# Characterizing the Biomechanical and Biochemical Properties of Mouse Uterine Tissue

**Eli Mondragon[1]\*, Kyoko Yoshida[2], M.S. Kristin Myers[2], Ph.D**
[1]Biomedical Engineering Department, Columbia University, New York, NY 10027
[2]Mechanical Engineering Department, Columbia University, New York, NY 10027

## Abstract

For a successful pregnancy, the uterus and the cervix work together as a biomechanical structure to protect the fetus until term. During gestation, typically 37 weeks, the uterus undergoes a growth transformation to accommodate the growing fetus and to prepare for labor. This uterine growth is characterized by an increase of its wet weight, elastin content, and collagen content. Then at parturition, the uterus must contract while the cervix ripens and dilates to allow the passage of the fetus. The transformation mentioned above is believed to be responsible for the contractions, and any deviations from the expected biochemical transformation put both the mother and baby in danger. The goals of this study are to quantify and compare the biochemical and biomechanical properties of uterine tissue from normal and abnormal mouse models of pregnancy. This study utilizes Anthrax toxin receptor 2 knock-out mice (*Antxr2 -/-),* which exhibit an accumulation of collagen in the cervix and uterus as a result of a defect in the maintenance of their extracellular matrix (ECM). Uterine tissues from non-pregnant *Antxr2 -/-* and non-pregnant wild type mice (*Antxr2 +/+*) were tested. Tissue samples were tested for collagen content, collagen crosslink strength (i.e. collagen extractability) and were subjected to tensile mechanical testing. Results from the biochemical assays revealed that the *Antxr2 -/-* uterine samples had significantly higher levels of collagen. It was also revealed that collagen extractability was region-dependent. Lastly, mechanical testing proved that *Antxr2 -/-* uterine tissue is mechanically stronger than *Antxr2 +/+* (peak stress 0.078 MPa and 0.04 MPa)*.* This study presents one of the first attempts to correlate the biochemical makeup of the uterus to its biomechanical properties.

Key Words: Anthrax toxin receptor 2*; extracellular matrix*

## Introduction

The United States has an annual preterm birth (PTB) rate of approximately 12%, making it the highest among developed nations [5]. Although the causes of PTB remain elusive, cervical insufficiency (CI) is a leading mechanism of PTB [5]. CI is the premature softening of the cervix in the absence of uterine contractions. In addition, a cervix that fails to dilate/ripen at term can be fatal to both the mother and child. These two medical conditions, a "soft" cervix and a "stiff" cervix, highlight the importance of the cervix's dual function. A need for a better understanding of cervical ripening has led to multiple studies on the biomechanical and biochemical properties of cervical tissues [6, 8-10]. However, for a successful pregnancy, the cervix and uterus must work together to retain the fetus during full gestation. Subsequently, at parturition, uterine contractions and cervical ripening occur to allow for the delivery of the fetus through the birth canal. An understanding of the biochemical and biomechanical relationship in uterine tissue is therefore equally as important as the cervix in building finite element models, reducing both the PTB rate and neonatal deaths. Future finite element models will help investigate how the uterus and

the cervix work together during gestation, and will require the input of material properties that can only be acquired through experimentation.

The mechanical properties of the uterus have been previously studied [2,3,11]. Early work done on human myometrium by Conrad, *et al.* has shown that pregnant tissue is more compliant than non-pregnant tissue [3]. More importantly, it has been shown that the connective tissue framework, not the muscle cells of the uterus, is responsible for the mechanical behavior of the organ [2]. Although Conrad, *et al.* conclude that the extracellular matrix (ECM) is crucial for a tissues' mechanical integrity, biochemical analysis was not performed. Other studies have investigated the ECM of uterine tissue, but these were qualitative [12-14]. Briefly, Peters, *et al.* worked with Capillary morphogenesis protein-2 knock-out mice (*CMG2 -/-*) and Reeves, *et al.* with *Antxr2 -/-* mice. Similar to the *Antxr2 -/-* mouse model, *CMG2 -/-* mice are characterized by the accumulation of collagen in the cervix and uterus. Both researchers used H&E (hematoxylin and eosin) and Masson's trichrome stains to reveal the accumulation of collagen in the uterus of their respective mouse model [12,13]. Spiess, *et al.* showed the distribution of different types of collagen (I, III, and V) in the endometrium of pregnant mice using immunofluorescence and confocal imaging [14]. Zhao, *et al.* also investigated the ECM of mouse uterine tissue, but their results are more quantitative. Zhao, *et al.* showed that both pregnant relaxin knock-out mice (*rlx -/-)* and pregnant wild type mice (*rlx +/+)* contain 6% of dry weight of collagen in their uterine tracts [16]. These studies on the biochemical makeup of mouse uterine tissue had no biomechanical information [12-14,16].

Previous studies have correlated biochemical makeup to biomechanical properties in other load-bearing soft tissues, but few studies have correlated biochemical variables to uterine mechanical properties. In particular, analysis of mutant and non-mutant mice Achilles tendon has shown that alteration of the ECM manifests in changes to its biomechanical strength [7]. This study aims to connect the biochemical composition of the uterus to its mechanical properties. Due to the difficulty in attaining human uterine tissue, mouse tissue was used throughout this research. The advantage of using mouse models is twofold. They provide timed pregnancy samples as well as an opportunity to investigate unique genetic models that may alter the maintenance of the ECM. For example, *Antxr2 -/-* mice experience a loss in both longitudinal and circular myometrial cell layers, causing inadequate uterine contractions for delivery [13]. As the *Antxr2 -/-* mice age, the cervices and uteruses thicken and accumulate collagen due to a defect in ECM homeostasis. Reeves, *et al*. have shown that the consequence of faulty ECM maintenance in Antxr2 -/- mice, morphologically different reproductive tracts, can be seen once the mice reach sexual maturity (3 months) [13]. The accumulation of collagen also leads to failed parturition. The nature of these effects, the abundance of collagen, provides the opportunity to investigate the relationship between the biochemical and biomechanical properties of the tissue (i.e. structure-function properties).

To determine uterine structure-function properties, collagen content, collagen extractability (relative strength of the collagen network), and tensile strength of uterine tissue specimens from both *Antxr2 -/-* and *Antxr2 +/+* non-pregnant mice were measured. *Antxr2 -/-* uterine tissue was found to have significant higher levels of collagen than *Antxr2 +/+*. Specific regions of *Antxr2 -/-* uterine tracts had significantly lower levels of extractabilities than *Antxr2 +/+* tissues, indicating *Antxr2 -/-* has a stronger collagen network (*25 ± 5 %* and 4*5 ± 4 %*). Lastly, *Antxr2 -/-* uterine tissue displayed stiffer properties than *Antxr2 +/+* (peak stress 0.078 MPa and 0.04 MPa).

## Methods

### Sample Preparation

All procedures followed the NIH Guide for the Care and Use of Laboratory Animals. All mice sacrificed were between 3-4 months old and were in the diestrus stage of their estrous cycle. The uterine tracts of 7 non-pregnant Antxr2 +/+ and 4 non-pregnant Antxr2 -/- were dissected from sacrificed mice. The two uterine tracts of the mouse were labeled Left (L) and Right (R), with the junction being evenly divided between the two (Figure 1A). L horns were used for mechanical testing and the R horns were used for biochemical assays. Once dissected and labeled, the uterus was separated at the junction of the two horns. The ovaries were left intact to determine orientation. Both horns were sliced open to expose the inner lining. While the L horn was immediately stored away in a -80 ºC freezer, the R horn was divided into regions A, B, C, and D, where region A is the furthest from the ovary (Figure1B). The samples were then stored in a -80 ºC freezer.
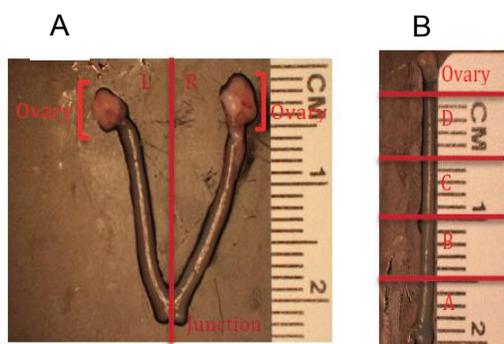


**Figure 1**: **A** Uterine tract of *Antxr2 +/+* is divided into horns R and L. **B** Division of an *Antxr2 +/+* R horn into regions A, B, C and D

### Biochemical Analysis

All tissue samples were homogenized prior to testing by flash freezing and pulverizing each sample using liquid nitrogen. The wet weights of all regions after pulverization were measured. The dry weights of all regions were measured after freeze-drying. The hydration of a sample is defined as the fraction of the difference between wet weight and dry weight over the wet weight. The equation for hydration is

$$\frac{Wet\ Weight - Dry\ Weight}{Wet\ Weight} \tag{1}$$

All regions, A through D, were assayed for collagen content using a standard hydroxyproline assay described in Myers, *et al* [8]. Briefly, a colorimetric procedure determined hydroxyproline content. Collagen content was then obtained by using a mass ratio of hydroxyproline to collagen of 1:7.47, and normalizing by the dry weight of the tissue.

Collagen extractability was measured for regions A and D. The collagen extractability of a tissue determines the solubility of the collagen network. It was measured by extracting the pulverized tissue in 0.5 M acetic acid with 0.5 mg ml$^{-1}$ of pepsin (150 µl mg$^{-1}$) for 3 days at 4 ºC. After centrifuging the samples for 1 hour at 15,000g, the tissue pellet and the supernatant were separated. The hydroxyproline content was measured in both the supernatant and pellet. The extractability of a sample is defined as the fraction of hydroxyproline in the supernatant compared to the total amount of hydroxyproline. The equation for extractability is

$$\frac{OH\ Pro_{sup}}{OH\ Pro_{sup} + OH\ Pro_{Pellet}} \tag{2}$$

### Biomechanical Analysis

All mechanical tests were conducted on a universal mechanical testing machine (Instron Micro Tester 5948, Norwood, MA) equipped with a 5 N load cell and a custom fluid chamber. Two perpendicular CCD cameras (Point Grey Grasshopper, GRAS-50S5M-C 75mm f/4 lens) were used to capture tissue deformation. Immediately before testing, the L horns were thawed in PBS for approximately 30 minutes. Samples were clamped into custom-made tensile jaws using sand paper and cyanocrylate to ensure a tight grasp. All samples were oriented such that the outer lining faced the same front camera. Forceps were used to avoid

misalignment and the formation of wrinkles. Prior to loading, each samples was airbrushed with India ink to produce a speckle pattern for tracking purposes (Figure 2A). Once the ink dried, each sample was loaded under a regimen that included three load-unload cycles to 5% engineering strain with a 5-minute re-equilibration time between cycles. These cycles were followed by four ramp-hold tests. The four engineering strain levels were 10%, 15%, 20%, and 30%, each was held for 45 minutes. An engineering strain rate of 0.5% s$^{-1}$ was used for all loading. See Figure 2B for an illustration of the full loading regimen. During testing, images

of the tissue deformation were taken at a frequency of 0.2 Hz.
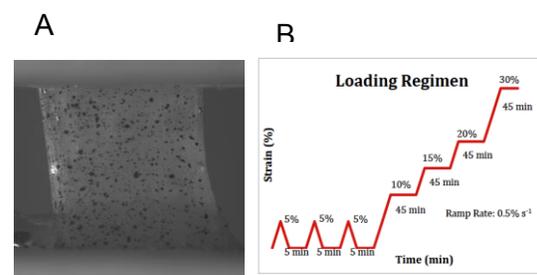


**Figure 2**: A. Speckle pattern on uterine horn
B. Loading regimen for tension test

## Results

### Biochemical Composition

The hydration levels for both *Antrxr2 +/+* and *Antxr2 -/-* mice uterine samples were 76 ± 11% and 75 ± 10%. A Student's t-test revealed that were no significant differences in hydration levels between regions in *Antxr2 +/+* and *Antxr2 -/-* mice (p>0.05). In addition, hydration levels were not significantly different between *Antxr2 -/-* and *Antxr2 +/+* samples. (Figure 3A). It was found that collagen makes up 29 to 37% of *Antxr2 -/-* uterine tract (n=4), and only 12 to 17% of *Antxr2 +/+* uterine tract (n=7). A Student's t-test showed that the collagen content levels in all regions of the *Antxr2 -/-* samples were significantly higher than their *Antxr2 +/+* counterparts (p<0.05) (Figure 3B). Collagen extractability is region specific in the case of *Antxr2 -/-.* A Student's t-test also showed that region D of the uterine tract from *Antxr2 -/-* mice had significantly lower levels of extractability than region D of *Antxr2 +/+* samples (Figure 3C) (p<0.05). The differences in extractability between regions A and D from *Antxr2 +/+* and *Antxr2 -/-* samples were not significant.

**Figure 3**: **A** Uterine hydration levels were not significantly different between *Antxr2 -/- and Antxr2 +/+,* **B** Collagen content in all regions was significantly higher in *Antxr2 -/-* mice, **C** Region D for *Antxr2 -/-* was significantly less extractable than region D of *Antxr2 +/+* mice.
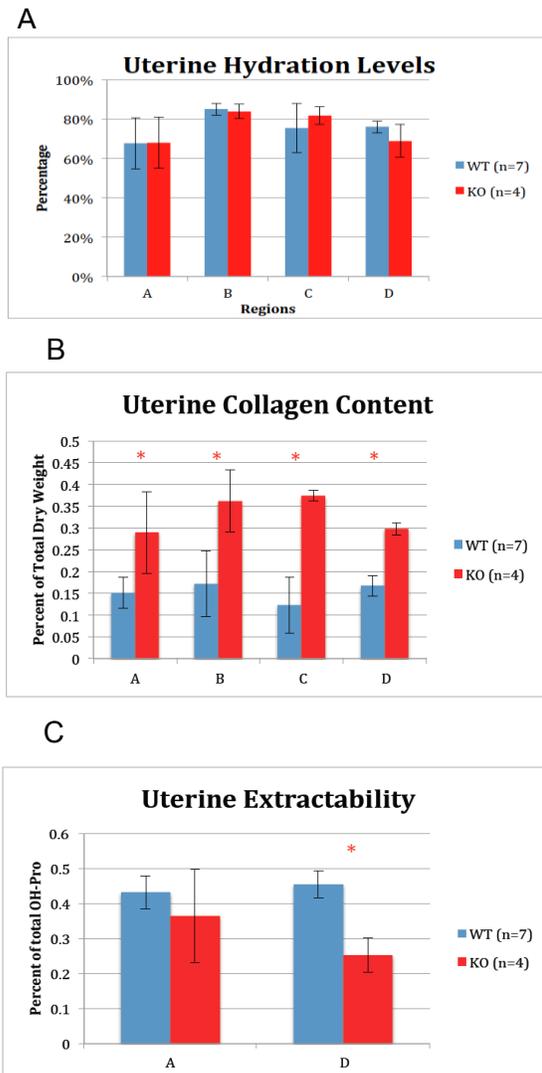
*Biomechanical Characteristics*

Tensile tests were performed on whole uterine horns (L horns). Figure 4A shows the averaged stress response of the tissue samples during the three load-unload cycles.

There was a trend towards higher stress peaks for all three cycles for *Antxr2 -/-* samples (n=3) compared to *Antxr2 +/+* samples (n=3), but they did not reach significance (p=0.1, 0.9, 0.18, Student's t-test). The tissues' stress-strain curve displayed non-linear behavior. In the case of the *Antxr2 -/-* samples, the first cycle, shown in light blue, was the stiffest peaking at 0.02 ± 0.01 MPa. The samples became more compliant with subsequent cycles, the peaks dropping to 0.018 ± 0.008 and 0.016 ± 0.008 MPa, respectively. This was the case for both the loading and unloading portions. This is usually referred to as "preconditioning", and it has been seen in other soft tissues [8]. Preconditioning, or softening, was not seen for the *Antxr2 +/+* samples (n=3) and all cycles peaked around 0.007 ± 0.002 MPa.

Figure 4B shows the averaged stress response for both *Antxr2 -/-* (n=3*)* and *Antxr2 +/+* samples (n=3) to three ramp-hold cycles. There is a trend toward higher peaks and equilibrium levels for the *Antxr2 -/-* samples. Briefly, at 10% strain a peak stress of 0.035 ± 0.01 MPa and an equilibrium stress of 0.0164 ±0.003 MPa were found for the *Antxr2 -/-* samples. For the *Antxr2 +/+* samples, peak stress response was 0.018 ± 0.002 MPa and equilibrium stress response was 0.012 ±0.003 MPa. At 15% strain, the *Antxr2 -/-* samples had a peak and equilibrium stress response of 0.051 ± 0.007 MPa and 0.029 ± 0.002 MPa, respectively. For the *Antxr2 +/+* samples, peak and equilibrium stress response were equal to 0.027 ± 0.003 MPa and 0.019 ± 0.006 MPa. Lastly, at 20% strain, the *Antxr2 -/-* samples had a peak and equilibrium stress response of 0.078 ± 0.008 MPa and 0.046 ± 0.002 MPa. The *Antxr2 +/+* samples had peak and equilibrium stress response of 0.04 ±0.01 MPa and 0.03 ± 0.01 MPa. *Antxr2* -/- samples had significantly

higher peak stress responses at 15 and 20% strain (p<0.05, Student's t-test). Figure 4C shows a table with the peak and equilibrium stress responses seen in both the *Antxr2 -/- and Antxr2 +/+* samples.

A
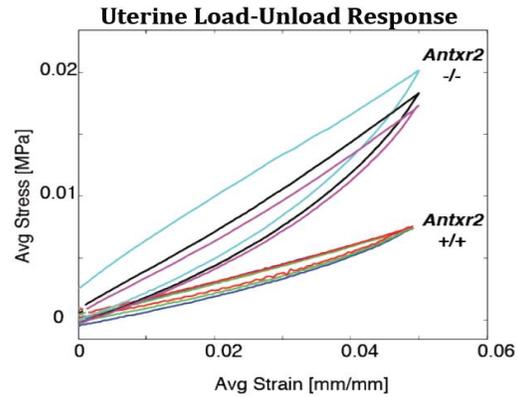


B
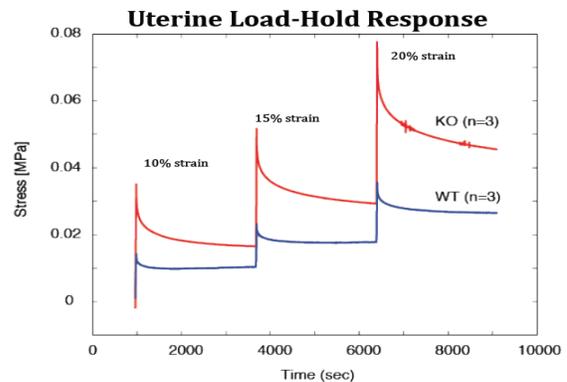


C

| Strain Level (%) | Avg Stress Peak *Antxr2 -/-* (MPa) | Avg Stress Peak *Antxr2 +/+* (MPa) | Avg Stress Equilibrium Level *Antxr2 -/-* (MPa) | Avg Stress Equilibrium Level *Antxr2 +/+* (MPa) |
|---|---|---|---|---|
| 10 | 0.035 ± 0.01 | 0.018 ± 0.002 | 0.0164 ±0.003 | 0.012 ±0.003 |
| 15 | 0.051 ± 0.007 | 0.027 ± 0.003 | 0.029 ± 0.002 | 0.019 ± 0.006 |
| 20 | 0.078 ± 0.008 | 0.04 ±0.01 | 0.046 ± 0.002 | 0.03 ± 0.01 |

**Figure 4**: **A** Graph of averaged stress response for *Antxr2 -/-* and *Antxr2 +/+* samples for load-unload tests, **B** Graph of averaged stress response for *Antxr2 -/-* and *Antxr2 +/+* samples for ramp-hold tests at 10, 15, and 20% strain, **C** Table of the averaged stress peak and equilibrium values for *Antxr2 -/-* and *Antxr2 +/+* samples for ramp-hold tests at 10, 15, and 20% strain.

## Discussion

The purpose of this study was to quantify the biochemical and biomechanical properties of uterine tissue from normal (*Antxr2 +/+)* and abnormal (*Antxr2 -/-*) mouse models of pregnancy. Uterine tracts from 3-4 month *Antxr2 -/-* mice were demonstrated to have altered biochemical composition and biomechanical properties compared to *Antxr2 +/+* mice. In addition, the differences in biomechanical properties were correlated to altered biochemistry. Few studies have correlated specific biochemical variables to uterine mechanical properties. Here, the accumulation of collagen, a load bearing protein, is correlated to the increase in the mechanical strength of the tissue. Despite the increase in its tensile strength, the uterus of *Antxr2 -/-* mice cannot produce adequate contractions for birth. The altered uterus cannot contract, highlighting the importance of the uterus' structure-function properties. Limitations were encountered and alterations were made during protocol development. Here the motivations behind protocol alterations, the significance of the experimental results, and future endeavors are presented.

### Biochemical

The collagen content of these *Antxr2 -/-* mice has been measured to be higher than their *Antxr2 +/+* counterparts through qualitative means, but here quantitative analysis in the form of percent of dry weight is presented [13]. Averaging at 33 ± 7% per dry weight, *Antxr2 -/-* uterine tracts were found to have twice as much collagen than *Antxr2 +/+* tracts (15 ± 5% per dry weight). Previous work by Zhao, *et al.* showed that mice contain around 8% of dry weight of collagen in their uterine tracts [16]. Taking into consideration the difference between protocols, breed, age, and the estrous stage in which the mice were sacrificed, reaching 15% of collagen is acceptable.

Knowing a tissue's collagen content is useful, but a more detailed analysis of the existing collagen network is needed. The extractability of the tissue measures the solubility collagen network is in a solvent, and is an improved measure of the mechanical quality of the uterine collagen. Regions A and D were chosen for extractability analysis because it was easy to guarantee that samples would not overlap with each other. A lower extractability, as seen in region D of *Antxr2 -/- (25 ± 5 %),* signifies a stronger network than region D of *Antxr2 +/+* (45 ± 4%). It can be inferred that the accumulation of collagen is responsible for extractability differences seen between region D of *Antxr2 -/- and Antxr2 +/+.*

Extractability was found to be region specific in *Antxr2 -/-* uterine tissue. Regions D, closest to the ovaries, were found to be less extractable than region A of *Antxr2 -/-.* There are a few reasons why samples from region A of *Antxr2 -/-* mice (36 ± 13%) were not significantly less extractable than samples from region A of *Antxr2 +/+* mice (43 ± 5%). It was noticed that the relatively large variability in region A of *Antxr2 -/-* was caused by one specific sample. Using Grubb's test showed that the individual value was close to being an outlier (P=0.09). If this individual value is excluded from the samples, region A from *Antxr2 -/-* is found to be significantly less extractable than region A from *Antxr2 +/+ (*P=0.012). Note that the particular sample is included in Figure 3C. Another explanation for the higher extractability value of region A from *Antxr2 -/-* tracts could be due to inclusion of the junction. The junction, or the area where the two uterine horns meet, could have inherently different extractability properties. A final explanation could be the close proximity of region A to the cervix. The collagen fiber architecture of the cervix is different than the uterus'. The ultrastructure of region A could be a combination between the upper regions of the uterus and the cervix, resulting in these extractability values.

### Biomechanical

For the load-unload test, *Antxr2 -/-* samples had stiffer (higher averaged stress response) than *Antxr2 +/+* samples. In addition, *Antxr2 +/+* tissues displayed no difference in

their averaged peak stress response between the three cycles (0.007 MPa all three), meaning pre-conditioning was not observed. It should be noted that *Antxr2 +/+* tissues tend to be very compliant, and at 5% strain levels, the difference between cycles could be below our instrument's sensitivity.

For the ramp-hold test, *Antxr2 +/+* had lower peak and equilibrium stress response than *Antxr2 -/-,* meaning *Antxr2 +/+* uterine tissue is more compliant. The 45 minutes in the ramp-hold test was not enough time for the tissues to equilibrate and reach their steady value. In initial tests of *Antxr2 +/+* uterine tissues, "drifting" was seen during the 45 minutes following the 10 and 15% strain levels. This led to a seemingly increasing stress reading during resting periods instead of reaching a steady value. This was not seen at 20% strain. However, limiting the initial gauge length of the samples to 4 cm eliminated the drifting. Despite the drifting, the *Antxr2 +/+* samples never reached the stress values for *Antxr2 -/-* samples.

## Future Work

### Biochemical

In conclusion, uterine tracts from *Antxr2 -/-* mice are stiffer than *Antxr2 +/+* tracts due to the accumulation of collagen. The change seen in the uterus' structure-function properties leads to failed pregnancies. Having biochemical data on these non-pregnant tissues has laid the foundation for future work, and will be essential when comparing to pregnant tissues. It is known that the uterus increases in wet weight, elastin, and collagen content during pregnancy [2]. This transformation has not been proven in *Antxr2 -/- mice*. Future work will analyze the biochemical makeup of pregnant uterine tissue from both *Antxr2 -/-* and *Antxr2 +/+* mice.

In the future, the extractability of regions B and C will be investigated in order to get a complete understanding of the uterine tracts. In addition, the sample preparation protocol will no longer include the junction since it is suspected to affect the extractability of region A.

Avery, *et al.* describes a more in-depth analysis of the collagen network [1]. Briefly, identifying and determining the concentration of the different types of collagen cross-links provides insight into collagen turnover. Future work will focus on analyzing collagen cross-links using the protocol described by Avery, *et al* [1].

### Biomechanical

The 45 minutes in the ramp-hold test was not sufficient for the tissues to equilibrate and reach their steady value. Consequently, testing times in the future will be increased. Weiss, *et al.* revealed that the uterus has zones of anisotropy [15]. A layer immediately surrounding the uterine cavity has collagen fibers oriented in a circular fashion [[15]. Outside this layer there are no easily identifiable holistic structures. Mechanical testing on the uterine tracts was performed in a longitudinal orientation. Due to the anisotropy found in both human and mouse uterine tissue, future mechanical tests will be performed in a different orientation.

Uterine samples were airbrushed to produce a speckled pattern. Using Correlated Solution Vic 2-D DIC software, the anisotropy of the uterus will be investigated. Vic 2-D software will find local strain values and material properties that will be used in future finite element models to investigate the interaction between the uterus and the cervix of the mouse in gestation.

## References

1. Avery, *et al (2009).* Quantitative determination of collagen cross-links. Methods in molecular biology (Clifton, N.J.) 522: 103-121.

2. Conrad, *et al*. (1966) Passive stretch relationships in human uterine muscle

3. Conrad, *et al.* (1966) Stress relaxation in human uterine muscle. American Journal of Obstetric and Gynecology 95.2: 254-265.

4.  Fata, *et al.* (2000) Cellular turnover and extracellular matrix remodeling in female reproductive tissues: functions of metalloproteinases and their inhibitors. Cellular and Molecular Life Sciences, CMLS 57.1: 77-95.

5.  Lawn, *et al.* (2012) Save the Children, WHO. Born Too Soon: the global Action Report on Preterm Birth. Geneva.

6.  Mahendroo, M. (2012) Cervical remodeling in term and preterm birth: insights from an animal model.

7.  Mikic, B. (2000) GDF-5 deficiency in mice alters the ultrastructure, mechanical properties and composition of the Achilles tendon. Journal of Orthopaedic Research 19.3: 365-371.

8.  Myers, *et al* (2008) Mechanical and biochemical properties of human cervical tissue, Acta biomaterialia 4.1: 104-116.

9.  Obara, *et al.* (2001) Changes in molecular weight of hyaluronan and hyaluronidase a activity in uterine cervical mucus in cervical ripening. Acta obstetricia et gynecologica Scandinavica 80.6: 492-496.

10. Oxlund, *et al.* (2010) Cervical collagen and biomechanical strength in non-pregnant women with a history of cervical insufficiency. Reproductive biology and endocrinology 8.1: 92-102.

11. Pearsall, G.W. and Roberts, V.L. (1978) Passive mechanical properties of uterine muscle (myometrium) tested in vitro. Journal of Biomechanics 11.4: 167-176.

12. Peters DE. Capillary morphogenesis protein-2 is required for mouse parturition by maintaining uterine collagen homeostasis. Biochemical and biophysical research communications. 2012-06; 422:393-397.

13. Reeves et al (2011) Anthrax Toxin Receptor 2 functions in ECM homeostasis of the murine reproductive tract and promotes MMP activity, in press at PLoS One.

14. Spiess K. Distribution of Collagen Types I, III, and V in Pregnant Mouse Endometrium. Connective tissue research. 2007-01;48:99-108.

15. Weiss, *et al*. (2006) Three-dimensional fiber architecture of the nonpregnant human uterus determined ex vivo using magnetic resonance diffusion tensor imaging. The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology 288A.1: 84-90

16. Zhao L. Collagen Studies in Late Pregnant Relaxin Null Mice. Biology of reproduction. 2000.9;63:697-703.