Cervical Mucus Properties Stratify Risk for Preterm Birth
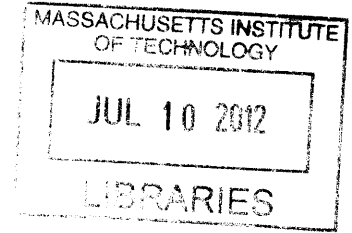
by

Grace Yao

S.B. Biology
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ABSTRACT

Preterm birth impacts 15 million babies every year, leading to morbidity, mortality, significant health care costs, and lifelong consequences. The causes of preterm birth are unknown, resulting in ineffective treatment, but it is correlated with ascension of vaginal bacteria through the cervix, which is normally protected by a dense mucus plug during pregnancy. This mucus plug, consisting of a tight meshwork of glycoproteins called mucins, should prevent pathogens from accessing the sterile uterine environment.

Cervical mucus from women at high risk and low risk for preterm birth was collected and compared. The aim of this study was to discover differences that will lead to clues about why preterm birth occurs, and ultimately what can be done about it in terms of prevention and intervention.

Using rheological techniques and a translocation assay, we found that cervical mucus from women at high risk is more translucent and more elastic under both elongational and shear stress, than cervical mucus in normal pregnancies. These properties more closely resemble mucus during ovulation, when spermatozoa can most easily penetrate the barrier, than mucus in normal pregnancy. Furthermore, high risk mucus is more permeable to beads of comparable size to viruses, suggesting the barrier is weakened and foreign particles may harmfully traverse it to cause intrauterine infection. The techniques in this paper have not been previously used to study cervical mucus in the context of preterm labor, but their results may have important implications. If these mucus properties in women indeed permit increased bacterial infection through the cervix, then they can be used to stratify patients, allowing for more personalized prenatal care to lower the rate of preterm birth.

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Chapter 1: Introduction

Mucus is a complex biomaterial that lines and lubricates moist mucosal surfaces in the body, including the lungs, gastrointestinal tract, eyes, and vagina, serving to shield us from infectious agents and other environmental particles while allowing selective passage to nutrients, ions, gases, and proteins. It has a vital protective function in the cervix, particularly during pregnancy. The majority of research on cervical mucus has been on its properties in fertility, and its role throughout pregnancy is understudied despite its prominent location within the cervical barrier. By elucidating its properties during pregnancy, we can better understand what part it plays in the occurrence of preterm birth, the leading cause of neonatal death with over 1 million children dying each year worldwide and many survivors facing a lifetime of disability[1].

1.1 Preterm Birth

Preterm birth, or birth before 37 weeks of gestational age, is usually preceded by preterm labor: multiple uterine contractions in an hour, accompanied by cervical dilation and effacement, or shortening of the cervix[2]. Preterm birth is often used synonymously with premature birth. While a preterm baby does not necessarily have underdeveloped organs at birth, the defining characteristic of premature babies, there is a significant overlap between the two so a preterm baby is generally also premature.

1.2.1 Impact

Preterm birth affects about 1 in every 8 babies in the US, resulting in major pediatric morbidity and mortality, as well as $26.2 billion of healthcare costs[2]. Average

first-year medical costs are about 10 times greater for preterm infants than for infants carried to full term[3]. Infants are often born underweight, less than 6 pounds, with underdeveloped organs, especially lungs, and are at high risk for multiple complications, such as growth and mental disabilities which can have lifelong effects[4]. Many have respiratory distress syndrome and apnea, which require breathing assistance. Cardiovascular problems are also common, such as when an artery called the ductus arteriosis that allows blood to bypass the lungs (while the fetus acquires oxygen through the placenta) fails to close before birth, resulting in breathing difficulty, poor weight gain, and possibly heart failure. Further complications include hemorrhaging in the brain, temporary vision loss, chronic lung disease, jaundice, anemia, and immunological abnormalities. An underdeveloped immune system can lead to serious infections such as meningitis, pneumonia, and sepsis[3]. Although care of premature infants has progressed, the rate of preterm birth has not significantly decreased and it is still a major problem in obstetrics, accounting for 70% of perinatal mortality[5].

1.2.2 Etiology

Since the causes of preterm birth are largely unknown, prevention and treatment options for it have remained extremely limited and often ineffective. There are likely many pathways in the etiology of preterm birth, but in a significant number of cases, vaginal flora seems to be the primary culprit. *Ureaplasma urealyticum, Mycoplasma hominis, Gardnerella vaginalis, Trichomonas vaginalis Escherichia coli* or *Streptococcus agalactiae* (Group B streptococcus) pass through cervical canal and invade the normally sterile intrauterine environment, suggesting that the cervical mucus barrier to infection is impaired[6,7]. This ascending bacterial infection promotes inflammation in the fetal

membranes and placenta and can invade the amniotic fluid, initiating a complex cascade of events ultimately resulting in uterine contractility and preterm birth[7]. These events may include the release of endotoxins and cytokines and the stimulation of a response by the decidua (uterine lining) during pregnancy. This decidual release of contraction-stimulating prostaglandins or matrix-degrading enzymes that weaken the fetal membranes can lead to premature rupture[7].
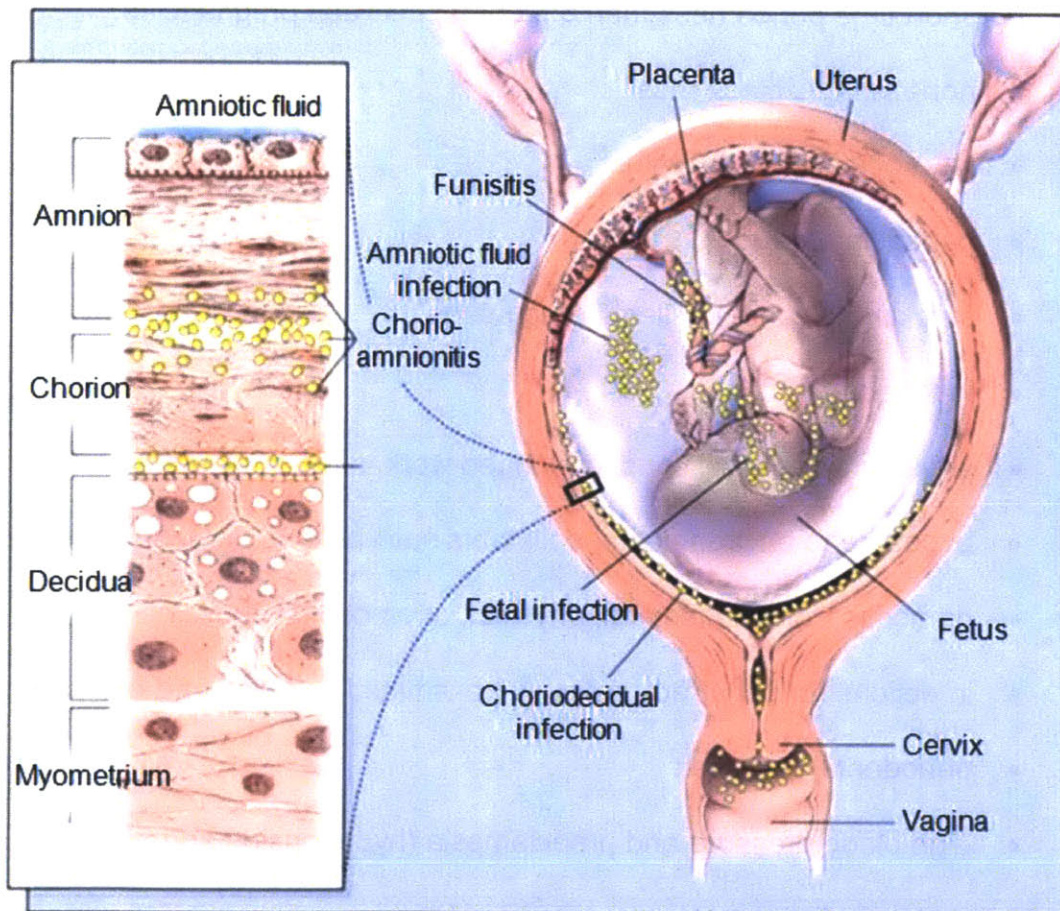
Figure 1-1. Potential sites of infection within the uterus include the choriodecidual space, chorion, amnion, placenta, amniotic fluid, the umbilical cord, or the fetus. Adapted from Goldenberg *et al.* (2000)[7].

Several factors in the maternal background have also been correlated with a higher risk of preterm birth. Women who have previously had premature birth, who have

uterine or cervical abnormalities, or who are expecting twins, triplets, or more rather than a singleton birth are at greatest risk[8,9,10].

Additional risk factors may include demographic factors, lifestyle factors, and medical conditions:

- women who are at the lowest (younger than 18) and highest (older than 35) ends of their reproductive years[5,11]
- short time period (less than 6 months) between pregnancies[12,13]
- non-hispanic black race[5]
- low socioeconomic status[5]
- genetic make-up[14,15,16,17]
- smoking, alcohol, or illegal drug usage[4,18]
- inadequate nutrition
- stressful work conditions, prolonged work hours or standing[5,19,20]
- exposure to environmental pollutants such as lead, mercury, or pesticide[4]
- domestic violence (sexual, physical, or emotional abuse)[21]
- infections (urinary tract, sexually transmitted, vaginal, and others)
- periodontal disease[22]
- high blood pressure and preeclampsia (hypertension and high amounts of protein in the urine)
- diabetes[23]
- being underweight or obese before pregnancy
- anxiety and depression[5,24]

1.2.3 Diagnosis

Preterm labor, in addition to contractions, is often accompanied by a change in vaginal discharge, pelvic pressure, backache, and cramps. Although it is sometimes difficult to distinguish between false labor and actual preterm labor, obstetric ultrasound of the cervix has been helpful in identifying women at risk of delivering preterm. It is uncertain whether a short cervix facilitates bacterial ascension or whether cervical shortening is a result of an infection that has already occurred. A cervical length of less than 25 mm indicates high risk especially in women with a previous preterm birth, while women whose cervical length is greater than 30 mm are unlikely to deliver within the week[25,26]. However, ultrasonography is not generally used in evaluation of patients who do not have any risk factors for preterm birth[27].
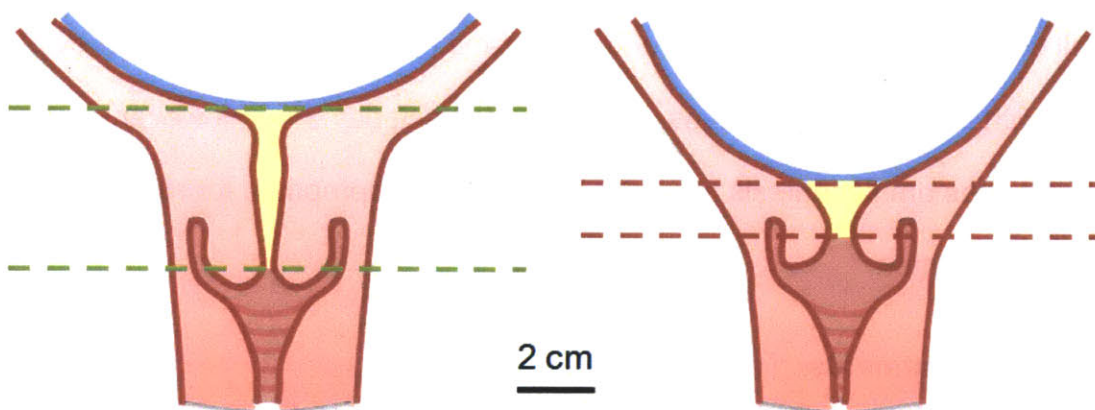
Figure 1-2. Left, cervix during healthy pregnancy. Right, a shortened cervix.

In some cases of cervical insufficiency or incompetence, which is more likely in women with shorter cervixes, the cervix begins to dilate before full term, without the onset of labor or other symptoms[28]. Again, vaginal ultrasounds are not usually recommended unless a woman has one or more of the above risk factors.

In both asymptomatic women and those in preterm labor, a fetal fibronectin test can be indicative of high risk for preterm delivery. The presence of fibronectin, a glycoprotein product of the placenta, in cervicovaginal secretions is strongly associated with neonatal sepsis and chorioamnionitis[7]. A negative result is also highly predictive of non-preterm delivery in symptomatic women[29]. Although this test is the strongest biomarker for increased risk of preterm birth, a positive result implies that an infection has already disrupted the membrane between the chorion and deciduas, leading to leakage of the protein into the cervix and vagina[30].

Bacterial vaginosis, or imbalance of the normal flora, detected in vaginal secretions is also associated with intrauterine infection. If an amine odor, loss of acidity, or cells covered by bacteria are detected, the woman is at high risk for preterm delivery[31]. Another well-studied infection site is the amniotic fluid, which has higher white cell counts, lower glucose concentrations, and higher concentrations of cytokines in women with intrauterine infections, in addition to bacteria[32,33,34]. However, studying this fluid requires amniocentesis, which currently is not appropriate for routine tests in asymptomatic women.

1.2.4 Current Treatments

In women who have known risk factors, several measures of preterm birth prevention have been studied. Antibiotic treatment trials have had mixed results in women who have bacterial vaginosis. Conflicting results in dose, delivery, and timing have been reported, as well as in different antibiotics – most commonly metronidazole or clindamycin (typically used for vaginosis)[35,36,37,38,39,40]. Unfortunately, not enough

evidence has been collected to make a conclusion about the effectiveness of antibiotic treatment, possibly because there are too many other interfering factors, such as patient behavior or inflammatory response.

The current gold standard of progesterone is usually recommended, as injections for women with a previous premature delivery, or as a vaginal gel for women with short cervixes[22,41,42,43]. Progesterone is an anti-inflammatory hormone that relaxes muscles, keeping the uterus from contracting, and plays a role in the control of cervical ripening[37,44,45]. It may have an effect on mucus production, as it is a hormone that typically rises after ovulation and changes mucus properties. Unfortunately, its mechanism is unclear, and it is not always effective.

Another treatment is cervical cerclage, or suturing the cervix shut in an attempt to prevent cervical shortening and dilation in women who already have incompetent or short cervixes and a history of preterm birth[46]. However, some women are not good candidates for cerclage, such as those with intrauterine infection, inflammation, active bleeding, premature membrane rupture, or in preterm labor, and the value of this procedure is unpredictable[14].
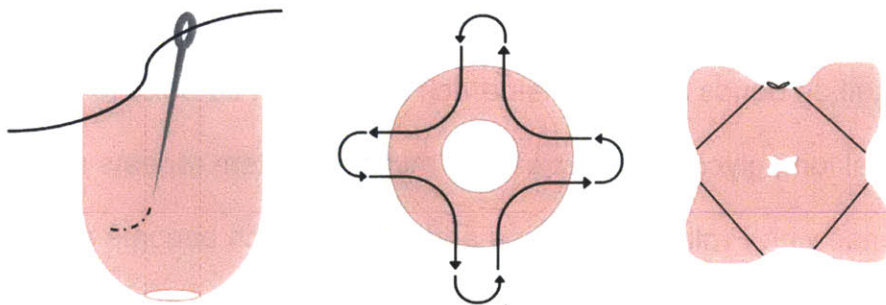
Figure 1-3. Cervical cerclage involves stitching the cervix after it has started to efface. There are three types of cerclage: in the one shown, a pursestring suture is placed in the upper part of the cervix and the cervix is cinched shut. The suture is cut before birth.

Tocolytic treatment, or anti-contraction medication, is a last resort for women in preterm labor to prolong pregnancy for up to 48 hours, to allow for transfer to a specialized unit. It has not been shown to prevent preterm delivery but only provides time to administer corticosteroids to possibly reduce neonatal organ immaturity[47].

1.2 Cervical Mucus

One critical element of the cervical canal is the protective and lubricating mucus secreted by endocervical columnar epithelial cells called goblet cells, which each contain many secretory vesicles that occupy the majority of the cell[48]. Pre-ovulatory mucus is composed of up to 96% water. The aqueous component also contains organic compounds (e.g. glucose, amino acids), enzymes, soluble proteins, trace elements, and electrolytes (calcium, sodium, and potassium).

The structural foundation for the mucus is provided by mucins, or large (10^6-10^7 Da) glycoproteins which entangle to form a tight meshwork and may have both biochemical and physical attributes that make for an effective defense mechanism[49]. The major mucin genes in the endocervical epithelium are MUC4 and MUC5B, while MUC5AC and MUC6 are more weakly expressed[50,51]. The first gene codes for a transmembrane glycoprotein, while the others are gel-forming mucins. Mucins are crosslinked by disulfide bonds at the cysteine-rich amino- and carboxy-terminal regions. They have a central long glycosylated stretch of multiple tandem repeats containing PTS domains, dense with *Proline*, *Threonine* and *Serine*, which become saturated with O-linked N-acetyl galactosamine, N-acetyl glucosamine, fucose, and galactose[52,53]. The O-glycans, contributing as much as 80% of the dry weight of mucus, have terminal sialic

acid groups, lending a strong negative charge to the molecule[54,55]. Resulting charge repulsion increases rigidity and stability within the network of the biopolymer, and sialomucin and carbohydrate content have been correlated to viscoelasticity as well as ability to allow sperm migration[56,57].
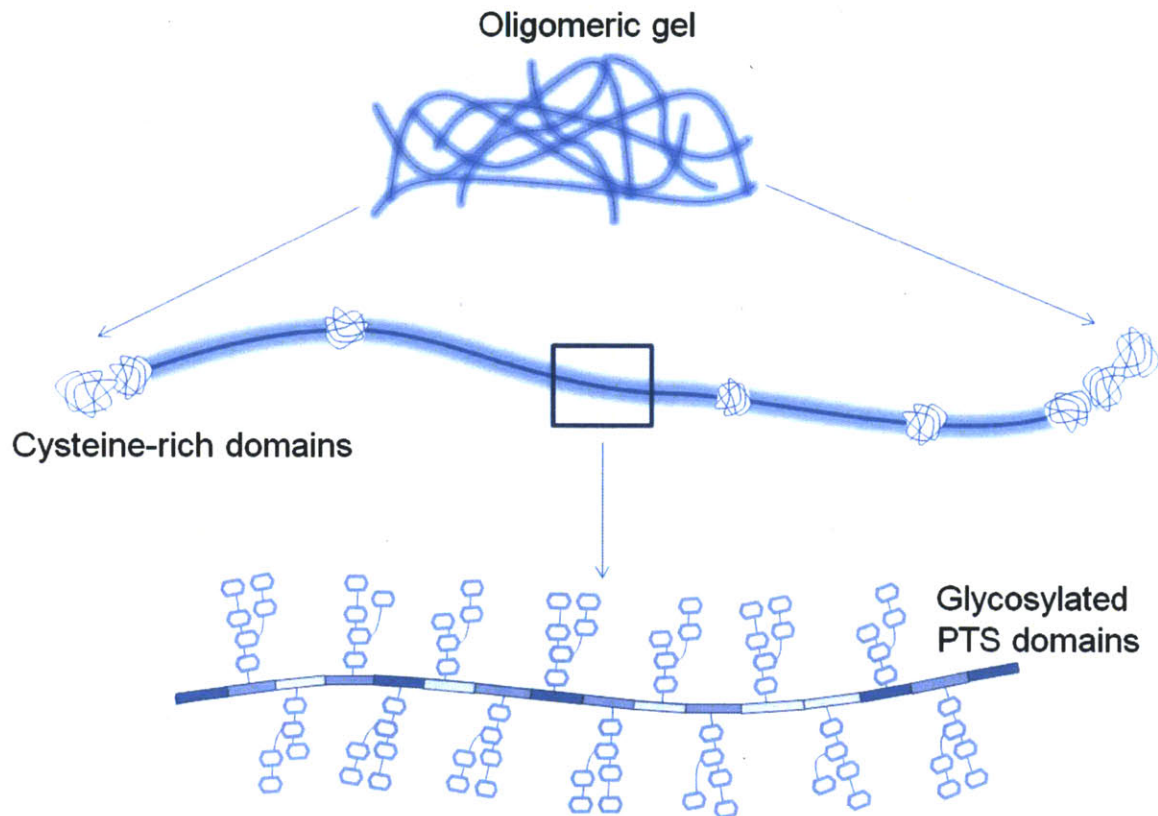
Figure 1-3. Major features of gel-forming mucins. Mucin monomers are linked together in an oligomeric gel. Each monomer contains cysteines in disulfide-rich domains at the termini and interspersed along the fiber. Individual mucin fibers are densely glycosylated in PTS domains with glycans negatively charged with sialic acids.

Although cervical mucus can generally be described by these components, it does undergo significant changes throughout the menstrual cycle under the influence of hormones. Due to an increase in estrogen, water content increases to over 97.5% in midcycle ovulatory mucus, with a strong correlation between hydration, viscosity, and

sperm penetrability[58,59]. The level of electrolytes such as sodium is highest at midcycle, resulting a characteristic pattern known as ferning, caused by salts crystallizing around organic matter when mucus dries[60,61].
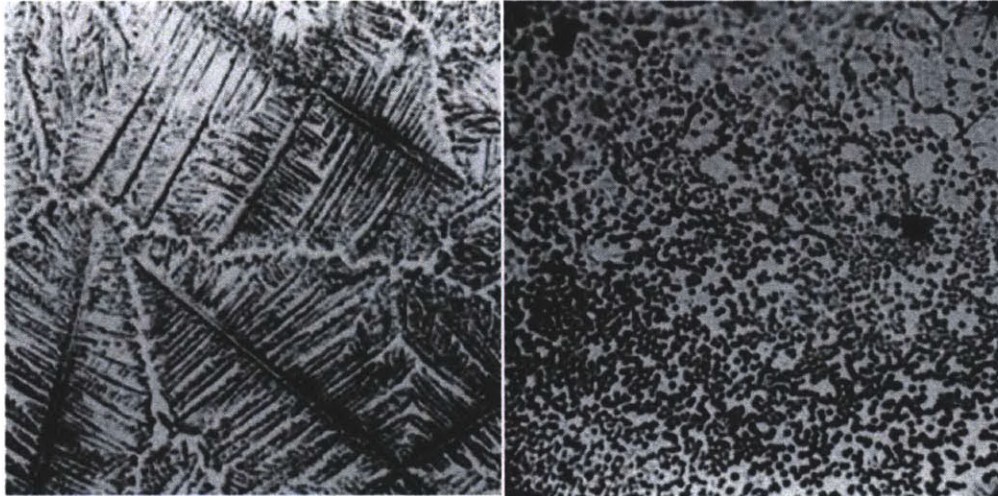
Figure 1-4. Presence (left) and absence (right) of ferning in dried mucus, from Moghissi (2008)[62].

Glycerol, a known lubricant which is also naturally present in cervical secretions, increases in amount during sexual activity[63]. MUC5B concentration is also at a maximum during ovulation, corresponding to midcycle observations of cervical mucus as thin, translucent, less acidic, more abundant, and less viscous than mucus at other times, with a texture similar to egg white[64,65]. Mucus at this time, like egg white, exhibits a characteristic known as spinnbarkeit ("spinnability" of a substance), or the ability of a liquid to be stretched or pulled into filaments, which has been used as a test for fertility. This mucus is most easily penetrated by sperm; in fact, egg white has been used to successfully improve the results artificial insemination in bovine experiments[65].

After ovulation, under the influence of progesterone, cervical mucus becomes scant, thick, acidic, tacky, drier, and more viscous[33,66,67]. It also becomes more opaque,

while ferning and spinnbarkeit are absent[65]. This mucus is recognized as "infertile," as it prevents spermatozoa, which become quickly enmeshed in the mucin glycoprotein network, from entering the uterus[68].

(a) " Other times " of the cycle.
Areas of translucency.

(b) Mid-cycle mucus.   Translucent throughout.

(c) Pregnant.   Homogeneous opacity.

A.

B.
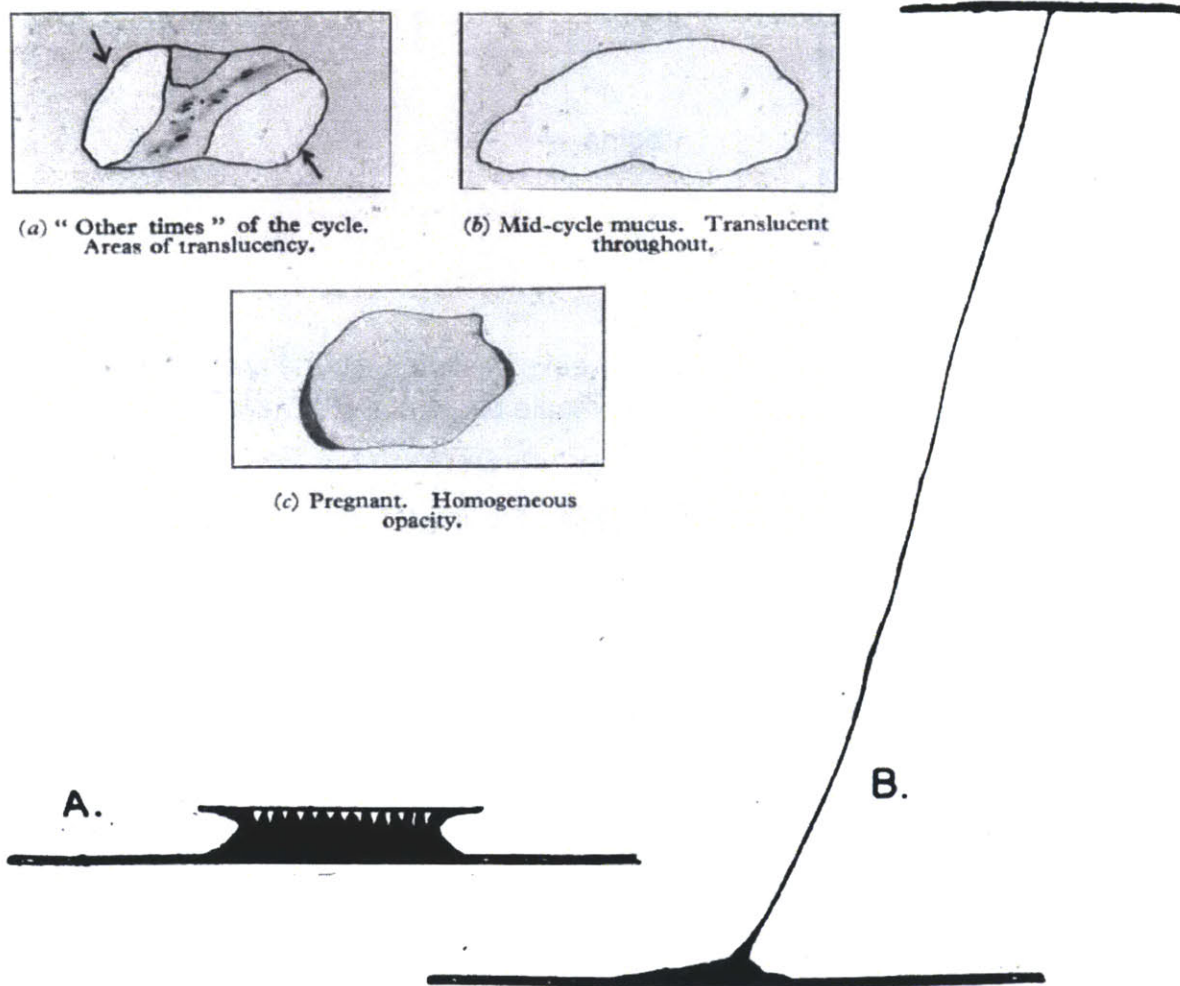
Figure 1-5. (A) Diagram of tack, typical in pregnancy cervical mucus). (B) Diagram of spinnbarkeit, normally seen in mid-cycle cervical mucus. Inset: Representation of macroscopic appearance at different times, from Clift (1945)[65].

During pregnancy, these characteristics remain the same or become even more pronounced. The mesh size decreases to form a dense protective cervical mucus plug that sterically or otherwise prevents microbial ascension, acting as a barrier between the vagina and the sterile intrauterine environment[69,70].
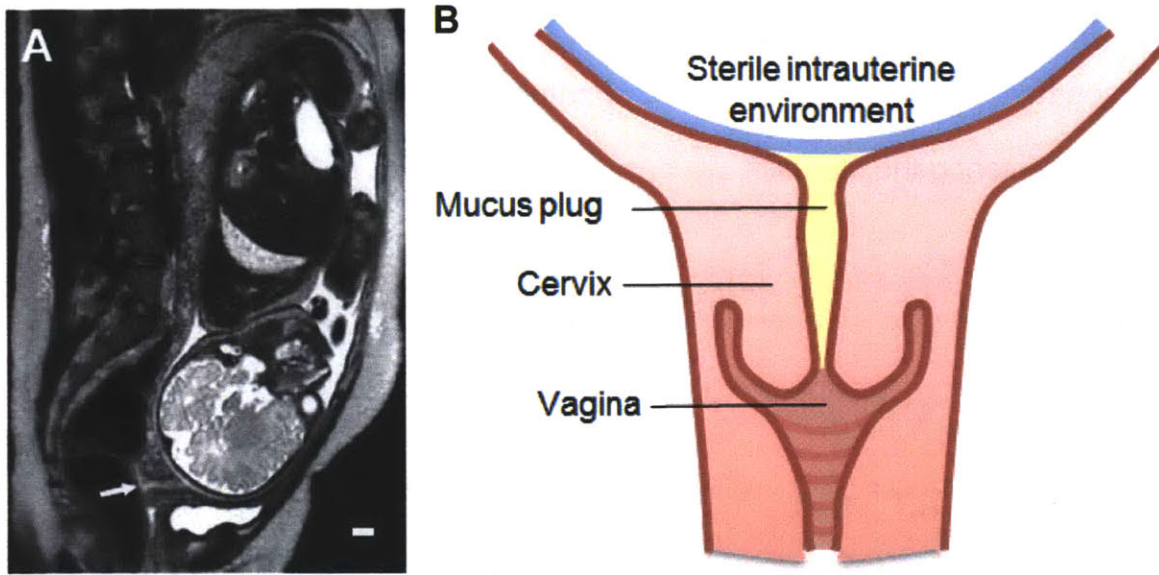
Figure 1-6. (A) MRI image of woman at 34 weeks gestation, courtesy of Dr. Michael House. Arrow points to cervical mucus plug. Scale bar, 1 cm. (B) Diagram of cervical mucus plug.
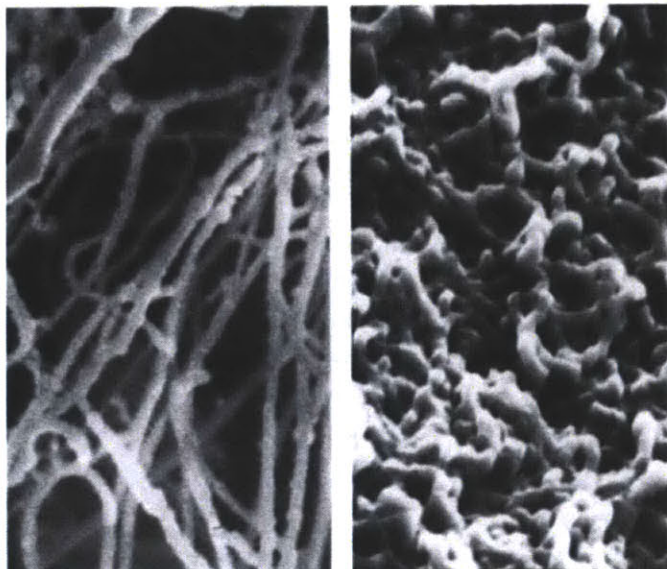
Figure 1-7. Scanning electron microscope images of cervical mucus during the last week of pregnancy, from Chrétien (1978)[70].

In rare pregnancy cases such as the one on the left, areas of heterogenous mucus presented a "typical preovulatory aspect," with long and smooth filaments. 13000x.

In most cases, mucus framework appears clotted and extremely dense, as on the right. 10800x.

Although pore size and arrangement may affect pathogen transport, the mucin fibers themselves also participate in the barrier function, making interactions with foreign particles so that even viruses smaller than the average mesh size move significantly more slowly compared to non-mucoadhesive particles of the same size[71,72].

The normally protective nature of the mucus and the occurrence of preterm birth lead to several open questions about whether the barrier properties of cervical mucus are different between women in healthy pregnancies and women who are at high risk for preterm birth. One such question is what accessible methods can be used to determine high risk in women? Here, we looked at 4 assays to discover if they could quantify differences in cervical mucus, starting with antimicrobial properties to see if low risk mucus had more biological activity against bacteria than high risk mucus. We furthermore examined the macro-rheology of the mucus in two ways: extensional properties and shear properties, which in addition to having the possibility to be easily employed in clinic, are also relatively well-studied in mucus in the context of fertility and could provide valuable insight. Finally, we inspected the actual permeability properties to get the most direct readout of whether high risk mucus would actually let more foreign material pass through it than low risk mucus. Together, these properties may help stratify women based on their risk level, which can lead to more personalized care.
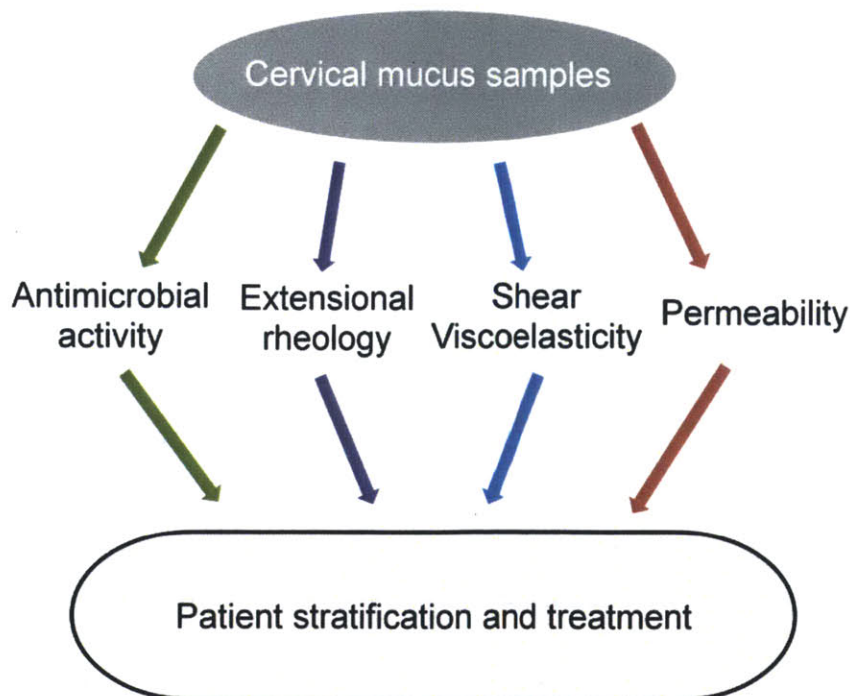
Figure 1-8. Overview of experimental strategy for investigating properties of cervical mucus.

Chapter 2: Study Group

The objective of this study was to identify and quantify differences between mucus from women at high risk of preterm birth and mucus from women having healthy pregnancies at the same gestational age, which may suggest a mechanism for the etiology of preterm birth. Women at high risk were classified as such if they were hospitalized because of preterm labor and had preterm cervical dilation (3.6 ± 1.1 cm).

2.1 Demographics

Cervical mucus samples were collected at Tufts Medical Center by Agatha Critchfield, M.D., and I was blinded to all demographic characteristics before testing samples. Interestingly, though African Americans are statistically more vulnerable to preterm birth, we did not happen to enroll any high risk patients in that demographic.

Table 1. Patient characteristics

Characteristic	High Risk (n = 22)	Low Risk (n = 24)	P – value
Age (Years)	27.18 (+/- 6.5)	29.4 (+/- 6.0)	0.05
Previous pregnancies	2.7 (+/- 1.6)	2.6 (+/- 1.8)	0.73
Previous deliveries	1.0 (+/- 0.9)	1.0 (+/- 1.1)	0.60
Race (%)			
White	33	54	
African American	0	16	
Hispanic	45	22	
Other	22	16	
Gestational Age (wk)	30.7 (+/- 3.3)	30.2 (+/- 3.4)	0.57
Dilation (cm)	3.4 (+/- 1.2)	0.04 (+/- 0.2)	<0.01
Prior Preterm Birth (%)			NS
Yes	22	20	
No	78	80	
GBS status (%)			NS
Positive	27	16	
Negative	73	84	
Amt Mucus collected (µl)	247.7 (+/- 123.9)	178.2 (+/- 59.9)	0.02

*Shown as Mean (+/- SD) or percentage; GBS = Group B Strep infection; NS = not significant

2.2 Exclusion Criteria

Due to the complexity of preterm labor etiology, we used a number of inclusion and exclusion criteria to limit the possibility of confounding variables. A patient had to have a singleton pregnancy, be between the ages of 18-50, and have a gestational age of 20 weeks to 33 weeks and 6 days. Preterm labor patients had to have documented cervical change and be dilated more than 2 centimeters, while control patients were at the same gestational age and not experiencing cervical change. Patients could not have significant medical conditions predisposing them to preterm labor, such as gestational diabetes, hypertension, collagen disorder, systemic infection, etc.

Additionally, they must have never smoked or had any history of drug abuse, abnormal Pap smear, or cervical procedure. A recent (within 6 months) vaginal or urinary tract infection would disqualify a patient, as would amniocentesis, or chorionic villus sampling within 4 weeks, and intercourse or a bimanual or speculum exam within 48 hours. At the time of sample collection, if rupture of membrane or vaginal bleeding occurred, the sample would not be used. Placenta previa (when the placenta grows in the lowest part of the uterus instead of at the top), progesterone use in the current pregnancy, and current antibiotic use were also all exclusion criteria.

2.4 Sample Collection

If a qualified patient consented, cervical mucus samples were obtained by sterile speculum exam using a CooperSurgical Endocervical Curette with a 12cc Vacu-Lok Syringe placed at the external cervical os (opening). Cervical mucus samples not used within 2 days were snap frozen in liquid nitrogen and stored at -80° Celsius.

Chapter 3: Antimicrobial Activity

Human cervical mucus has been shown in some cases to have antimicrobial activity[73,74,75]. Lysozyme has been proposed as an important factor that causes immune bacteriolysis: in one study, solubilized cervical mucus samples containing the enzyme were able to inhibit growth of *Micrococcus lysedeikticus*[73]. In a similar study, cervical mucus plugs from healthy women in labor were able to completely inhibit growth of *Staphylococcus saprophyticus, E coli,* and *Pseudomonas aeruginosa* and partially inhibit *Enterococcus faecium, Staphylococcus aureus, Streptococcus pyogenes,* and Group B Streptococcus[74]. Another experiment showed that both plugs and their aqueous extracts exhibited antibacterial activity toward Group B Streptococcus, and to a lesser degree, *E. coli* and *Candida albicans*, suggesting that secreted proteins such as leukoprotease inhibitor, lysozyme, lactoferrin, and neutrophil defensins were present at high enough concentrations to be considered antimicrobial factors[75]. In light of these studies, we decided to test the antimicrobial activity of preterm labor mucus samples.

3.1 Assay Design

A growth inhibition assay was designed by spreading Group B Streptococcus, one of the most common bacteria implicated in preterm birth, across the surface of an agar plate using glass beads. A small hole about 1 cm in diameter was then made in the center of the agar. The hole was filled with 25 µL penicillin/streptomycin (10%) for the positive control, phosphate buffered saline (PBS) as the negative control, or mucus sample, and the plates were incubated at 37°C overnight. The next day, the diameter of the zone of inhibition was measured.

3.2 Results

Unfortunately, neither high risk samples nor low risk samples showed any inhibition compared to the positive control, which had a zone of inhibition with a diameter of 2.5 cm. Sonification (3 x 10 seconds, with 10 second pauses in between) was tested on subsequent samples, to liquefy the mucus and possibly allow for more release of antimicrobial molecules, but the results were the same. Additionally, contamination was seen on several plates, most likely from native bacteria lodged in the cervical mucus.

A

B

High risk (n=2)	Low risk (n=2)	Pen/Strep	PBS
0	0	2.5	0

C

25 µL Pen/Strep　　　25 µL PBS　　　25 µL Cervical Mucus
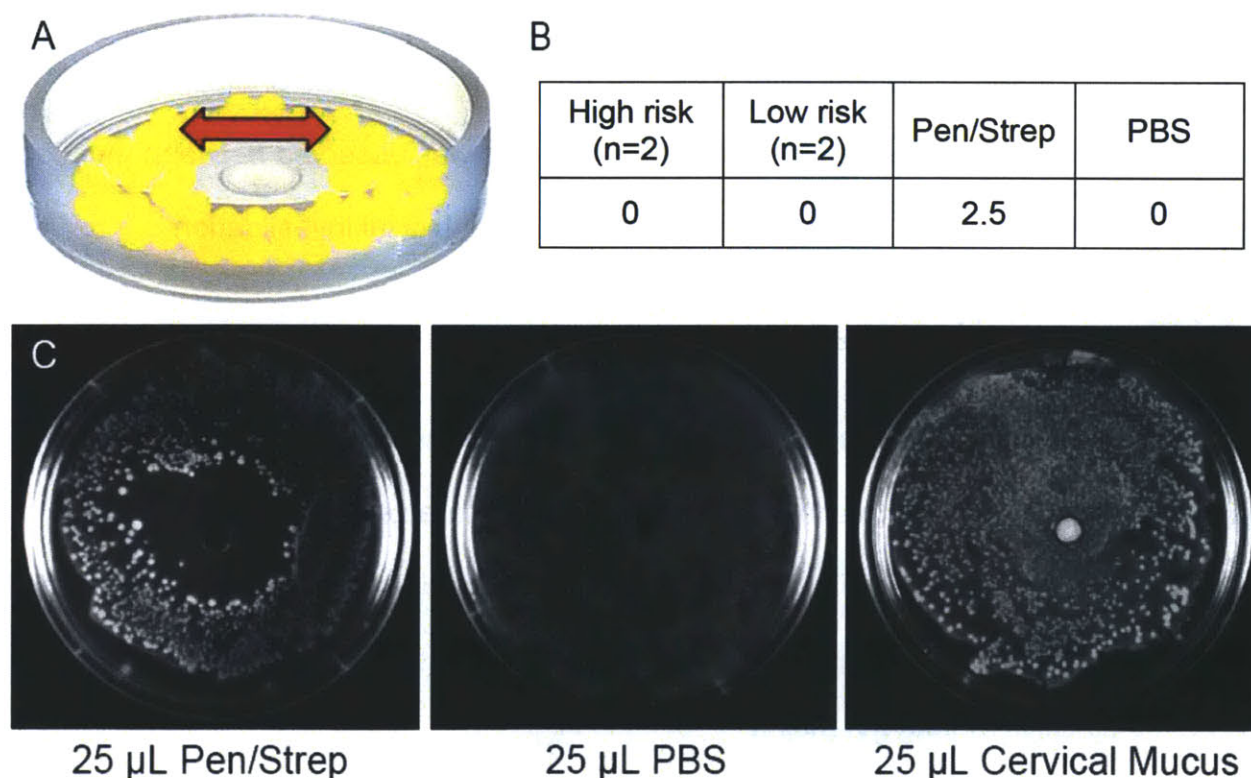
Figure 3-1 (A) Design of assay for antimicrobial activity. Bacteria were spread on an agar plate and cervical mucus was placed in the hole at the center. Red arrows indicate zone of inhibition. (B) Measured zone of inhibition diameters in cm. (C) Representative images of each condition.

Chapter 4: Rheology

Rheology, or the study of the flow and deformation of materials, describes two properties: viscosity and elasticity. Viscosity is resistance to flow – for example, honey or molasses are highly viscous, and difficult to pour out of a bottle. Elasticity, on the other hand, refers to ability to return to the original shape after deformation. After being stretched, a rubber band will return to its original conformation, unless the strain caused it to surpass its elastic limit, in which case it would undergo plastic (non-recoverable) deformation. A viscoelastic material exhibits both properties.

Rheology has been studied in cervical mucus since the 1930's, but attention has been focused greatly on fertility, with very little interest in the context of preterm labor. However, a marked difference in mechanical properties was observed between mucus samples from the two groups which merited detailed rheological investigation.

4.1 Extensional Rheology

Extensional rheology is the study of flow and deformation originating from pulling on a material, as opposed to shear rheology, which is characterization of flow and deformation resulting from a simple shear stress. In the course of this experiment, we identified and quantified spinnbarkeit, an interesting characteristic in cervical mucus that describes elasticity during extension.

4.1.1 Capillary Breakup Extensional Rheometer

A disparity in elasticity was postulated to be the main difference between the two sample groups, and we investigated it using a Capillary Breakup Extensional

Rheometer (CaBER) with ThermoHaake software[76]. Similar to a "filancementer" used to study spinnbarkeit of respiratory mucus, the CaBER draws a material apart into a filament at a fixed rate for rheological observation[77]. 200±100 µL (viscoelasticity caused great difficulty in getting a specific amount) of mucus sample was placed between two circular metal plates that were 6 mm in diameter, with an initial gap of 2 mm. Plates were then separated to a distance of 20 mm at a constant rate of 3.6 mm/s, and the separation distance at which the sample broke was recorded.

4.1.2 Spinnbarkeit Measurements

Healthy pregnancy mucus samples had a breaking length of 13.6 ± 2.3 mm. In contrast, most mucus samples from high-risk women remained intact at 20 mm after plate separation and clearly displayed spinnbarkeit, which should be present in ovulatory mucus but absent in pregnancy. One case in which the mucus did not stay intact could be the result of variation in the cause of preterm labor.

Additionally, while mucus from high risk women had a texture resembling raw egg white and was partially translucent, control mucus from low risk women was paste-like and homogeneously opaque, lacking spinnbarkeit. In two recorded cases (see images of samples 43 in the following figure, and sample 41 in Appendix) and several separate observations, low risk mucus was more easily separated into two parts; i.e. after the extension, part of the mucus would be on the top plate, and part would be on the bottom plate. In two other cases (samples 45 and 47, see Appendix), all the mucus ended up on the bottom plate, but these cases also contained a small amount of blood (around 10% of the total sample), which may have affected overall mucus properties.
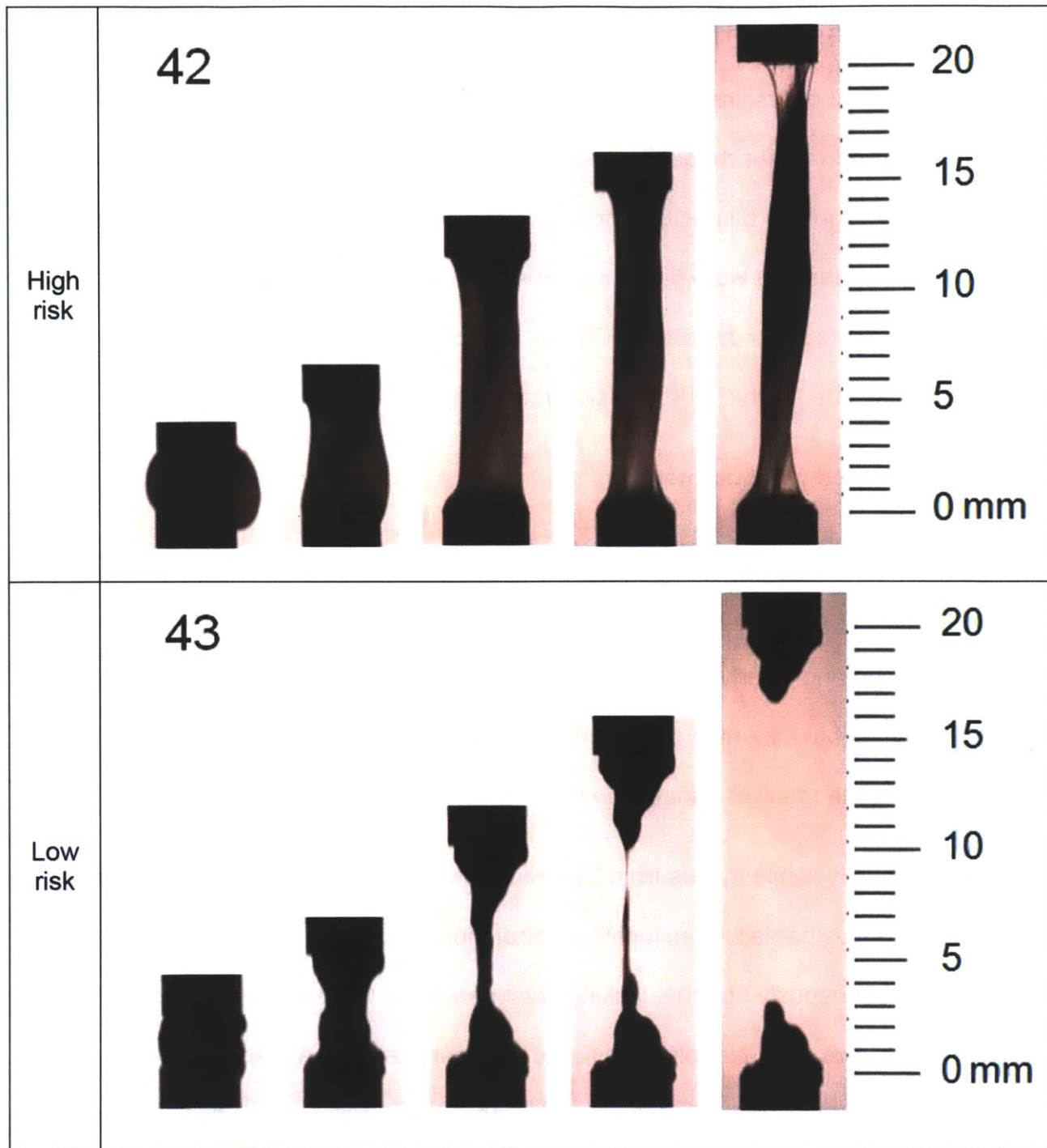
Figure 4-2. Time series of spinnbarkeit test at 2, 5, 10, 15, and 20 mm. Almost all high risk samples could be stretched to at least 20 mm without breaking. In contrast, mucus from low risk patients had an average break length of 13.6 ± 2.3 mm. Times series of remaining samples can be found in the Appendix.

4.2 Shear Viscoelasticity

In addition to extensional rheometry, we investigated viscoelasticity using a rotational shear force, measuring G' (storage modulus, quantifying the elastic, solid-like, recoverable property of a substance) and G" (loss modulus, quantifying the viscous, liquid-like, non-recoverable property). A perfectly viscous material would have G'=0, while a perfectly elastic material would have G"=0. These variables will reveal the macro-rheological properties of cervical mucus, which have been previously studied during the menstrual cycle: in non-pregnant women, viscosity and elasticity have been correlated with success of cervical mucus as the first line of defense against infections of the upper genital tract by vaginal flora[78,79]. Therefore, it is likely that there will be a difference in rheological properties of high risk and low risk mucus.

4.2.1 Parallel Plate Rheometer

In a TA instruments ARG2 parallel plate rheometer, about 75 µL of mucus was placed in a 1.5 mm gap between an 8 mm diameter steel plate, and a Peltier plate whose temperature was controlled at 25°C.

Upper rotational plate

Cervical mucus

Lower stationary plate
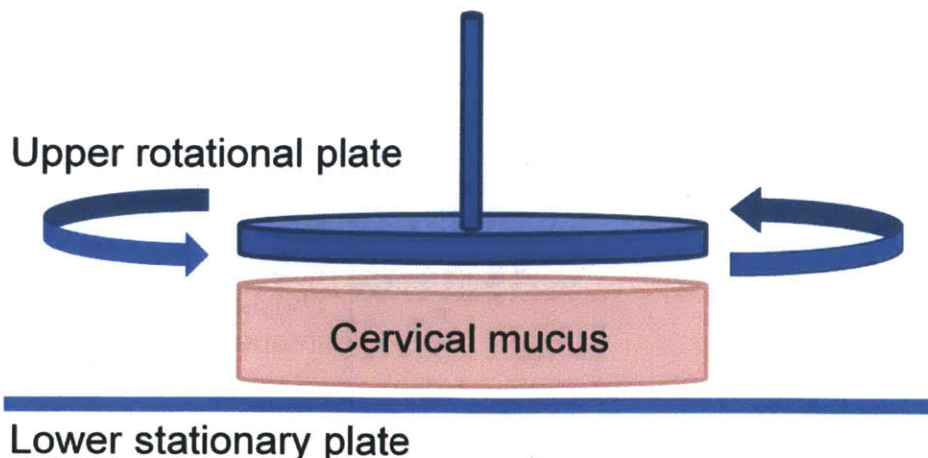
Figure 4-3. Diagram of parallel plate rheometer.

Again, due to the viscoelasticity of the mucus, it was difficult to get an exact amount loaded, but after lowering the upper plate, excess mucus around the sides would be removed with a spatula. During the test, the oscillating rheometer applied a time-varying sinusoidal stress to the mucus, resulting in periodic deformation of the material which was measured. The computer processed this responding strain into an output of several parameters, including G' and G".

4.2.2 Viscoelastic Spectra

It was necessary to ensure that the strain amplitude at which we oscillated the upper plate was in the linear viscoelastic regime for both mucus samples by first performing a strain sweep with a fixed frequency. This helped guarantee that we were examining mucus in as close to its native form as possible, with little breakdown of overall structure. Looking at when G' and G" started dropping off quickly, we saw that the yield stress, or the amount of stress applied that will cause the polymer to deform plastically, was about 3%. Thus a strain amplitude of 1% in the middle of the linear regime was chosen to be the fixed strain.
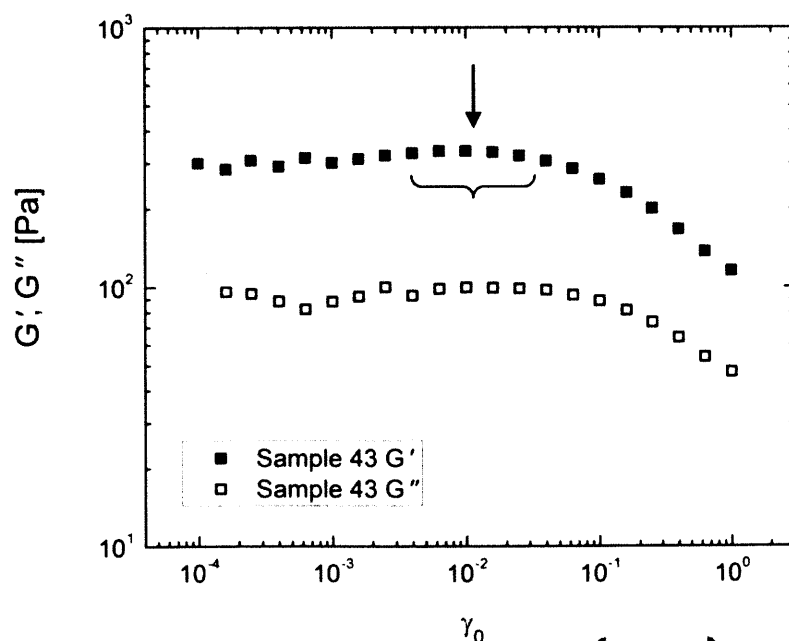
Figure 4-4. Strain sweep of a representative sample. From within the linear viscoelastic regime marked by the brace between $\gamma_0 = 0.4\%$ to 3%, a fixed strain of $\gamma_0 = 1\%$ was chosen to be used in the frequency sweep.

Next, a frequency sweep was performed with the chosen fixed strain, and the viscoelastic moduli were measured under small-amplitude oscillatory shear at applied frequencies from $\omega = 0.5$ to 12.56 rad s^{-1}.

It should be noted that these measurements were made after subjecting the same samples to the spinnbarkeit test, since so little cervical mucus is available. Although rheological measurements are influenced by handling of the material, by eye, the spinnbarkeit procedure did not appear to appreciably affect viscoelasticity and the mucus did not seem to undergo any breakdown of structure. However, again the heterogeneity of some samples was very extensive. A few of the samples seemed to have different phases of paste-like parts and more gel-like portions.

Both high risk and low risk mucus had a higher storage (or elastic) modulus than loss (or viscous) modulus, indicating that they both are more solid-like than liquid-like.
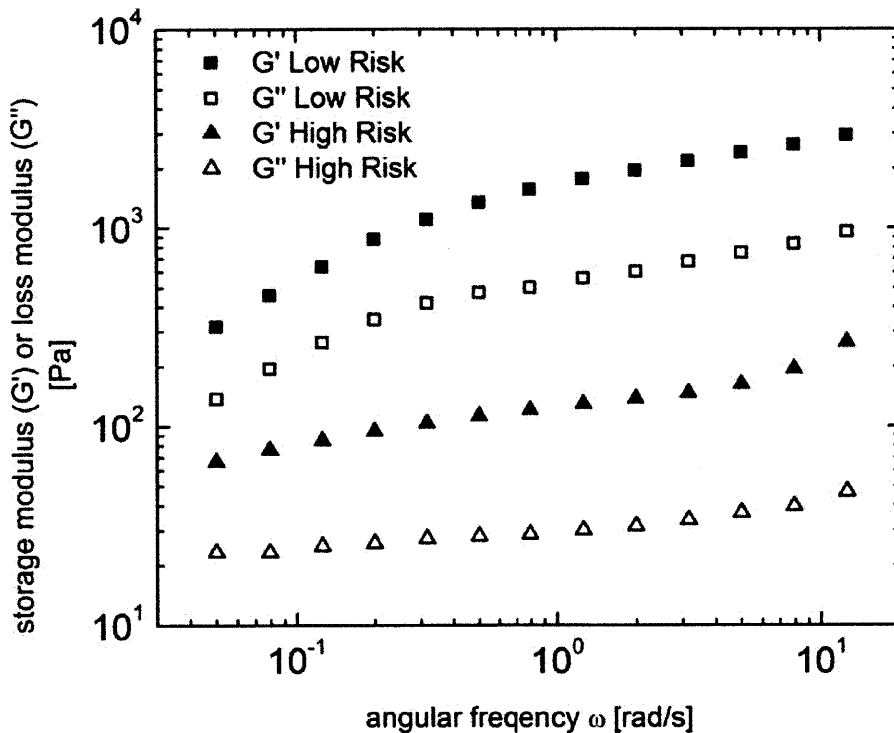
Figure 4-5. Linear viscoelastic spectra of a pair of mucus samples (42 and 43). Storage modulus G' of low risk mucus is an order of magnitude greater than that of high risk mucus, as is loss modulus G", indicating that high risk mucus is more weakly crosslinked than low risk mucus.

The storage modulus of high risk mucus was found to be an order of magnitude lower than that of low risk mucus, suggesting that the molecules forming the gel of high risk mucus are less effectively crosslinked. Interestingly, during ovulation (mid-cycle, about 14 days after menses), the apparent viscosity of mucus is likewise much lower than viscosity at other times of the cycle, further suggesting that mucus in high risk patients is similar to ovulatory mucus and may be more hospitable to incoming pathogenic organisms.
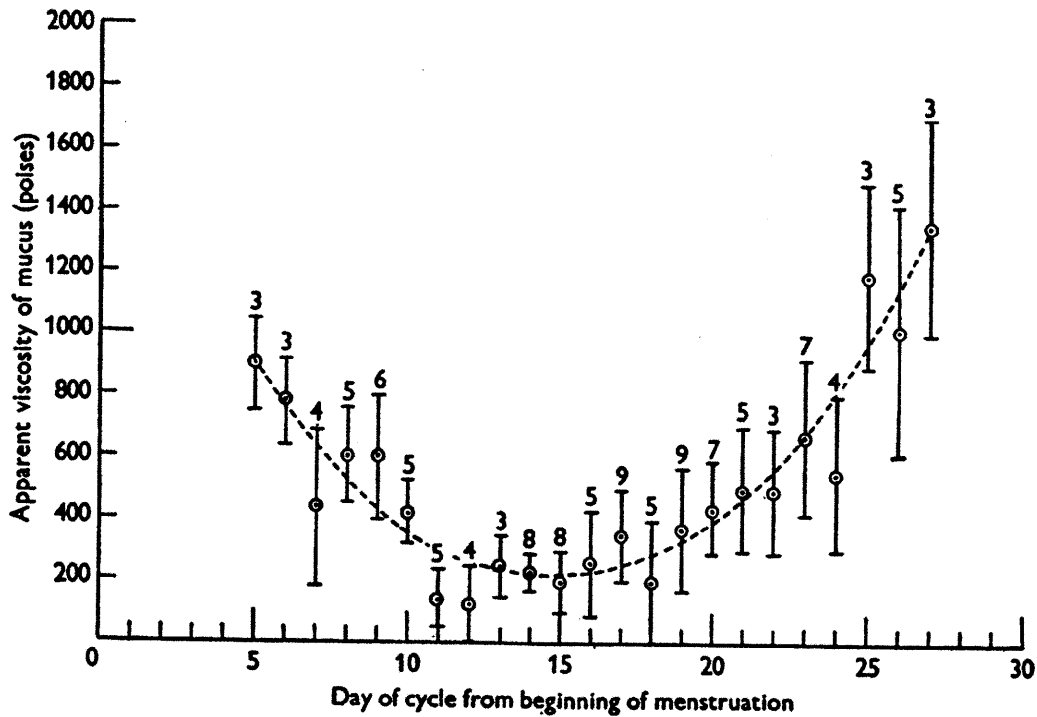
Figure 4-6. Variations in viscosity of cervical mucus from healthy, non-pregnant subjects with day in menstruation cycle. The number above each point indicates number of samples averaged, from Clift (1953)[80].

Chapter 5: Permeability

Drawing a parallel between optical and rheological properties of ovulatory mucus, whose elasticity is correlated with much higher penetrance by spermatozoa than mucus during the rest of the menstrual cycle, and mucus from high risk women, we hypothesized that high risk mucus is more permeable than mucus from low risk women.

5.1 Bioinfectivity

The selective barrier property of mucus can also be considered its microrheology (as opposed to the rheology discussed above, which is really macrorheology). The Ribbeck lab has developed several assays to probe this property and has shown that mucins are able to protect an underlying cell layer from infection, partly by slowing the diffusion of particles such as viruses inside the matrix[81].

5.1.1 Assay Design

To investigate mucus permeability, we first attempted an *in vitro* bioinfectivity assay. HeLa cells were seeded in 96-well plates with DMEM and were confluent the following day. A layer of mucus was spread on top of the HeLa cells, or 1% mucin and HEPES buffer in control cases. Next, 5 μL of virus particles at a concentration of 3×10^{10} particles/mL was dropped on top of the mucus and allowed to diffuse during 2 hours of incubation. The particles were HPV-16 pseudovirions with the endogenous DNA removed and replaced by a GFP expression vector[82]. After the 2 hours, the virus and mucus were washed off the cells, and the cells were grown in media for two days to allow the GFP to express. Cells were then trypsinized, and percentage of infected

(fluorescent) cells was counted by FACS, with a background fluorescence established from uninfected cells.

Figure. 5-1. Diagram of bioinfectivity assay during incubation period, adapted from Vladescu (2012)[83].

MUCUS

EPITHELIAL CELLS
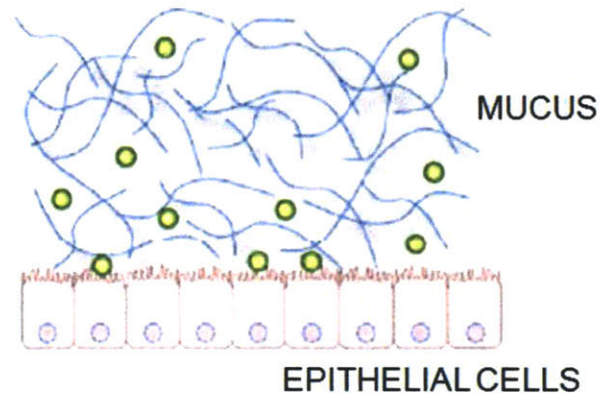
5.1.2 Results

The cervical mucus did prevent more virus particles from reaching the cells compared to the negative control buffer case. Additionally, we saw that snap freezing the mucus and storing them at -80°C overnight did not significantly affect infectivity rate.

H7 (buffer only)...73.3%

1% mucin...16%

cervical mucus...4.7%

frozen cerv.mucus...2.3%
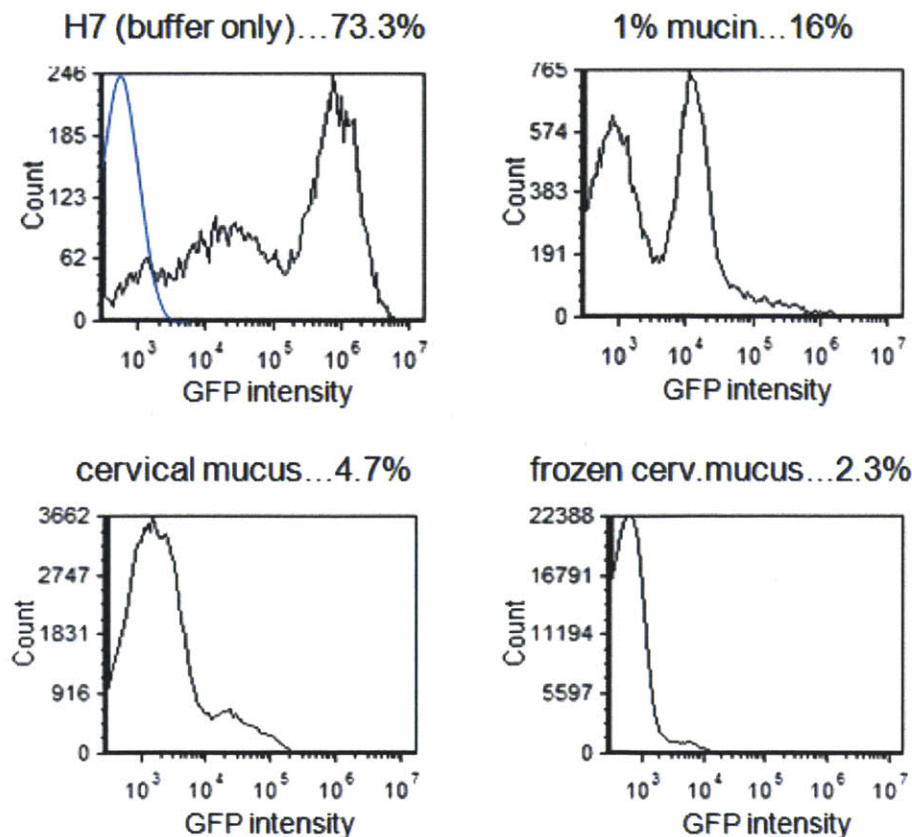
Figure 5-2. Positive control cells, covered with only buffer, were infected at a rate of 73.3%. The blue line indicates background fluorescence measured from uninfected cells. Reconstituted 1% mucin gel lowered infection to 16%, and cells covered with cervical mucus were infected at a much lower rate of 4.7%. Mucus stored at -80° also maintained a very low rate of infection.

5.2 Bead Translocation

Unfortunately, covering the HeLa cells with mucus for 2 hours resulted in adverse effects where the cells would frequently detach. This introduced a variability which was extremely undesirable for long-term use, so we turned to another translocation assay that mimicked the bioinfectivity assay but removed the dependence on HeLa cells, creating a more controlled environment.

5.2.1 Assay Design

A bead translocation assay was performed in triplicate, spreading 25 µL of sample in a well on an Arrayit glass slide that was activated with streptavidin, pre-incubated for 30 minutes in 0.5% BSA to eliminate non-specific binding, and encased in an Arrayit 24-well multiplex microarraycassette[81]. 20 mM HEPES buffer without any mucin was used as a control, and 5 uL Invitrogen biotinylated Fluospheres (0.2 µm) at a concentration of 5×10^6 particles/mL was added on top the mucus or buffer and allowed to diffuse for 2 hours at room temperature.
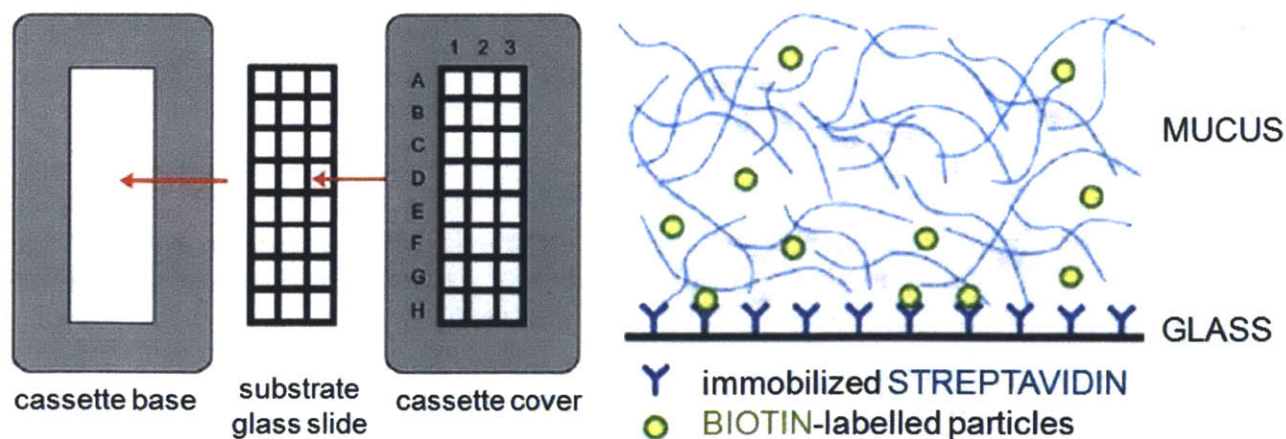
Figure 5-3. Diagram of bead translocation assay set-up (left) and during the incubation period (right). The substrate glass slide is enclosed in the cassette to prevent dehydration of the mucus. From Vladescu (2012)[83].

The glass slides were washed of the mucus three times in washing buffer, with 0.1% Triton-X detergent added to the first washing step. Next, 3 images per well were acquired with a fluorescence microscope at 10x to quantify the number of streptavidin-bound biotin beads that had passed through the mucus to the underlying surface. The mean of the 9 images per sample was taken to represent the number of beads that passed through each sample.

5.2.1 Permeability Measurements

Taking the average of 9 pairs of samples, we found that 2.7 times more beads reached the surface of the slide through high risk mucus than through control samples ($p < 0.01$). In 2 pairs, there was little difference between the two; this could be attributed to the complexity of preterm birth causation. Overall, this set of data suggests that high risk mucus is more easily penetrated by particles such as bacteria than mucus in normal pregnancy conditions.
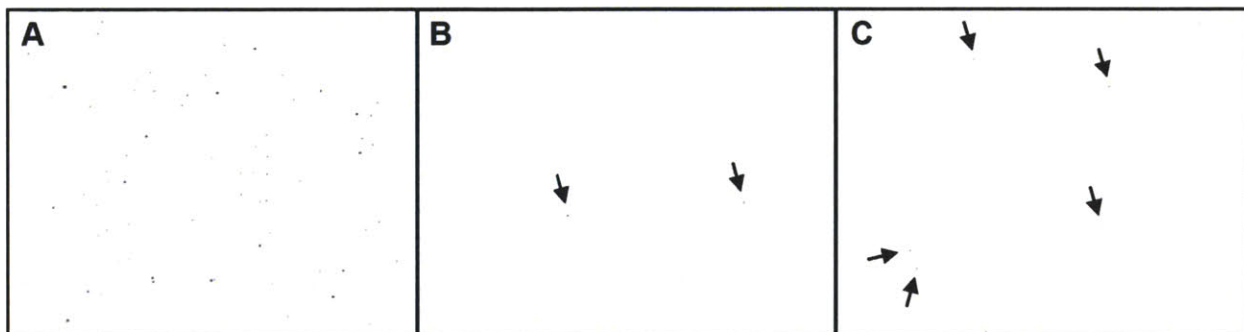
Figure 5-4. Example images of biotin beads on streptavidin slides (color inverted and a portion of microscope field enlarged to 22x for ease of viewing). Detection is difficult due to small bead size, so arrows are shown pointing to bead locations.

(A) Buffer only conditions averaged about 250 beads per field.

(B) This sample of low risk mucus had 1-9 beads per field.

(C) This sample of high risk mucus had 6-15 beads per field.
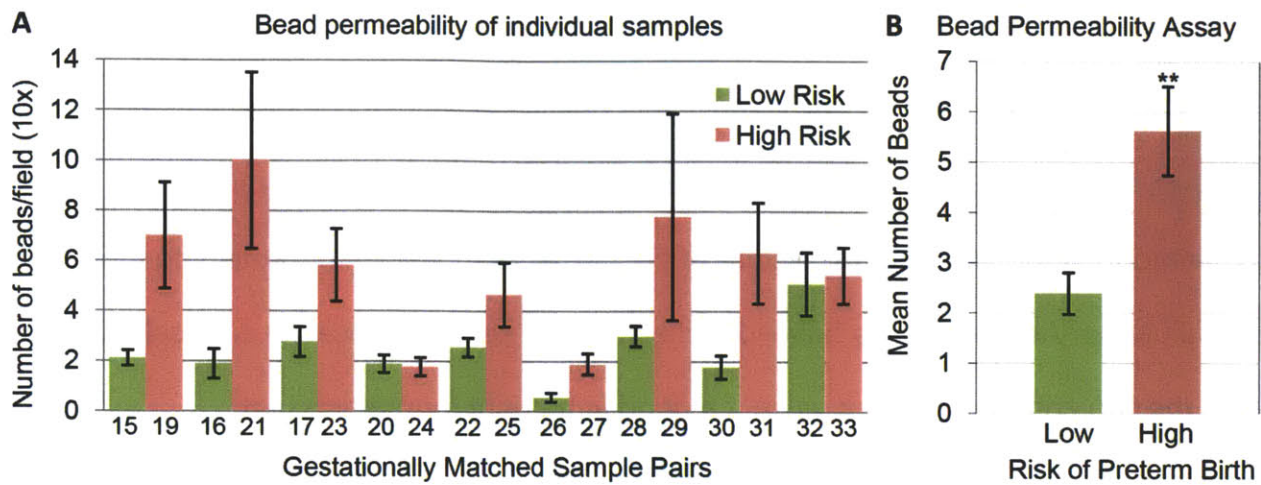
Figure 5-5. (A) Average number of beads per microscope field, with individual samples paired with their controls. Some large standard deviations are present due to the heterogeneous nature of mucus. Numbers along x-axis denote patient number. (B) Number of beads averaged across all samples was 2.7 times higher for high risk mucus than for low risk mucus.

Chapter 6: Discussion and Future Directions

In the course of this project, subjecting native cervical mucus from healthy pregnant women and women who have experienced preterm labor to a series of experiments provided a clearer picture of the barrier responsible for protecting the sterile uterus. As a result, we have identified several key differences in cervical mucus from the two cohorts that may make it a good fingerprint for preterm birth.

One initial difference in cervical mucus between the two cohorts is the optical properties: in a majority of samples, mucus from low risk patients was less heterogeneous and opaque, while high risk mucus was more heterogeneous and translucent when they were spread thinly. Although this finding provided a qualitative view for discussion, this quality is difficult to quantify. Theoretically, a spectrophotometer could be used, but the heterogeneity of both low risk and high risk mucus may prevent accurate measurements. Another challenge was to consider the possibility of contamination by cellular debris and small amounts of blood, which would also interfere with analysis of the samples. Though we attempted to avoid this, it was nevertheless often present in samples and could not be removed without risk of disturbing other mucus properties.

Another disparity between the healthy and high risk group, which is easily quantifiable, is the spinnbarkeit property. An elongational viscosity test on the CaBER showed that the average break length of healthy pregnancy mucus is about 13.6 mm, while high risk mucus does not break even at 20 mm. Spinnbarkeit, which may rely on the molecular arrangement of mucins, helps provide optimum conditions for

spermatozoa penetration during ovulation[70]. Its presence in mucus from pregnant women could be indication that the mucus is not properly blocking bacteria from passing through the cervix. We can speculate that there is a hormone imbalance causing the mucus to be more similar to ovulatory mucus than normal pregnancy mucus, or perhaps there are external signals from foreign organisms changing the mucus properties. Though we cannot say yet whether it is the mucus changing that allows bacteria through, or bacteria causing changes in the mucus making it more vulnerable, we can be sure that there is a difference. A long-term prospective study in pregnant women, keeping track of spinnbarkeit starting from the middle of the second trimester, could be the key to solving this question.

A third property that can distinguish low risk from high risk mucus was found from observations of the viscoelastic spectra. Using a parallel plate rheometer, we saw that storage modulus G' and loss modulus G" are an order of magnitude higher in low risk mucus than in high risk mucus, indicating that the low risk mucus has a higher degree of crosslinking. Together with the spinnbarkeit results, this could imply that high risk mucus is more susceptible to alignment of mucin fibers to the axis of the cervix during ordinary bodily movement, while low risk mucus is so well crosslinked that it cannot be pulled into alignment. This alignment, seen in ovulatory mucus, may serve to guide foreign particles towards the uterus instead of ensnaring them in a dense meshwork[84]. Typically in mucus, a rough calculation of mesh size can be made with the viscoelasticity measurements, estimating the size of the holes or pores in the mucus: when the complex modulus G* (equivalent to $\sqrt{G'^2 + G''^2}$) decreases, as it does in high risk mucus compared to low risk mucus (25 nm versus 10 nm), the effective mesh size increases,

leading to an increase in permeability. However, in these mucus samples, this calculation could be misleading, as mucin fibers are often densely bundled into clot-like formations, resulting in a very skewed distribution of spacing within the mesh. There is little to no spacing within bundles, while between the bundles, there are much larger spaces on the order of 500-1000 nm. Hence, while the effective mesh size seems smaller than a bacterium, the actual viscosity that a bacterium might encounter would probably be closer to that of the water filling the pores. Thus, we wanted to develop a more direct way to measure permeability.

This leads us to the fourth distinction: in the bead translocation assay, about 2.7 times more beads can pass through the high risk mucus than the low risk mucus. Although this may seem like a straightforward and obvious result if we already suspect that one is more weakly crosslinked than the other, mucus permeability is much more complex than pore size. For example, some viruses (HSV, HIV) which are smaller than the average pore size of a mucin network, move much more slowly through the mesh, impeded not by steric hindrance but by the interactions they made with the glycoproteins[85,86]. Additionally, acidic pH improves interactions between particles and charged mucins, causing a more selective barrier, while high ionic concentrations lead to shielding effects on particles and weaken interactions[87]. Thus, seeing a difference in bead permeability between mucus from high risk and low risk patients may imply that there is a fundamental change that brings about the change in crosslinking and the weakened defense.

To take this analysis a step further would require a dissection of the high risk

mucus to look for any differences at the molecular level, possibly looking at the

composition and glycosylation of the mucins by mass spectrometry. This could be done

by separating the mucins, blotting them to polyvinylidene fluoride membranes, releasing

glycans by beta-elimination, and analyzing with HPLC-MS/MS. This method has been

used to identify substantially different O-glycosylation in mucins of cervical mucus

before and during ovulation: neutral oligosaccharides became relatively more abundant

than acidic ones during ovulation[88].
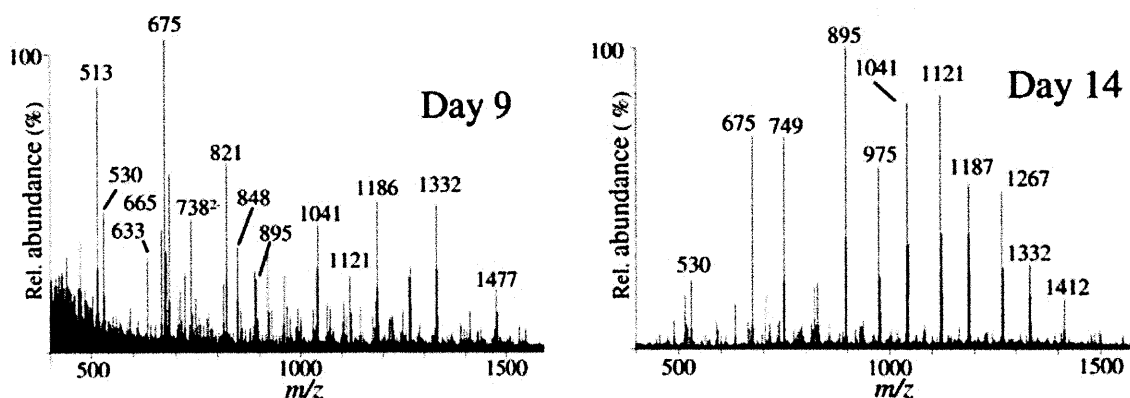
Figure 5-1. Examples of total ion spectra of O-linked oligosaccharides from mucins
from Andersch-Björkman (2007). A clear difference can be seen in mucins collected
before ovulation (Day 9) and during ovulation (Day 14) [88].

Another promising direction for characterizing the differences is by scanning

electron microscope (SEM). In fact, this is the path we are currently taking to visualize

the microstructure of the mucus, following the protocol used to take the images in

Figure 1-7. Fresh samples are fixed on a cover slip with glutaraldehyde, frozen in liquid

nitrogen, dried in a lyophilizer, sputter coated with gold, and imaged[89]. The greatest

difficulty may be preserving the structure of the matrix due to the hydration and salts, as

well as the stress it undergoes during freezing and drying, which may cause changes in

alignment due to shear and flow. Being able to see the glycoprotein mesh would be very

useful in determining how micro-architectural changes are related to preterm birth. Both the elongational viscometry, showing that spinnbarkeit is present in high risk but not low risk mucus, and the parallel plate rheometry, indicating a more weakly crosslinked meshwork in high risk mucus, support the hypothesis that the high risk mucus is compromised in structure. Furthermore, the bead permeability assay seems to confirm the idea that the high risk mucus allows more pathogens to pass through. While optical properties and spinnbarkeit are more easily discernable, permeability is clinically significant, as it can allow for an increased number of microorganisms to harmfully traverse the barrier of the cervical mucus plug. Since the two appear to be correlated, rheology could be used clinically, perhaps even in a bedside setting, to infer mucus permeability properties.

Moreover, all of these traits point to high risk cervical mucus resembling translucent, fertile ovulatory phase mucus instead of opaque, impenetrable pregnancy mucus. In fact, this seems to be the opposite problem of "thick mucus syndrome," in which women who were trying to conceive were unable to, having mucus that "never became transparent and…viscosity remained high," with lack of ferning. In one case, a woman had a normal hormonal profile including the pattern of progesterone, but required estrogen therapy to overcome her infertility[90]. If biomarkers, perhaps related to the hormones involved in the menstrual cycle such as an excess of estrogen or deficiency of progesterone, to indicate the differences in high risk mucus are found, a prospective study with pregnant women could then be done to investigate their predictive power. If preterm labor and birth can be predicted, then measures such as local antibiotics, hormones, and cervical cerclage, could be taken. More importantly,

these biomarkers may provide critical insights in elucidating the mechanism of effect, which can direct the design of better prevention and intervention strategies.
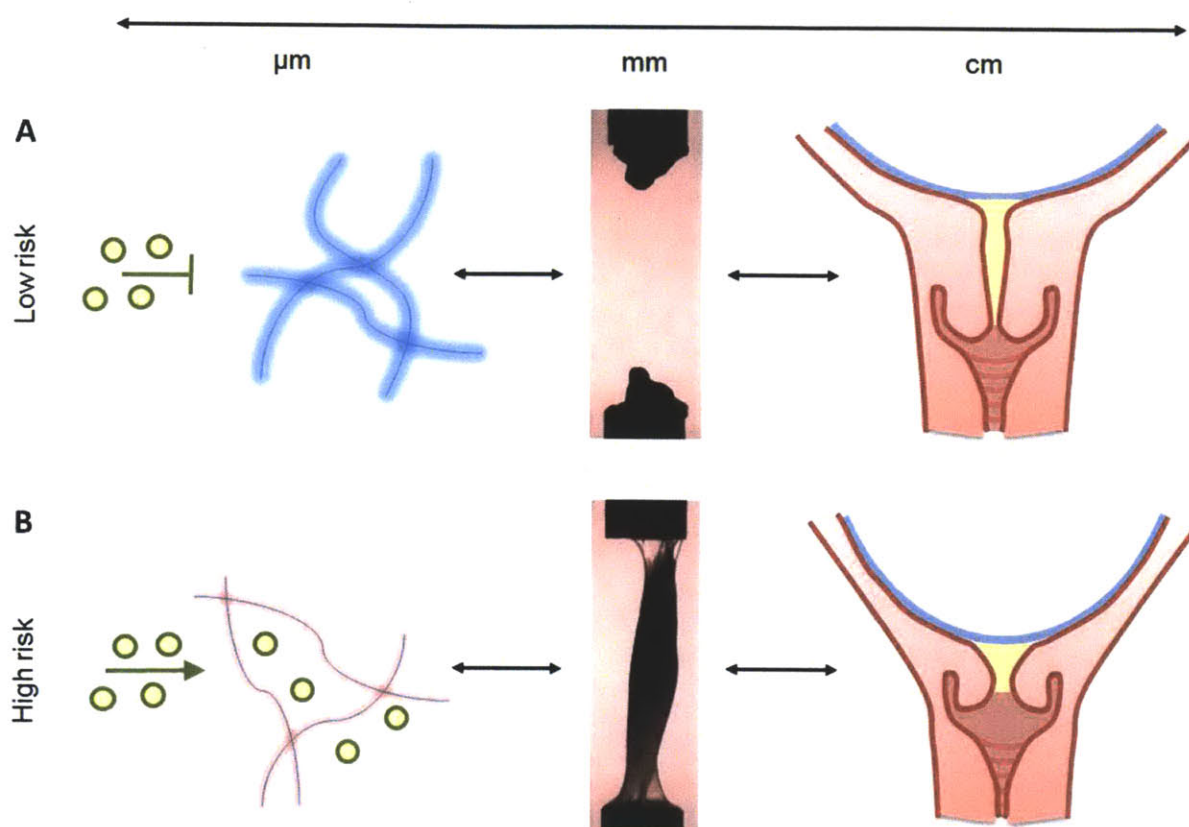
Figure 5-2. (A) Cervical mucus in normal pregnancy should entrap bacteria in the meshwork of mucin fibers. The high degree of crosslinking may also account for the macro-rheological properties such as absence of spinnbarkeit. Effective protection would allow for a normal cervical length. (B) Cervical mucus in a high risk situation may be more permeable to bacteria, since it is not as well crosslinked. This lack of cross-linking may result in alignment of the mucin fibers under strain, which may even help guide the bacteria through the cervix. It might also account for the spinnbarkeit seen in high risk mucus. If bacteria reach the uterus, they can cause an infection leading to shortening of the cervix and eventually preterm birth.

Although there is much more to be discovered about the etiology of preterm birth, this study has revealed that high risk for preterm birth is significantly correlated with cervical mucus being more translucent, extensible, and permeable than it is in healthy pregnancies. The similarity of high risk mucus to ovulatory mucus is alarming since the

latter is intended to allow spermatozoa to easily pass through the cervical canal and is probably more susceptible to microorganisms, while mucus in pregnancy should be an effective gatekeeper against pathogens. The differences we found can help in stratifying patients and lead to personalized prenatal care to lower the rate of preterm birth.

Appendix

Time series of mucus at 2, 5, 10, 15, 20 mm and at break lengths (arrows) if applicable.

High Risk   **Low Risk**
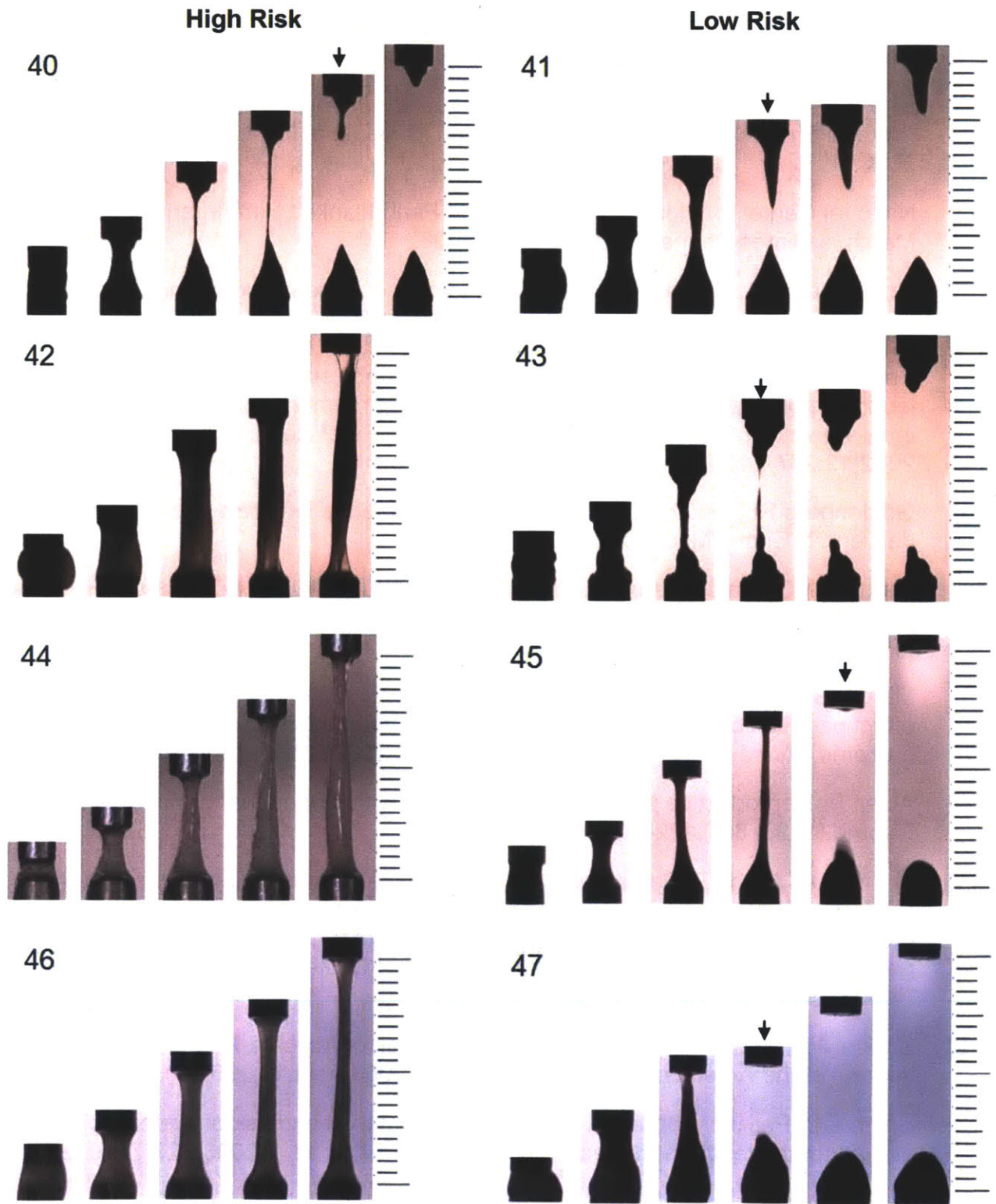
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