% nominal strain). The overall averaged initial stiffness of the preconditioned state is with a value of $(5.9 \pm 2.1) \cdot 10^{-2}$ MPa higher than the corresponding value of $(2.1 \pm 0.7) \cdot 10^{-2}$ MPa of the virgin response. As can be seen in Figure A.1 the differences in the mechanical response between the preconditioned state and the virgin response do also influence the quality of the Rubin-Bodner model optimization, i.e. optimization of the virgin response works better than for the preconditioned state.

Correlations between parameters characterizing the mechanical response and parameters quantifying the microstructural constituents were calculated by means of linear regression. Table A.2 shows the resulting coefficients of correlation ($R$ value). Only the correlation between the high strain stiffness $E_2$ for the preconditioned state and the elastin content is statistically significant ($p < 0.05$). No correlation between the collagen and elastin content could be observed ($R = 0.17$).
Table A.1: Mechanical parameters from the stress-strain curves of the membranes used for uniaxial tension testing. Parameters are given as membrane specific average ± standard deviation and evaluated as nominal values. Evaluation is performed for the preconditioned state and the virgin condition.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>$E_1$ [· 10⁻²MPa]</th>
<th>$E_2$ [MPa]</th>
<th>$\frac{q}{\mu_0}$ [MPa⁻¹]</th>
<th>$E_1$ [· 10⁻²MPa]</th>
<th>$E_2$ [MPa]</th>
<th>$\frac{q}{\mu_0}$ [MPa⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1</td>
<td>6.6 ± 0.1</td>
<td>1.53 ± 0.04</td>
<td>81.7</td>
<td>3.0 ± 1.1</td>
<td>731</td>
<td></td>
</tr>
<tr>
<td>U2</td>
<td>6.8 ± 1.4</td>
<td>1.74 ± 0.53</td>
<td>25.7</td>
<td>1.7 ± 0.2</td>
<td>2620</td>
<td></td>
</tr>
<tr>
<td>U3</td>
<td>4.8 ± 2.7</td>
<td>2.38 ± 0.29</td>
<td>74.3</td>
<td>1.8 ± 0.6</td>
<td>1364</td>
<td></td>
</tr>
<tr>
<td>U4</td>
<td>6.6 ± 2.4</td>
<td>2.46 ± 0.82</td>
<td>70.1</td>
<td>2.9 ± 1.1</td>
<td>488</td>
<td></td>
</tr>
<tr>
<td>U5</td>
<td>3.8 ± 1.2</td>
<td>2.74 ± 0.63</td>
<td>103.5</td>
<td>1.7 ± 0.8</td>
<td>2771</td>
<td></td>
</tr>
<tr>
<td>U6</td>
<td>3.6 ± 0.6</td>
<td>2.42 ± 0.42</td>
<td>32.6</td>
<td>2.1 ± 0.7</td>
<td>1478</td>
<td></td>
</tr>
<tr>
<td>U7</td>
<td>4.1 ± 1.1</td>
<td>1.55 ± 0.63</td>
<td>98.4</td>
<td>0.7 ± 0.4</td>
<td>7791</td>
<td></td>
</tr>
<tr>
<td>U8</td>
<td>7.9 ± 3.9</td>
<td>4.83 ± 0.28</td>
<td>24.6</td>
<td>3.2 ± 1.0</td>
<td>628</td>
<td></td>
</tr>
<tr>
<td>U9</td>
<td>10.6 ± 6.3</td>
<td>2.56 ± 0.32</td>
<td>30.7</td>
<td>1.9 ± 1.2</td>
<td>944</td>
<td></td>
</tr>
<tr>
<td>U10</td>
<td>5.1 ± 4.2</td>
<td>2.79 ± 0.41</td>
<td>62.3</td>
<td>2.4 ± 0.2</td>
<td>2034</td>
<td></td>
</tr>
</tbody>
</table>

Figure A.1: Optimization of the isotropic, elastic, and incompressible formulation of the Rubin-Bodner model to the averaged uniaxial response of the preconditioned (a) and virgin (b) state.

Table A.2: Coefficients of correlation $R$ between microstructural constituents and mechanical parameters. The value printed in bold is statistically significant ($p < 0.05$).

<table>
<thead>
<tr>
<th></th>
<th>preconditioned state</th>
<th>virgin state</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_1$</td>
<td>Elastin 0.513</td>
<td>Collagen 0.455</td>
</tr>
<tr>
<td>$E_2$</td>
<td>0.724</td>
<td>0.166</td>
</tr>
<tr>
<td>$\frac{q}{\mu_0}$</td>
<td>-0.482</td>
<td>-0.261</td>
</tr>
<tr>
<td></td>
<td>0.266</td>
<td>0.509</td>
</tr>
<tr>
<td></td>
<td>-0.455</td>
<td>-0.341</td>
</tr>
</tbody>
</table>
Discussion

Preconditioning has a large impact on the initial stiffness of the tissue, as already discussed in section 3.11. Overall averaged values of the initial stiffness change from 
\((2.1 \pm 0.7) \cdot 10^{-2}\) MPa for the virgin response to \((5.9 \pm 2.1) \cdot 10^{-2}\) MPa after five cycles of preconditioning. However, only the initial stiffness of the virgin response agrees with the findings of Jabareen et al. (2009) who have determined a corresponding value of 
\((1.8 \pm 0.5) \cdot 10^{-2}\) MPa. The reason for this deviations might be related to a different implementation of the preconditioning cycles. Similarly, a suitable result of the Rubin-Bodner model optimization can only be achieved based on the virgin response, although for a smaller strain level. Coefficients of the Rubin-Bodner model parameter \(q/\mu_0\) show similar deviations as the initial stiffness. Parameter values of \(q/\mu_0\) are within \(488 \text{ MPa}^{-1}\) to \(7790 \text{ MPa}^{-1}\) for the virgin response and within \(24.6 \text{ MPa}^{-1}\) to \(104 \text{ MPa}^{-1}\) for the preconditioned state, whereat the values of the virgin response are closer to the parameter range of \(363 \text{ MPa}^{-1}\) to \(2141 \text{ MPa}^{-1}\) as determined by Jabareen et al. (2009).

The present results do not allow a reproduction of the correlations previously found by Jabareen et al. (2009). The strong correlations between parameters \(E_1\) as well as \(q/\mu_0\) and the elastin content as observed by Jabareen et al. could not be proved in this study. However, at least weak tendencies (\(R\) in the range of \(\pm 0.5\)) toward a similar relationship between the parameters could be observed. Although the mechanical parameters of the virgin response agree better with the previous findings, the corresponding correlations are even weaker. Only one correlation (\(E_2\) vs. elastin) was found to be statistically significant in the present work. However, this might mainly be explained by an extreme value (outlier) of the parameter \(E_2\) of membrane U8.

Conclusions

The present findings point to difficulties involved in both the determination of the initial stiffness of soft biological tissues and the determination of the elastin content with biochemical assays. Uncertainties related to both disciplines complicate a reproduction of previous results. At least similar tendencies between some parameters could be observed, although with much lower coefficients of correlation. The outcome of the present study is still satisfying especially when considering that the present study was conducted by using different experimental devices and protocols as compared to the previous study of Jabareen et al. (2009).
Appendix B

Effect of freezing on the uniaxial response

The study partially summarized in this chapter was originally conducted to investigate the membrane specific mechanical response to uniaxial and pure-shear loading. However, the pure-shear tests were not successful due to experimental difficulties involved in the sample fixation. Nevertheless, the uniaxial tests were performed with an experimental protocol similar to the one used in Jabareen et al. (2009). In contrast to the results presented in section 3.7.1, the five preconditioning cycles were defined as 20\(^\circ\) nominal strain with respect to the initial free sample length of 40\,mm. Therefore, the present implementation might be more similar to the one used in Jabareen et al. (2009). Since the membranes were frozen at \(-20^\circ C\) prior to mechanical testing, the results obtained in this study enable a qualitative evaluation of the effect of freezing on the uniaxial response of FM tissue if compared to the data of Jabareen et al. (2009) obtained by tensile testing of fresh FM samples.

\textbf{Table B.1}: Mechanical parameters from the stress-strain curves of the membranes used for uniaxial tension testing. Parameters are given as membrane specific average \pm standard deviation and evaluated as nominal values. Evaluation is performed for the preconditioned state.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>(E_1 \cdot 10^{-2}\text{MPa})</th>
<th>(E_2) [MPa]</th>
<th>(\varepsilon_{\text{crit}}) [%]</th>
<th>(\sigma_{\text{crit}}) [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>UF1</td>
<td>2.5 \pm 0.9</td>
<td>1.34 \pm 0.57</td>
<td>21.3 \pm 5.4</td>
<td>45.6 \pm 12.0</td>
</tr>
<tr>
<td>UF2</td>
<td>2.0 \pm 0.5</td>
<td>0.58 \pm 0.28</td>
<td>18.4 \pm 9.2</td>
<td>56.1 \pm 3.8</td>
</tr>
<tr>
<td>UF3</td>
<td>2.0 \pm 1.0</td>
<td>1.67 \pm 0.70</td>
<td>29.9 \pm 13.8</td>
<td>41.7 \pm 7.4</td>
</tr>
<tr>
<td>UF4</td>
<td>1.6 \pm 0.2</td>
<td>0.95 \pm 0.07</td>
<td>15.0 \pm 3.2</td>
<td>44.6 \pm 6.8</td>
</tr>
<tr>
<td>UF5</td>
<td>2.5 \pm 0.7</td>
<td>1.78 \pm 1.20</td>
<td>42.0 \pm 16.8</td>
<td>54.3 \pm 6.0</td>
</tr>
<tr>
<td>UF6</td>
<td>3.0 \pm 0.9</td>
<td>1.78 \pm 1.11</td>
<td>31.5 \pm 19.2</td>
<td>36.6 \pm 5.8</td>
</tr>
<tr>
<td>UF7</td>
<td>2.8 \pm 0.4</td>
<td>2.02 \pm 0.70</td>
<td>38.7 \pm 14.2</td>
<td>43.7 \pm 9.8</td>
</tr>
<tr>
<td>UF8</td>
<td>1.8 \pm 0.7</td>
<td>1.55 \pm 1.64</td>
<td>21.1 \pm 10.6</td>
<td>44.6 \pm 20.9</td>
</tr>
</tbody>
</table>
Appendix B. Effect of freezing on the uniaxial response

25 samples from eight different membranes were tested within this study. Table B.1 summarizes the membrane specific averages of the common scalar parameters. Extraction of the parameters $E_1$ and $E_2$ was performed on the stress-strain level for consistency with the previous study of Jabareen et al. (2009). Overall averaged data are: $(2.3 \pm 0.8) \cdot 10^{-2} \text{MPa}$ for the low strain stiffness $E_1$, $(1.5 \pm 0.9) \text{MPa}$ for the high strain stiffness $E_2$, $(46 \pm 11) \%$ for the maximum engineering strain $\varepsilon_{\text{crit}}$, and $(0.28 \pm 0.15) \text{MPa}$ for the maximum stress $\sigma_{\text{crit}}$.

The mechanical parameters characterizing the low and high strain stiffness and the tensile strength are within the range reported by Jabareen et al. (2009). Corresponding overall averaged data from Jabareen et al. (2009) are: $(1.8 \pm 0.5) \cdot 10^{-2} \text{MPa}$ for $E_1$, $(1.7 \pm 0.9) \text{MPa}$ for $E_2$, and $(0.27 \pm 0.17) \text{MPa}$ for the tensile strength. Only the strain at rupture of the current study is somewhat smaller as the reported value of $(54 \pm 13) \%$.

In conclusion, no effect of freezing on the mechanical response of FM tissue under uniaxial loading can be observed within this study. Differences of the overall averaged mechanical parameters between the fresh and frozen samples are within the general scatter of the data. For a more accurate analysis of the effect of freezing, samples from one membrane should be cut and randomly assigned to two test groups “fresh” and “frozen” and the significance of the differences should be evaluated for these two sample groups. Also evaluation of the in-plane kinematic response of the two tests groups should be performed to investigate possible effects of freezing on the mechanisms of fiber reorientation under loading and unloading. Evaluation of several membranes with this protocol would enable a statistically more relevant analysis.
Pure-shear testing of FM samples

Pure-shear testing (Ogden, 1972) offers a simple and efficient way of testing tissue samples within a biaxial stress state. It requires an adequate (low) aspect ratio of the sample (length:width $\approx 1:6$) to ensure a restricted lateral contraction during testing. Pure-shear tests are highly defined in terms of the in-plane kinematics ($\lambda_1 = \lambda$, $\lambda_2 = 1$, with $\lambda$ being the applied stretch), however the corresponding stress component $\sigma_{22}$ in lateral direction is unknown. A first study conducted on FM samples showed a higher compliance of the membrane specific pure-shear response compared to uniaxial data of the same membrane. The cause for this unphysical response can be explained by experimental difficulties involved in the sample fixation. The inhomogeneity of the FM sample complicated a uniform clamping over the whole width of the sample, which resulted in remarkable slippage of the sample within the clamping, see Figure C.1(a).

Figure C.1: Illustration of a pure-shear test on a FM sample with the original clamping, similar to the one used in Hollenstein et al. (2010) (a). The original clamping consisting of two rigid bars of length 60 mm lead to pronounced sample slippage. A modified fixture (b) was designed which is able to account for the varying thickness of FM samples by subdivision of the clamping area into single segments.
Appendix C. Pure-shear testing of FM samples

Figure C.2: Comparison of the membrane specific averaged tension-stretch response of membrane U2 (a) and U3 (b) obtained in uniaxial tension and pure-shear configurations.

A modified clamping was developed (see Figure C.1(b)) which is able to account for the varying sample thickness. Five samples (free length of $10 \times 60$ mm) from two different membranes were tested with the modified clamping. The experimental protocol, consisting of five cycles of preconditioning and a subsequent monotonic tension to failure test, was identical to the one used for the uniaxial tension tests in section 3.3.2. Moreover, the samples used for pure-shear testing were extracted from membranes U2 and U3 used within the uniaxial study (section 3.3.2). Therefore, the pure-shear data can be compared to uniaxial data from the same membrane. Only the virgin response of the measurements is evaluated for consistency with other data reported in this thesis. The force threshold ($0.04$ N) used for the normalization of the pure-shear data accounts for the larger sample width. Figure C.2 illustrates the membrane specific averaged pure-shear and uniaxial response of the two membranes. Membrane specific averages of the initial stiffness within pure-shear $K_{PS}^1$ and uniaxial $K_{UA}^1$ configuration are: $(1.6 \pm 0.1) \cdot 10^{-2}$ N/mm and $(0.8 \pm 0.1) \cdot 10^{-2}$ N/mm for membrane U2, as well as $(1.9 \pm 0.8) \cdot 10^{-2}$ N/mm and $(0.9 \pm 0.3) \cdot 10^{-2}$ N/mm for membrane U3. The data indicate pronounced differences in the initial stiffness depending on the state of deformation. Based on isotropic linear elasticity a factor of $K_{PS}^1/K_{UA}^1 = 4/3$ would be expected. The present ratios are somewhat higher, which can be attributed to the pronounced lateral contraction of FM tissue that is restricted in pure-shear testing. Although, the modified clamping allows a more suitable sample fixation than the original one, difficulties of positioning the short and wide sample without differences in the prestress over the width still remain.
Investigation of the zone of altered morphology by inflation testing

The results presented in this chapter are a summary of two attempts undertaken to demonstrate the mechanical changes in the zone of altered morphology by inflation testing.

Motivation

Several studies investigated the biochemical changes occurring in FM tissue during gestation. Skinner et al. (1981) and MacDermott and Landon (2000) found a general reduction of the collagen content in the FM with increasing gestational age. Moreover, Skinner et al. observed that samples from PROM membranes had a reduced collagen content compared to samples from patients without PROM. More recent, Stuart et al. (2005) proved the general reduction of collagen with gestational age but found no regional variations of the collagen content between the rupture and non-rupture site. Malak and Bell (1994) and McLaren et al. (1999a) have identified a zone of altered morphology (ZAM) overlying the cervical region. This region is characterized by swelling and disruption of the connective tissue and a reduction of the thickness of chorion’s sublayers. El Khwad et al. (2005) were the first who demonstrated that the structural changes within the discrete ZAM are related to mechanical weakening of the FM tissue. El Khwad et al. observed in puncture tests that samples from the ZAM region have a reduced strength, stiffness, and ductility. However, the state of deformation induced by puncture testing, where a spherical metal probe deflects the membrane sample, deviates from the in vivo loading. Inflation testing best mimics the natural deformation state of the FM and enables a more reliable determination of membrane strength. Therefore, the present study aims to demonstrate the mechanical changes in the ZAM by inflation testing.
Appendix D. Investigation of the zone of altered morphology by inflation testing

Method

FM samples were collected from planned cesarean sections according to the protocol described in section 3.2. The region of the fetal membrane overlying the internal cervical orifice was marked by the surgeon after delivery of the newborn but before the expulsion of the placenta. Samples for mechanical testing were obtained by following the procedure explained in section 3.4.2. Typically one to two samples were obtained from the marked region and classified as “ZAM” samples and one to five samples were obtained from the remaining area of the FM. In a first part of the study the conventional clamping with a diameter of 50 mm was used for the inflation tests, while in a second part a clamping with a diameter of 25 mm was used for more consistency with the study of El Khwad et al. (2005) and a more localized detection of the ZAM region. Inflation tests were performed and evaluated according to the methods described in section 3.4. In addition to the mechanical tests also the thickness and the collagen content were measured for both sample types.

Results

A total of 31 membranes were tested on the inflation device for the analysis of the mechanical changes in the ZAM region, i.e. 18 membranes for study 1 (Ø 50 mm clamping) and 13 membranes for study 2 (Ø 25 mm clamping). Table D.1 summarizes the mechanical and histological data of both studies and for both sample types (ZAM and remaining). For the analysis of the critical membrane tension only the samples which ruptured in the middle or away from the clamping site were considered. Values of maximum pressure and displacement were evaluated as system parameters, independently from the rupturing site, due to their similarity to the values of maximum force and displacement as determined in El Khwad et al. (2005).

The values in Table D.1 show that there is no reduction of strength and deformation capacity of the ZAM samples compared to samples from the remaining area for neither of the two studies. Only the histological data of thickness and collagen content show a reduction in the ZAM area, whereat only the differences in the thickness are statistically significant ($p < 0.05$). The collagen content is evaluated as mg of collagen per area, since this measure accounts for the changes in the sample thickness. Original values as extracted by the biochemical assays are: for study 1 (178 ± 78) mg/g in the ZAM and (134 ± 49) mg/g in the remaining area, and for study 2 (157 ± 48) mg/g in the ZAM and
Table D.1: Summary of the mechanical and histological data obtained in the two studies, investigating the mechanical and biochemical changes in the zone of altered morphology (ZAM) compared to the remaining area of the FM. No information about the stretch in the apex region can be obtained for the tests performed with the smaller clamping, since the corresponding stretch curves are missing.

<table>
<thead>
<tr>
<th></th>
<th>Study 1 (Ø 50 mm)</th>
<th>Study 2 (Ø 25 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZAM</td>
<td>remaining</td>
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<tr>
<td>Crit Tension [N/mm]</td>
<td>0.28 ± 0.09</td>
<td>0.26 ± 0.11</td>
</tr>
<tr>
<td>Crit Stretch [−]</td>
<td>1.24 ± 0.07</td>
<td>1.20 ± 0.04</td>
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<tr>
<td>Max Pressure [mbar]</td>
<td>184 ± 97</td>
<td>138 ± 52</td>
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<tr>
<td>Max Displacement [mm]</td>
<td>9.3 ± 2.5</td>
<td>8.6 ± 1.7</td>
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<tr>
<td>Thickness [µm]</td>
<td>292 ± 148</td>
<td>497 ± 265</td>
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<tr>
<td>Collagen [mg/cm²]</td>
<td>0.42 ± 0.24</td>
<td>0.51 ± 0.24</td>
</tr>
</tbody>
</table>

(99 ± 20) mg/g in the remaining area, thus giving the impression of a higher collagen content in the ZAM region.

Discussion

The results obtained in the present studies do not allow to verify the findings of El Khwad et al. (2005) of a reduced strength and failure stretch of samples from the ZAM region. Although the remarkably reduced thickness of the ZAM samples indicates that the marking procedure allowed to detect a zone of structural changes, the mechanical parameters do not point to any weakening of the ZAM samples.

The mechanical characteristics measured in this study cannot directly be related to the values previously been published in El Khwad et al. (2005) by puncture testing. However, analysis of the method of puncture testing in chapter 5 has shown that the clamping used in El Khwad et al. (2005) might be related to particular sample slippage. Moreover, it has been demonstrated that slippage of the amnion layer can lead to a unique rupture sequence and an overall more compliant response in terms of the force-displacement behavior. Malak and Bell (1994) have shown that the structural changes occurring in the ZAM region are characterized by a relatively thicker compact layer and a swollen and disintegrated spongy layer. Recently, Mauri et al. (2013) observed a behavior close to the findings of Malak and Bell and have shown by multiphoton microscopy that the interface layer of the ZAM samples shows an altered collagen structure. It seems that the structural changes occurring in the ZAM facilitate sliding and relative movement between amnion and chorion, and thus contribute to an increased compliance of the tissue in the puncture...
Appendix D. Investigation of the zone of altered morphology by inflation testing

tests of El Khwad et al. (2005) (and similarly in vivo). On the other hand, utilizing a dedicated clamping technique and sample preparation, within inflation or puncture testing (see section 4.5 and 5.4), limits the influence of possible relative movement of amnion and chorion on the measured mechanical response. The present findings indicate that strength reduction of ZAM samples might be related also to changes in the interface layer, which contribute to a localized weakening of the FM tissue in the cervical region in addition to the biochemical degradation.

The histological parameters determined in this study are in agreement to findings reported in the literature. El Khwad et al. (2005) report that the cervical region was thinner when amnion, chorion, and the maternal decidua were considered. Already Artal et al. (1976) had observed that the thickness of the FM was reduced near the rupture site. The measured collagen content between 9.8 %DW to 17.6 %DW is in good agreement to our earlier findings in section 3.8. Also the observation of approximately similar collagen content in the samples from the ZAM and remaining area is in agreement to findings of Stuart et al. (2005) who found no regional variations of the collagen content.

Conclusions

The present findings attribute a relevant role to the intermediate (spongy) layer of the FM for the observed weakening of the FM region overlying the cervix. Mechanisms of relative sliding between amnion and chorion due to an altered structure of the intermediate layer might also contribute to an increased compliance of the ZAM samples in addition to the biochemical degradation. The strength of adherence of amnion and chorion has previously been measured for vaginal deliveries, term cesarean sections, and preterm deliveries (Kumar et al., 2009; Strohl et al., 2010). However, corresponding investigations of the regional variations are still missing but should be performed in future to verify the present interpretation.
Effect of swelling on the mechanical behavior of FM

Biological tissues are usually tested in phosphate buffered saline solution to mimic the in vivo conditions. The tissue samples are expected to swell by water uptake due to differences in the osmotic pressure if the tests were performed in water. However, experimental data illustrating the effects on the mechanical behavior are missing.

Seven additional tension tests on an amnion layer originating from one membrane were performed for a preliminary investigation of this effect. The tests were performed and evaluated according to the protocol described in section 4.2. The only difference is that the amnion layer was immersed in DI water for about 1h prior to testing and that the tests were performed in a bath filled with DI water.

Figure E.1 shows the experimental results from the tests performed in water and compares the tension-stretch curves as well as the kinematic response to the data obtained in saline solution. As can be seen in Figure E.1 there is a pronounced right shift of the curves obtained in DI water. It seems that swelling of the sample hinders the mechanisms of fiber reorientation in particular at smaller longitudinal stretches. Maximum values of incremental Poisson’s ratio are still in the range of 5.7 to 7.8 and maximum values of membrane tension are comparable to the data obtained in saline solution.

However, these are preliminary data and more experiments should be performed for a more reliable analysis of this effect.
Appendix E. Effect of swelling on the mechanical behavior of FM

Figure E.1: Comparison of amnion’s uniaxial mechanical response if tested in PBS solution or DI water. The figure illustrates the tension-stretch (a) and the kinematic response (b) for the two cases investigated. Data were normalized by a force threshold of 5 mN.
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