Developing an *In Vitro* Model to Modulate Molecular Transport in Uterine Myometrial Tissue

A Major Qualifying Project Submitted to the Faculty of Worcester Polytechnic Institute In partial fulfillment for the Degree of Bachelor of Science

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Abstract

Intramural uterine fibroids are non-cancerous tumors within the myometrium of the uterus. Fibroids affect over 75% of women, yet few in vitro models exist. Therefore, little research has been done to investigate how altered molecular transport (e.g., oxygen, hormones, small molecules) within fibroids affects their behavior and that of the surrounding tissue. To recapitulate fibroid associated changes to molecular transport in vitro, the team developed two in vitro modelling approaches— one utilizing a chemical induction of hypoxia in 2D cultured cells via Cobalt Chloride supplemented media and one that uses an alginate overlay to modulate molecular transport to cells encapsulated within an underlying 3D hydrogel. We evaluated the stiffness of the hydrogel formulations, the viability of the cells under model conditions and the diffusion of representative salts through the hydrogel materials.

Key words: fibroid, myometrium, hypoxia, in vitro modelling

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1.0 Introduction

Uterine leiomyomas, also known as uterine fibroids, are monoclonal tumors of the smooth muscle tissue layer (myometrium) of the uterus (McWilliams & Chennathukuzhi, 2017). There are three different types of uterine fibroids: subserosal, intramural, and submucosal (Sefah et al., 2023). Intramural fibroids, the second most common sub-type, are located within the myometrium. Symptoms of uterine fibroids may include pelvic discomfort, painful and heavy menstrual bleeding, anemia, urinary incontinence, recurrent pregnancy loss, preterm labor, and infertility in some cases. Although uterine fibroids are considered benign, they are the cause of major quality-of-life issues for approximately 25% of all women. Despite the population-wide prevalence of uterine fibroids, there are few affordable treatments that address long-term symptoms while leaving fertility intact (McWilliams & Chennathukuzhi, 2017). The most common treatment for uterine fibroids is a hysterectomy—the complete or partial removal of the uterus (Ramdhan et al., 2017). Other treatments include a myomectomy, hormone therapy, or uterine artery embolization (McWilliams & Chennathukuzhi, 2017; Stewart et al., 2016). Uterine fibroids are also found to disproportionately affect African descent women. Aside from incidence alone, these women also appear to have larger fibroids, earlier onset (around 10-15 years earlier), and more clinically significant symptoms (McWilliams & Chennathukuzhi, 2017).

The lack of understanding of the etiology of uterine fibroids contributes to the scarcity of medical therapies available (McWilliams & Chennathukuzhi, 2017). More research needs to be done to understand how and why uterine fibroids form. Current research includes using xenografts to identify potential treatments for the fibroids. Uterine fibroid xenografts — in which a human fibroid tumor sample is surgically implanted into a non-human species — typically utilize murine models (Serna & Kurita, 2018). While xenografts offer *in vivo* conditions for observing uterine fibroid development, the differences between human and mouse anatomy limit the accuracy of the model (Serna & Kurita, 2018). Additionally, the ethical implications of using animal models are debated. *In vitro* models are also utilized to study uterine fibroids, with both 2D and 3D conditions. While 2D *in vitro* models, such as 2D cell culture within a well plate, are inexpensive and easy to execute, the behavior of cells in a 2D format is not fully representative of their behavior *in vivo* (Kapałczyńska et al., 2018). The use of 3D *in vitro* models introduces an element of structure and architecture that better approaches *in vivo*-like conditions

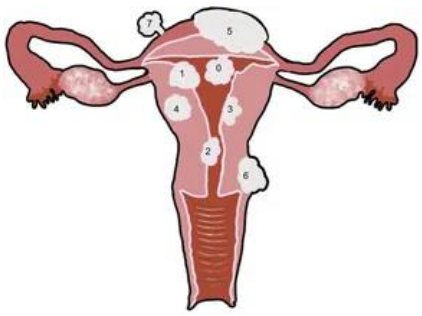
(Kapałczyńska et al., 2018). Current 3D *in vitro* models include organ on a chip, tissue ring models, and tumorspheres. While many of these 3D model approaches have yielded promising results in other fields, applying these techniques to create an accurate 3D model of uterine fibroid is an ongoing process.

This project aims to modulate molecular transport in uterine myometrial tissue in a 3D, *in vitro* model. This model should mimic the physical and chemical interaction between myometrium and fibroids. It should also be accurate to fibroid extracellular matrix (ECM) composition, be cost efficient and be reproducible. The group used literature and advisor guidance to develop a final design of a 3D *in vitro* intramural uterine fibroid model. The group performed a literature review to develop alternative designs that reflect components of uterine fibroids and the surrounding myometrium. Expansive clinical studies review of uterine fibroids, polycystic ovarian syndrome, endometriosis and adenomyosis is included at the end of this report.

2.0 Literature Review

2.1 Anatomy and Pathophysiology

Uterine fibroids are benign tumors that vary in size from less than 5 millimeters to greater than 10 centimeters (Bérczi et al., 2015). Fibroids grow on smooth muscle cells in the uterus, also known as myometrium tissue. Myometrium grows in a consistent pattern, whereas the fibroids grow disorganized and in ball shape. There are three classifications for the locations of the fibroids: subserosal, intramural, and submucosal. Figure 1., shows the varying locations where each type of uterine fibroid can grow. Subserosal fibroids grow outside of the myometrium, shown in Figure 1. Intramural fibroids grow within the myometrium (Figure 1). Finally, submucosal fibroids grow inside the uterine cavity (Figure 1). There is another type of fibroid classification, called pedunculated (typically subserosal or submucosal), that can grow inside or outside of the uterine cavity with no specific location (Sefah et al., 2023).



Classification of uterine fibroids by anatomical positioning		
FIGO	Subtype	Positioning
0	Submucosal - Subtype 0	100% endometrial cavity or intracavity
1	Submucosal - Subtype 1	> 50% intramural
2	Submucosal - Subtype 2	< 50% intramural
3	Intramural	In contact with the endometrium
4	Intramural	100% intramural
5	Intramural	Subserosal >50% intramural
6	Subserosal	Subserosal <50%
7	Subserosal	Pedunculated

Figure 1. Demonstrative image of the uterus and locations that uterine fibroids can grow, with fibroid subtype key. Image sourced from Sefah et al, 2023. Image used with permissions under Creative Commons BY license

2.1.1 Genetic Drivers of Fibroids

At a genetic level, studies indicate that mutations in the MED12 gene-a transcriptional regulator-are present in around 80% of uterine fibroids. Gain of function mutations in the MED12 gene, which may contribute to genomic instability, are found in multiple reproductive cancers, including breast cancer, prostate cancer, and cervical cancer (Machado-Lopez et al., 2021). Mutations to MED12 have not been discovered in any healthy myometrium samples

(Ciebriera et al., 2018). Additionally, genes that encode ECM proteins were found to be overexpressed in uterine fibroids (Brakta et al., 2015).

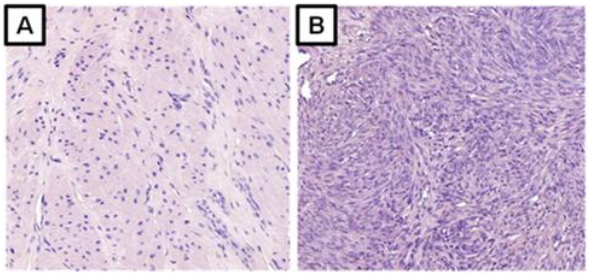


Figure 2. Microscope slides showing the difference in healthy myometrium (A) and a myometrium with uterine fibroids (B). Sourced from Ciebriera et al., 2018. Image used with permissions under Creative Commons.

2.1.2 Fibroid Microenvironment

On a tissue level, uterine fibroids are characterized by their excessive ECM accumulation, with levels of collagen I and glycoproteins such as fibronectin, and laminin that are elevated relative to normal myometrium (Islam et al., 2018). There is approximately 50% more ECM in the tissue of a uterine fibroid compared to regular myometrial tissue in surrounding areas (Ciebiera et al., 2018). Additionally, fibroids are shown to have reduced organization of collagen in the ECM (Maekawa et al., 2022). Mechanical properties of uterine fibroid ECM, specifically stiffness, are significantly altered compared to normal myometrium. Uterine fibroid tissues are shown to be 2 to 3-fold stiffer than unaffected myometrium, with respective averages of 4.9 kPa and 18.6 kPa respectively (Rogers et al., 2008). This accumulation and stiffening of the ECM are known as fibrosis. While the source of uterine fibroid initiation is not yet known, in fibrotic diseases, the normal wound healing process requires a positive feedback loop of continual ECM remodeling.

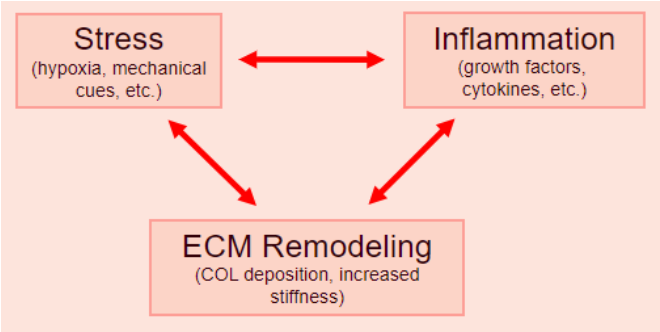


Figure 3. Positive feedback loop of fibrosis in the uterine fibroid tumor microenvironment.

In healthy wound healing, alterations to the cells' homeostasis contribute to inflammation, wherein cytokines, growth factors, exosomes, and other cell signals are produced. These signals instruct the surrounding cells, namely fibroblasts to begin ECM remodeling, accumulating ECM proteins like collagen (Islam et al., 2018). However, in some cases the process of remodeling can continue uninhibited, and the resulting stress to the cells promotes even greater degrees of inflammation and subsequent ECM deposition, eventually resulting in a uterine fibroid tumor.

2.1.2 Molecular Transport in Fibroids

Another crucial factor in the development of uterine fibroids is reduced molecular transport through the bulk of the fibroid. During the development of a fibroid, the accelerated growth and subsequent lack of proper vascularization creates an environment where important molecules struggle to transport to the cells. This lack of vasculature can manifest in several ways including hypoxia, in which a cell does not receive the necessary oxygen to maintain homeostasis. This hypoxic condition produces reactive oxygen species (ROS) which promote uncontrolled proliferation of fibroid cells. The production of ROS, as well as the lack of available oxygen in the fibroids, contributes not only to proliferation, but also to angiogenesis-the formation of new vascular networks. This cascade of events inhibits apoptosis of fibroid cells, thus creating a tumor-friendly microenvironment (Fedotova et al., 2023). Hormones integral to the female reproductive system, like estrogen and progesterone also play a key role in the fibroid microenvironment. While the exact function of estrogen and progesterone in uterine fibroids is not fully known, reduced transport of reproductive hormones into the core of the uterine fibroid may have greater effects on the pathogenesis of uterine fibroids .Previous studies have shown progesterone to be essential to the maintenance of uterine fibroids, and estrogen has been shown to increase the expression of progesterone receptors-a known risk factor for developing Uterine fibroids (Machado-Lopez et al., 2021).

2.1.4 Surgical Treatments

Symptoms of fibroids are determined based off the size, location, and number of tumors. The most common treatment for fibroids is a hysterectomy, though other treatments are available. For submucosal fibroids, and in some cases, intramural fibroids, a myomectomy is the

recommended treatment (De La Cruz & Buchanan, 2017). Hysterectomies are the only treatment that fully eliminates uterine fibroids and can be performed as a full or partial removal. While removing the uterus may remove current fibroids and prevent the formation of new fibroids, the surgery permanently removes the patient's fertility, and can often result in secondary conditions such as vaginal prolapse (*Vaginal Prolapse*, 2021). Myomectomies — a procedure in which the fibroids are surgically removed while keeping the uterus intact — are a potential treatment, but uterine fibroids may re-appear. Myomectomies may allow the patient to retain their fertility but are often only a temporary solution. For myomectomies, there is a 15-33% reoccurrence rate of fibroids, and approximately 10% of women who receive myomectomies need a hysterectomy within 5-10 years of the myomectomy. Another treatment is uterine artery embolization (UAE), which embolizes the uterine arteries to cause fibroid devascularization. Although an UAE will help some patients, its main goal is to decrease the size of the fibroids by approximately 50% in volume and treat the symptoms (Bérczi et al., 2015). UAE does not fully eliminate uterine fibroids in most women. For individuals with uterine fibroids, there are several treatments aimed at symptom management, including medical therapies as well as surgical procedures. UAE, in which a catheter delivers small particles that block blood flow to the fibroid, and myolysis, in which the fibroids are targeted via a targeted energy system that utilizes heat, laser, and magnetic resonance-guided focused ultrasound surgery (De La Cruz & Buchanan, 2017).

2.1.5 Non-surgical Therapies to Manage Symptoms

Some of the medical therapies offered to patients include, oral contraceptives, levonorgestrel-releasing intrauterine device (IUD) such as Mirena, non-steroidal anti-inflammatory drugs, anti-fibrinolytics such as tranexamic acid, and hormone therapies (De La Cruz & Buchanan, 2017). Many of these therapies reduce the severity of blood loss during menses, and some therapies such as non-steroidal anti-inflammatory drugs offer pain relief. Hormone therapy, such as gonadotropin-releasing hormone agonists and selective progesterone receptor modulators, may offer temporary relief from uterine fibroid symptoms. It is often only prescribed to post-menopausal women, or those about to enter menopause, due to the severity of its side effects. Some such side effects of hormone therapy include hot flashes, vaginitis, and even changes to breast size if used long-term.

2.2 Current Research Models

2.2.1 Current 2D Models

A common method used in preclinical trials of tumor research is 2D *in vitro* models. Fibroids are classified as non-malignant tumors (Tinelli et al., 2021). Tumor microenvironments (TMEs) are hard to reproduce outside the body due to numerous changing factors such as diverse cell types, hormones and inflammatory responses (Mu et al., 2023). Despite its advantages, 2D tumor models do not account for all characteristics of *in vivo* conditions (Mu et al., 2023). A common characteristic of cultured cancer cell-lines is modeling the artificial culture conditions, instead of reflecting behavior in patients (Yada et al., 2018). Over time (long term passage), these cells may shift phenotypic characteristics or genetic expression to reflect the TME of the culture plate. Common applications of 2D *in vitro* modeling within the uterine fibroid research space include genomic analysis of fibroid and myometrial tissue, exposure to environmental pollutants, and treatment with biologically relevant growth factors (Yang et al., 2021).

2.2.2 Current 3D Models

A common type of 3D model is a tumorsphere, a solid, spherical formation developed from the proliferation of one cancer stem/progenitor cell (Johnson et al., 2013). They are created by placing cells into suspension colonies in a scaffold-free environment. They do not have the aid of ECMs or other physical supports (Mu et al., 2023). Tumorspheres can be distinguished because they become fused together so single cells cannot be recognized. Due to the lack of structure, tumor spheroids are less complex and cannot completely rebuild the system *in vivo*, but they are more convenient for co-culture with immune cells. Tumorspheres are typically used for cancer modeling because some cells do not properly form tumorsphere structures (Johnson et al., 2013).

The 3D microfluidic based culture method is when tissue pieces-matrix mixture is injected into the central channel, and culture medium with/ without immune cells is added into the parallel channel (Mu et al., 2023). This model is used in cancer research and has not been explored for uterine fibroids. Some advantages of this model are that multiple parameters are like *in vivo* status, small amount of tumor tissue is required, and high customizability. Some limitations are that the 3D microfluidic culture method can require special equipment, be

expensive, be limited by the volume of the device, and small size samples cannot reflect the heterogeneity of the TME (Mu et al., 2023).

Recent developments in 3D modeling include the use of smooth muscle cell (SMC) tissue rings. Tissue ring formation allows engineered vascular tissue fabrication entirely from cells and cell-derived matrix (Adebayo et al., 2013). Similar methods have been explored with scaffold-free tissue engineering approaches such as rolling cultured cell sheets, or assembly and fusion of clustered cells (Tracy, 2011). These self-assembled cellular constructs exhibit a greater cell density, enhanced ECM production and tissue strength, and lower susceptibility to degradation and infection (Strobel et al., 2017).

Aside from using cells in 3D culture, other methods describe the use of affected tissue samples in uterine fibroid research. Organotypic culture typically consists of removing an explant of an affected tissue and cultivating it in a controlled environment to mimic in vivo conditions. Explants are not models, rather ex vivo culture methods (Powley et al., 2020). Explants provide insight into the functional components of complex biological systems (Szczesny, 2020). Though they are not models themselves, they can be used in models, such as organotypic 3D models, and can be analyzed directly. This method has been successfully applied to affected patient uterine fibroids and used in cancer models. Uterine fibroid samples were placed on alginate scaffolds to maintain the structural integrity and mechanical properties. Studies showed these samples should not be kept in culture past 7 days, or a decrease in cellularity and hormone receptors, and a large increase of ECM was observed (Salas et al., 2020). This modeling system successfully demonstrated driver MED12 mutations at exon 2 in most organotypic fibroid samples. The 3D structure of the uterine fibroid along with cellular interactions remains intact through this model, as demonstrated through H&E staining of organotypic cultures (Shved et al., 2022). This model allowed for observation during the addition of ovarian hormones such as estradiol and progesterone, where gene expression was heightened through the addition of these hormones (Salas et al., 2020). While this model is not particularly difficult to maintain, a large drawback in fabrication lies in obtaining samples from the uterine fibroids. The stiff, compact nature of these tumors makes it difficult to consistently remove thin samples of the relative same size. Another drawback to this method is that explants are difficult to obtain due to their viability and cost, even though they supply researchers with insights into the physiology of the tumor (Stackhouse et al., 2021). Where many 2D culture models seem to

lose the MED12 genetic mutation characteristic to uterine fibroids, organotypic culture successfully expresses it. This could be due to the loss of soluble factors that are not present in 2D *in vitro* culture media or the lack of interactions throughout the ECM (Salas et al., 2020). These findings suggest that using a 3D model where ECM is present is essential to maintaining and successfully modeling the MED12 mutation.

The last type of model is a xenograft, a model that implants human tissue into a targeted specimen, typically a mouse or other small animal. In cancer models, cancerous cell lines are transplanted into a mouse. These models better reflect the physiological environment of a human patient but may still lack some true characteristics due to exposure to *in vitro* cultures (Yada et al., 2018). To transplant the cell lines, a tumor or a cell suspension is collected from a human patient and are typically inserted subcutaneously. Xenografts are more successful when in organ systems similar to that of the patients. They can successfully be used to model uterine fibroids as well as treatment options and therapies (Borahay et al., 2015). Although these model systems seem to accurately demonstrate uterine fibroid behavior, they lack myometrial controls due to differences between human and mice myometrial cells (McWilliams & Chennathukuzhi, 2017). This type of modeling has been used to measure drug efficacy related to uterine fibroids such as patient-derived xenograft model for uterine fibroid by sub-renal capsule grafting. In this study, it was determined that uterine fibroids are dependent on ovarian steroid hormones. Steroid pellets containing 17β-estradiol (E2) and progesterone were implanted with uterine fibroid tissue into the animal and the growth rate of the tumor was measured. Results of the experiment show that tumor cells became dormant by hormone withdrawal (Serna & Kurita, 2018). Another study used xenografts to investigate the impact of simvastatin on ER-α signaling in fibroid cells, including its expression, downstream signaling, transcriptional activity, post-translational modification, trafficking, and degradation. They found simvastatin significantly reduced E2-induced proliferation and PCNA expression. In addition, simvastatin reduced total ER-α expression in fibroid cells and altered its subcellular localization by inhibiting its trafficking to the plasma membrane and nucleus. Simvastatin also inhibited E2 downstream signaling, including ERK and AKT pathways, E2/ER transcriptional activity and E2-responsive gene (Afrin et al., 2023).

2.2.3 The gold standards in 2D and 3D culture:

The current 2D gold standard in *in vitro* uterine fibroid modeling is separate 2D cultures of patient matched fibroids and myometrium cells (Dimitrova et al., 2009). Traditional culture is used to culture both fibroids and cells in a flat well plate. This standard is cheap, reproducible, and models cell to cell interactions. The interface between the fibroid and myometrium is not modeled within this gold standard and it is not clear how these two cultures affect each other. A major limitation is that this model lacks clinical and engineering translations as it does not accurately represent *in vivo* conditions. Cell differentiation is poor, while cell proliferation is typically higher than true *in vivo* conditions. This model also lacks demonstrating cell interactions with ECM, a major component of uterine fibroids. Despite its ease of manipulation and low costs, this model shows little insight into the *in vivo* behavior of myometrium cells or fibroids.

The current 3D gold standard model uses fibroid cells themselves or harvested fibroids to do organotypic culture. Organotypic culture has been successfully done on lung, prostate, colon, gastric, and breast cancer malignancies (Salas et al., 2020). The organotypic culture is placed onto an alginate scaffold (Salas et al., 2020). Limitations of this method are that the fibroids' availability is low, and cost is high.

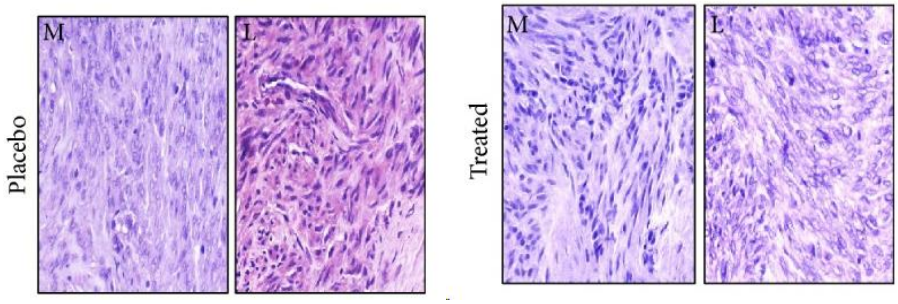


Figure 4. Comparison of patient matched fibroid (L) and myometrium (M) cells in treated and placebo conditions. Image taken from (Lewis et al., 2018). Imaged used with Permissions through Creative Commons Attribution License.

2.3 Clinical/Scientific Significance

Approximately 77% of women will develop uterine fibroids in their lifetime. However, this number may be skewed as many asymptomatic uterine fibroids will go undiagnosed. In younger patients, uterine fibroids are typically only diagnosed through ultrasounds during early pregnancy (Laughlin et al., 2010). Of this significant percentage, around 25% of these will lead to major quality of life issues. This entails women who undergo symptoms that are clinically significant. These can include pelvic discomfort, dysmenorrhea, menorrhagia, anemia, urinary incontinence, and infertility. Symptoms vary depending on the location of the fibroids. There is also a difference in symptoms between the distinct types of fibroids. For example, submucosal and intramural fibroids present with abnormal uterine bleeding whereas subserosal and pedunculated fibroids present with pelvic pain and bowel and bladder issues (Havryliuk et al., 2017). Women who become pregnant with uterine fibroids are at a higher risk for miscarriage and complications with pregnancy (McWilliams & Chennathukuzhi, 2017). Most uterine fibroid diagnoses occur at the perimenopausal age. They are often not found before then unless clinically significant symptoms are present. Symptoms impact the quality of life greatly, those who do experience symptoms may miss important events in their life due to pain and discomfort (Havryliuk et al., 2017). Another impact on their quality of life is the emotional distress that is often associated with uterine fibroids, including depression and anxiety from having to deal with constant symptoms (Giuliani et al., 2020). Based on the low incidence rate for older women, it appears that women who have not developed uterine fibroids by this perimenopausal age are a low-risk group (Laughlin et al., 2010). Diet, exercise, and sex hormones also are contributing factors to uterine fibroids.

Uterine fibroids are found to disproportionately affect African descent women. Aside from a higher incidence alone, these women also have larger fibroids at diagnosis, earlier onset (around 10-15 years earlier), and more clinically significant symptoms. Where white women above 50 years old have a uterine fibroid incidence of 70%, women of African descent have an 80% incidence and seek treatment more than any other ethnic group in America. Due to the clinically significant symptoms faced more often, women of African descent are more likely to miss work or have major quality of life issues due to their diagnoses. Women of African descent are 2.4 times more likely to have a hysterectomy for treatment of fibroids and have a 6.8-fold higher rate of myomectomy treatment for removal of fibroids compared with Caucasian women

(McWilliams & Chennathukuzhi, 2017). Although it is understood that these women are disproportionately affected by uterine fibroids, the cause for this is not understood (McWilliams & Chennathukuzhi, 2017). These numbers reflect African women in the United States, data from countries with other black populations is needed to determine the true high incidence rate in black women (Laughlin et al., 2010).

2.4 Limitations in the Field and Clinical Gaps

2.4.1 Limitations

In 2021, the Society for Women's Health Research held a meeting to review the current knowledge about uterine fibroids and future work that needs to be done to help women live healthier lives (Aninye & Laitner, 2021). Aninye and Laitner discuss how because of the complex pathophysiology of fibroids, the therapies we currently use do not remove or prevent the growth of new fibroids. In this meeting the Society for Women's Health Research held, there was a discussion on how the environmental risk factors are unknown and have not been studied. Studies have been completed but they are based off self-reported data and were not focused on fibroids, which yields limited longitudinal data available to researchers. The limits forced onto this research field based on outside resources not being sufficient in the field will continue to prevent new research and treatments being developed.

2.4.2 Clinical Gaps

Uterine fibroids have had limited research on them since they were discovered. This lack of research has left some large gaps in the clinical setting. Although there are classifications for the types of uterine fibroids, there are no measurements for classifications other than the location of the fibroid. The biggest clinical gap is that there is no uterine fibroid treatment that is successful in removing the fibroids entirely while maintaining the uterine function. More research needs to be completed on how the fibroids form in the uterus to better understand how we can either prevent them or safely remove them. Another clinical gap that is not addressed is how fibroids affect women of African descent more than another other population. There needs to be research into what causes this discrepancy to understand and prevent the African descent population from being disproportionally affected. One risk factor that has been found is obesity although it is unknown why obesity and uterine fibroids are linked. Obese patients with uterine

fibroids expressed higher amounts of upregulating associated factors (Afrin et al., 2023). Vitamin D deficiency has been found to affect the uterine fibroids and could be a cause for the disparities of those from African descent. An increase of vitamin D can reduce the uterine fibroid cell proliferation *in vitro* (Brakta et al., 2015). There are possibilities for new therapeutics using vitamin D to reduce uterine fibroids without hormones or other current therapies.

2.4.3 Engineering Gaps

A large gap in engineering is the lack of understanding about the interface between the myometrium and fibroids. There is limited literature regarding both the chemical and physical interface between the myometrium and uterine fibroids within the context of *in vitro* modelling. While there are a number of studies investigating the chemical interactions between fibroid cells and cells of the surrounding tissues, such as adipocytes, there is little research surrounding myometrium-fibroid crosstalk (Afrin et al., 2023). The physical interface between the myometrium and fibroid poses a challenge as well. While multiple methods of producing 3D tissue models of uterine fibroids are being developed, the vast majority are limited to the uterine fibroid itself, with no junction between the tumor and healthy myometrial tissue (Malik & Catherino, 2012; Winter et al., 2020). The limited understanding of the chemical and physical interface and interactions between the myometrium and uterine fibroids highlights the urgent need for more comprehensive *in vitro* design and development.

3.0 Project Strategy

3.1 Initial Client Statement

The initial client statement given to the group was to identify an *in vitro* model format (e.g., tissue rings, spheroids, encapsulated cells) and culture conditions that replicate the myometrium-fibroid tissue interface, promote, and maintain uterine smooth muscle cell phenotype, and support assessment of *in vivo*-like outcomes.

3.2 Technical Design Requirements

3.2.1 Objectives

To develop a project that meets the current gaps in uterine fibroid research, our team identified a few key design objectives.

Table 1. Key Design Objectives

Design Objectives
1. Models the Myometrium/ Fibroid Interface
2. Accurate to ECM composition
3. Accurate to cell composition
4. Cost efficient
5. Reproducible
6. Ease of Analysis
7. Achievable within time limit

The objectives 1-3 aim to ensure that any model developed is representative of the tissue(s) we aim to study. These objectives are important because without ensuring an accurate model environment, any conclusions reached through the model may have little to no impact on the actual condition. The inclusion of a myometrium/fibroid interface aims for a novel approach to fibroid research, given the lack of *in vitro* models that address the structural context of a fibroid in the uterus. An accurate model of the ECM is especially important for a condition that is driven by fibrosis-a stiffening of the ECM. Not only this, but it is also vital that the ECM matches in vivo conditions, for an accurate representative model. By keeping the model accurate

to the cell content, as well as that they represent mutations and sustain viability, we ensure that the project is investigating the proper component of the human body. Objectives 4-7 were identified to keep the project efficient and minimize negative impacts on the team. That is because the project must stay within budget and is reproducible to us and future teams. Ease of analysis and time refer to us obtaining results through time spent in the lab and spending time analyzing the data, though it might be difficult.

3.2.2 Technical Design Requirements:

With the goal to create an *in vitro* model of uterine myometrium to study uterine fibroids, several technical design requirements were identified. Cells used needed to be sourced from representative tissue, meaning myometrial SMCs could not be interchanged with SMCs from another source. Additionally, procedures needed to align with imaging techniques, sterility was to be ensured, and biocompatibility of all materials used also needed to be ensured.
In addition to the objectives, our team also developed a few functions as well as means to achieve those functions that correspond to each objective. Each objective and their corresponding functions and means are listed in the table below.

3.2.3. Design Functions and Means

Table 2. Design Objectives, Functions, and Means

Objective	Function	Means
(1) Models the Myometrium/ Fibroid Interface	Model mimics physical interaction between myometrium/fibroids	• Fibroid cells are directly cocultured with myometrial cells • A fibroid sphere is placed into a ring mold with the myometrial SMCs, and the ring self assembles around/integrating with the fibroid
	Model mimics chemical interaction between myometrium/fibroids	• Model includes cell co-culture between myometrial cells and cells in the fibroids • Myometrial and fibroid cells in indirect co-culture (transwell/ conditioned media)

Objective	Function	Means
(2) Accurate to ECM composition	ECM model component mimics native tissue biochemically	• ECM has collagen content necessary to sustain cell population (viability > 80%) • ECM includes hormone content (progesterone, estrogen) • ECM includes cytokine factors (tumor necrosis factor alpha, transforming growth factor beta, activin A, platelet derived growth factor
	ECM model component mimics native tissue functionally	• ECM provides structure for cells by stiffness value of 2-3x greater than myometrium (~18.6kPa compressive resistance to 0.1 strain) • Encapsulate cells in 3D matrix to mimic excessive ECM deposition and associated factors • Hydrogel composition contains enough alginate for structural properties and collagen for cell attachment
(3) Accurate to cell composition	Cell composition mimics native tissue biochemically	• Model is exposed to cell signals from surrounding tissues, including adipokines from adipose-tissue, growth factors like TNF-alpha from myocytes, estrogen and progesterone from hormone signaling (endometrial cells being exposed to fibroid cell signals, fibroid cells being exposed to adipocyte cell signals, etc.)
	Cell content mimics native tissue functionally	• Model encapsulates cell types present in normal fibroid (SMCs and fibroblasts) • Monoclonal cell lines for uterine fibroid cells • Cells maintain MED-12 mutation in culture, no loss of mutation (MED12 is a driving factor of fibroid development)
	Cell content mimics native tissue structurally	• Cells are encapsulated in a 3D structure • Fibroid cells are layered on top of myometrial cells
(4) Cost efficient	Project does not exceed allotted budget	• Alternative materials are considered before purchasing expensive items (~50$) • Evaluation of the costs of media and cell lines concluded that the uterine fibroid tissue was not feasible for this project • Limited the amount of specialized cell media purchased to run the first experiments to not overbuy

Objective	Function	Means
(5) Reproducible	Fibroid manufacturing results in consistent characteristics	• During experimentation, utilize cells of similar passage number and age to minimize differentiation effects of cells over time • Size of the fibroids manufactured in molds • Cell counts encapsulated (per cell type) is controlled when seeding
	Measured outcomes are statistically robust	• Utilize protocols with quick fabrication turnaround to enable a larger N size, utilize methods that can produce a large (>3) quantity of biological replicates at a time (favor molds that produce 6+ spheres vs 3 spheres) • Trials are done at statistically significant sample numbers (N>3, n>3) • Try to minimize subjective analysis (like using ImageJ with manual thresholding) by using quantitative assays (Western blot, immunostaining, etc.) • Morphology/viability is quantified via image
	Utilizes assays/techniques that give empirical data	• analysis of fluorescent stains, not just representative images • Use western blot for protein expression • Quantify/verify media components (exosomes) with DLS
(6) Ease of Analysis	Sources of variation in results are not confounding with one another	• Only one experimental condition is being varied at a time per experiment • Potential confounding variables are investigated in preliminary experiments before formal testing (e.g., $CoCl_2$6H20 and $CaCl_2$ preliminary viability)
	Model is compatible with various imaging techniques	• Model fits inside a microscope; cultured within a standard 96 well plate, custom well inserts are favored over proprietary plate designs • If using 3D encapsulation, various stain concentrations and incubation times are evaluated on the model in the event of failed stains
	Assay and other testing methods are optimized for the model format	• Favor assays that use media analysis (Alamar blue) to avoid 3D stain diffusion issues • Take slices of gels for 3D staining if 2D stains are failing to work in 3D format (smaller working gel thickness) • Favor smaller kDa stains for easier diffusion through 3D gels

Objective	Function	Means
(7) Achievable withing the time limit	Work is being conducted in the lab continuously	• Detailed lab schedule is made at the start of each term • Lab work is conducted multiple times a week to keep up with demands of the project
	Fabrication processes are being kept in mind	• It takes 7 weeks to develop a singular design • It takes 7 weeks to start research (for second semester of project) • It takes 1-2 weeks to fabricate rings • It takes 1 week to fabricate spheroids
	Results are collected before project end date	• Results are saved to the drive immediately after collection • Analysis of results is completed within a week of the data collection

3.2.4 Functions to accomplish modeling the myometrium/ fibroid interface

To develop a model that meets the objective of modeling the myometrium/fibroid interface, it is necessary to model both the physical and chemical interactions between the two tissues. Some potential means to model the physical interface may include growing fibroid-derived cells on top of myometrial-derived cells in a 2D layered model within a well plate. Another approach may be to use a smooth muscle cell (SMC) self-assembling ring model and place a small fibroid spheroid within the ring mold for the SMCs to assemble around, thus creating a fibroid integrated within the ring. For the chemical interface between the tissues, it may be necessary to utilize indirect-co culture to model the cross communication between myometrial and fibroid cells. A more direct co-culture, like in the 2D layered model, would also meet the need of modeling the chemical interactions.

3.2.5 Functions to accomplish an accurate ECM composition

Keeping the model accurate to the composition of the ECM in uterine fibroids is integral to making a useful model, as the changes to the ECM play a significant role in the condition's development. To accomplish this, we aim for the model to mimic the ECM biochemically, functionally, and structurally. An approach for mimicking the fibroid's ECM biochemically includes using a hydrogel comprised of the main ECM competent of fibroids, collagen. It may also be useful to include hormones or chemical factors relevant to the disease, such as the hormones estrogen and progesterone, or factors like tumor necrosis factor alpha, transforming growth factor beta, activin A, or platelet derived growth factor. To mimic the ECM functionally, suspending the cells in a 3D collagen is a means that allows the ECM to perform its normal role.

For the function of mimicking the ECM structurally, the stiffness of the ECM within the fibroid could be altered to a level that reflects the stiffness change in uterine fibroids. Literature suggests a 2-3x greater resistance to compressive strain of 0.1 as compared to myometrial tissue (roughly 18.6kPa) would be accurate (Rogers et al., 2008).

3.2.6 Functions to accomplish an accurate cell composition

By keeping the model accurate to the cellular composition of uterine fibroid, we are ensuring the results of any testing be applicable to fibroid tissue. Similarly, to the ECM, to accomplish this we will mimic the cell composition biochemically, functionally, and structurally. For biochemical mimicking, a means may be to include biochemical signals from cell types that are surrounding the myometrium and fibroid, such as adipocytes or endometrial cells. The cell signals may come from conditioned media, or direct co-culture. To mimic the cell composition functionally, one means would be to include all cell types present in the fibroid, namely uterine smooth muscle cells, and fibroblasts. Another means would be to utilize only monoclonal cell lines, as uterine fibroids are monoclonal in origin. Finally, to mimic the cell composition structurally, we may encapsulate cells in a 3D hydrogel to model the interspersion of the cells in normal tissue, or growth the fibroid cells on top of myometrial cells to mimic the proximity the myometrial wall.

3.2.7 Functions to accomplish a cost-efficient product

To keep the project cost efficient, it is especially important that our project does not exceed the allotted budget of $1,000. To avoid maxing out our budget, some potential approaches include keeping detailed records of any purchases made, as well as developing a team policy of investigating alternative purchases when faced with high budget items.

3.2.8 Functions to accomplish an easily reproducible product

To deliver reproducible results, it is necessary to manufacture fibroids with consistent characteristics, ensure statistical robustness of measured outcomes, and utilize techniques that deliver empirical data. For the manufacturing of fibroids, using molds and controlling encapsulation of cell counts will assist in obtaining this function. To ensure statistical robustness, written protocols will be kept consistent and performed by the same person, if possible, trials

will be done with statistical significance, and subjective analysis will be minimized when possible. To utilize techniques that deliver empirical data, florescent stains and representative images will be used to determine morphology and viability, western blot is to be used for protein expression, and media components will be quantified and verified with DLS.

3.2.9 Functions to accomplish an ease of analysis

To have the project be easy to analyze, sources of variation in the results must not be confounding with one another, which means that only one experimental condition should be varied at a time per experiment and that potential confounding variable should be investigated in preliminary experiments before formal testing occurs. Also, it is important to make sure that the model is compatible with various imaging techniques, such as that the model fits inside a microscope, can be cultured inside a standard 96 well plate, and various stain concentrations and incubations times are known. Finally, the assay and other testing methods are optimized for the model format. For example, assays that use media analysis are favored and kDa stains are also favored to allow for an easier diffusion through 3D gels.

3.2.10 Functions to accomplish a product within the time limit

For the project to be achievable within the time limit, it is important that project work is being continuously done, including being in the lab often. This can be managed by creating a detailed lab schedule at the start of each term. Another way to ensure the project is completed within the time limit is that fabrication processes are being kept in mind. For example, it takes 1-2 weeks to fabricate rings and 1 week to fabricate spheroids. Finally, to ensure the project is completed, results need to be collected well before the project end date to allow for appropriate time to analyze them. This can be done by continuously saving results to the lab notebook immediately after collection and analyzing results within a week of data collection.

3.3 Important Industry Standards

ISO standards are internationally agreed upon by experts within each field. For this model design the ISO standards that will be followed are ISO 10993-1:2018, ISO/AWI TR 4752, ISO/TC 150/SC 7. These standards are important to make sure that the design is ethical and safe

for future use if the project continues past Worcester Polytechnic Institute. Another standard we are following is an ASTM standard for the materials that we will be testing and working with. All these standards must do either with medical devices, materials, or treatments of cells. This is not a comprehensive list of all the standards that must be followed. These are the most important ones that were found and are viewed as important for this design project.

ISO 10993-1:2018-Biological evaluation of medical devices- Part 1: Evaluation and testing within a risk management process

ISO/AWI TR 4752- Inventory of methods for detection of microbiological contamination in mammalian cell culture

ISO/TC 150/SC 7- Tissue-engineered medical products

ASTM F813-20-Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices

3.4 Project Approach

3.4.1 Pairwise Comparison of Objectives

To establish a ranking of importance for the proposed objectives, the team completed a pairwise comparison chart. Across each row each cell represents the importance of the row header relative to the column header, where 0 represents lesser importance and 1 represents greater importance. For example, the value of 0 in the ECM row, cell column indicated the importance of the myometrium-fibroid interface is lesser than the importance of the ECM accuracy. This was chosen due to ECM deposition being a necessary characteristic associated with uterine fibroids. Column and row headers correspond to objectives listed in table (#) but are shortened for ease of formatting.

Table 3. Pairwise Comparison Chart

	Interface	ECM	Cell	Cost	Reproducibility	Ease of Analysis	Time	**Total**
Interface		0	0	1	0	1	1	**3**
ECM	1		0	1	1	1	1	**5**
Cell	1	1		1	1	1	1	**6**
Cost	0	0	0		0	0	1	**1**
Reproducibility	1	0	0	1		1	1	**4**
Ease of Analysis	0	0	0	1	0		1	**2**
Time	0	0	0	0	0	0		**0**

3.4.2 Revised Client Statement

The revised client statement, seen below, was remodeled to accurately depict the updated objectives. After researching each design concept in more depth, discussed below, three concepts were identified as most applicable to the established design objectives. The client statement was then revised to include the design concepts: the top layered diffusion, the layered 3D coculture, and the 2D hypoxia.

This project will identify an *in vitro* model format and cell culture conditions that specifically model the utilized primary uterine SMC as myometrial population, while also utilizing a hydrogel that reflects in vivo like stiffness properties without compromising cell viability and reproduces reduced molecular transport by altering hydrogel properties.

4.0 Design Process

4.1 Needs Statement

The current core problem within the scope of this project is the lack of reasonable treatments for uterine fibroids. Treatments offered are confined to full or partial hysterectomy, removing the patient's ability to produce biological offspring, and methods of symptom management that do not address the underlying condition. To develop effective treatments that address the source of uterine fibroids and offer reasonable consequences of treatment, fundamental understanding of the mechanisms of fibroid development must be achieved.

4.2 Preliminary Design Concepts

4.2.1 3D Fibroid

3D encapsulated fibroid modeling involves spheroids suspended in a collagen hydrogel. The spheroid would contain HUtSMCs and represent the uterine fibroid. This would be a continuation of a prior Worcester Polytechnic Institute Major Qualifying Project, which determined that HUtSMCs could form spheroids. The goal of this model would be to see what helps fibroids grow and will be able to mimic the tumor microenvironment, tumor growth, and cell proliferation. The gelatin hydrogel would mimic the properties of the ECM. The stiffness of the hydrogel could be modified by adding or removing collagen. Varied materials like vitamin D or sex hormones could be added to this model to see how the addition would affect the growth of the fibroid. Advantages of this design were that the spheroids are easy to manufacture and accurately mimic the 3D fibroid structure. The main disadvantage was that the design lacks myometrium interactions.

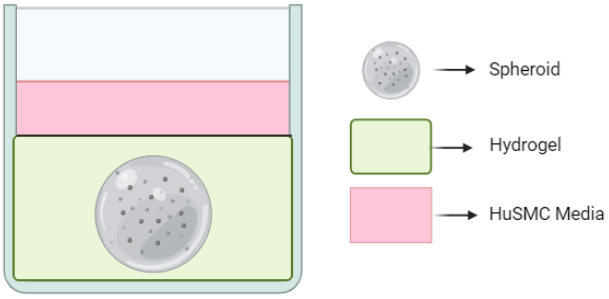


Figure 5. 3D Fibroid design concept. Image created in BioRender with WPI institutional license.

4.2.2 3D Ring

Literature has shown that when culture in a donut shaped mold, smooth muscle cells (SMCs) can self-assemble into the shape of a ring. Previous experiments conducted by prior MQP groups have established that this self-assembling ring is achievable with myometrium-derived SMCs. Additionally, these SMC rings possess the ability to deposit their own ECM. Modelling the myometrial wall with a myocyte SMC ring would allow for investigating the effects of various chemical factors on the behavior of myocytes in a context more in line with *in vivo* conditions. For the 3D ring design concept the advantage was that the concept was accurate to the myometrial tissue structure. The main disadvantage was that there was no fibroid interaction represented in the design.

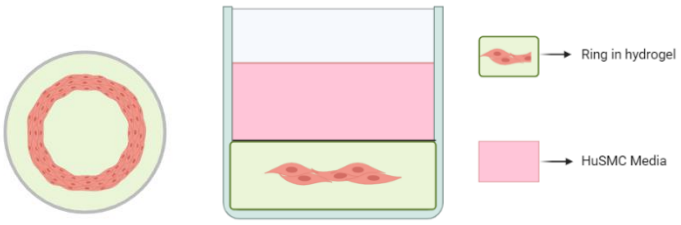


Figure 6. 3D Ring design concept with top (left) and side view (right). Image created in BioRender with WPI institutional license.

4.2.3 Ring and Spheroid Co-Culture:

This design would involve a HUtSMC self-assembled ring plus co-culture. The spheroid would contain fibroid cells and represent the uterine fibroid. The ring would be made of smooth muscle cells and would form into a ring using a mold. The sphere would be placed in the center of the ring and the entire goal of this design would be to look at myocyte signal changes 2D versus ring structure on fibroid sphere. The ring and spheroid design structure allowed for an accurate myometrial/fibroid interaction. The main disadvantage was the complexity of co-culture for multiple cell lines in the design.

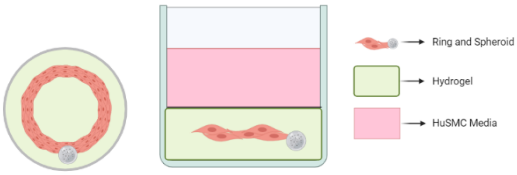


Figure 7. Ring and spheroid co-culture design concept with both top (left) and side views (right). Image created in BioRender with WPI institutional license.

4.2.4 Layered 3D Coculture:

With patient matched fibroids and myometrium cells in 2D culture being the current gold standard for models of uterine fibroids, a slightly different set up could be used to investigate the growth of the fibroids, how they adhere to the myometrium wall and the effect of the ECM. Layering multiple key factors such as a myometrium layer that along with smooth muscle cells, and fibroids can give a view into the interface. Media would be resting on top of the myometrium layer that can be adjusted for evaluating the growth of uterine fibroids under varying conditions like vitamin d deficiencies or different media types. After initial testing, the 3D model should be able to understand the representation of the interface or growth of uterine

fibroids. The layered 3D co-culture design allowed the advantage of an accurate representation of interactions between the fibroids and smooth muscle cells. The main disadvantage to this design was similar to the ring and spheroid design being the complexity of co-culture for two different cell lines.

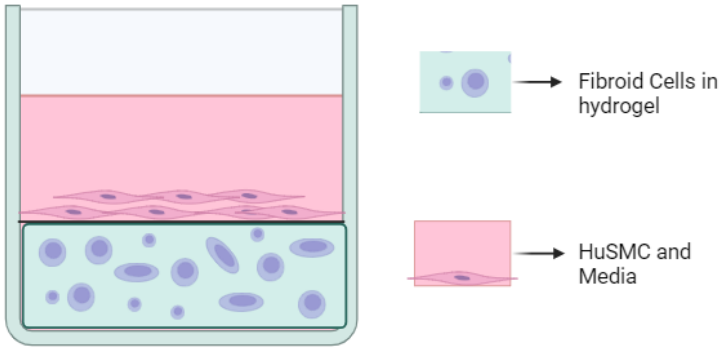


Figure 8. 3D Coculture design Concept. Image created in BioRender with WPI institutional license.

4.2.5 Top Loaded Diffusion:

Top loaded diffusion modeling involves layering media on top of 3D encapsulated hydrogel. The 3D hydrogel will be better to model *in vivo* cell conditions, along with ECM composition and stiffness and the interface. This is through improved myocyte to fibroid signaling. The main concept of this model revolves around trying to alter the matrix, either through pore size or density to get the simulation of how distinct factors can or cannot get through the fibroid. A downside of using this model is that it requires the use of collagen, which is both expensive and time consuming. Another downside of this model is that it is harder to image and analyze. There are also multiple factors to control, such as the thickness of the gel, pore size, concentration, and stiffness. This is done by manipulating the hydrogel to match myometrial stiffness while also allowing for top-down diffusion. Thickness is important because we need to ensure the 3D hydrogel is thick enough for the solution at the top to diffuse down. The top layer diffusion design provided the advantage of being able to alter the molecular transport through adjusting the myometrium replicate. The main disadvantage for this design was the complications with manipulating the overlaid material while maintaining an accurate myometrial replicate.

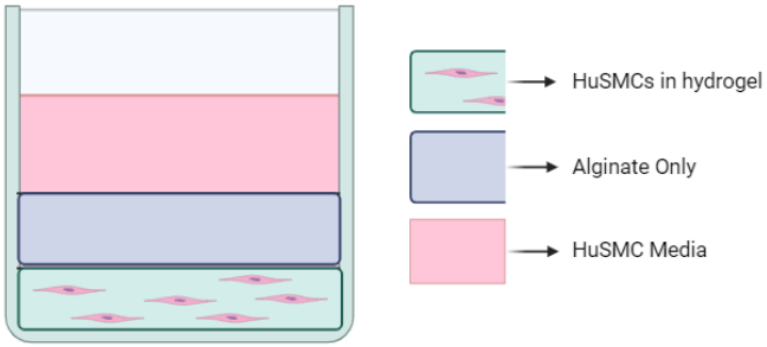


Figure 9. Top loaded diffusion design concept. Image created in BioRender with WPI institutional license.

4.2.6 2D Hypoxia Loaded Gel:

The use of cobalt chloride hexahydrate ($CoCl_26H_2O$) as a media supplement is an established method for modeling hypoxic conditions without altering the oxygen levels of any incubators in use (Wu & Yotnda, 2011).

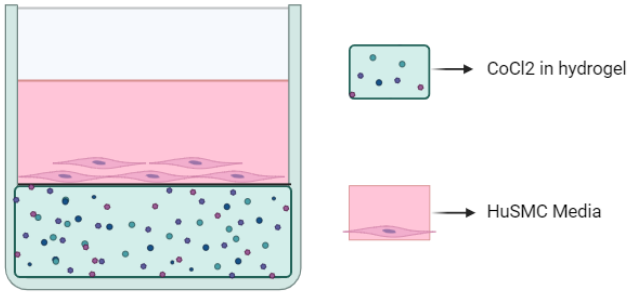


Figure 10. 2D hypoxia design concept. Image created in BioRender with WPI institutional license.

However, the negative effects of the cobalt chloride solution on cell viability limit the concentration of cobalt chloride safe to administer to the cells at a single timepoint, as well as limiting the duration of culture in such conditions. To circumvent this issue, it was theorized that a slow-release hydrogel that limits the instantaneous concentration of delivered cobalt chloride may allow for safe, long-term incubation of myometrial cells in hypoxic conditions. The composite hydrogel, made up of alginate and type I collagen, will be formed at the bottom of a 24 or 96 well plate, then loaded with the desired cobalt chloride solution. Myometrial cells will be cultured on top of the cell, where the cobalt chloride released over time by the gel will create a model of the hypoxic conditions present in uterine fibroids. The 2D hypoxia design allowed for

an accurate replication of a long-term hypoxic environment but a lack of prior research for the CoCl2 was a main disadvantage.

4.3 Alternative Design selection

Informed by the pairwise comparison (see section 3.4.1), a weight value of each objective was developed to be used in the Pugh Matrix analysis of each design concept, where a higher numerical value corresponds to higher importance within the design considerations.

Table 4. Objectives Weight Value

Objective	Weight Value
Time	1
Cost	1
Ease of Analysis	2
Interface	3
Reproducibility	4
ECM	5
Cell	5

To compare the relative value of our preliminary design concepts we evaluated each concept against the gold standard of patient matched 2D cultured fibroid cells across each design objective. Within each cell, the first score denotes the unweighted relative performance of each concept (as denoted in the column header) with regards to the objective (as denoted in the row header). The relative performance is represented by a -1, 0, or 1, indicating worse, same, or improved performance, respectively, as compared to the gold standard. The scores within the parenthesis are the weighted performance of each concept with respect to the objective, as calculated by the unweighted relative performance multiplied by the weight of the objective (denoted by the value within the parenthesis in each row header). The total score, shown at the bottom row of the matrix, is the sum of each weighted performance for the preliminary concept, and represents the relative "value" of each concept. Concepts with higher total scores pose more value as a design in accordance with our relative importance of objectives and the performance for each objective relative to the gold standard design. The gold standard design was patient matched 2D cultures of fibroid and myometrial cells which lacked representation of the ECM interface and not accurate to *in vivo* conditions. The Pugh matrix analysis suggested the top loaded diffusion, layered 3D coculture, 2D hypoxia, and 3D fibroid by itself have the greatest

relative value as designs. The motivation for this ranking was due to the advantages and disadvantages mentioned above. Moreover, they were the most suitable for fulfilling the established design objectives, making the front runners stand out in the decision matrix. The choice not to pursue additional investigation into the 3D fibroid by itself model stemmed from the significant disadvantage posed by the absence of myometrium interactions.

Table 5. Pugh Matrix

	Gold Standard – patient matched 2D	3D Fibroid	3D Ring	Ring and Sphere Coculture	Top Loaded Diffusion	Layered 3D Coculture	2D Hypoxia
Cell (5)	0	1 (5)	1 (5)	1 (5)	1 (5)	1 (5)	0
ECM (5)	0	1 (5)	1 (5)	1 (5)	1 (5)	1 (5)	0
Reproducible (4)	0	0	-1 (-4)	-1 (-4)	0	0	1 (4)
Interface (3)	0	1 (3)	0	1 (3)	1 (3)	1 (3)	1 (3)
Ease of Analysis (2)	0	-1 (-2)	-1 (-2)	-1 (-2)	-1 (-2)	-1 (-2)	1 (2)
Cost (1)	0	-1 (-1)	-1 (-1)	-1 (-1)	-1 (-1)	-1 (-1)	0
Time (1)	0	-1 (-1)	-1 (-1)	-1 (-1)	-1 (-1)	-1 (-1)	0
Total	0	9	2	5	9	9	9

4.4 Final Design Selection

To verify the leading design concepts, several criteria were examined to ensure design objectives and specifications were met. These criteria pertain to stiffness, viability, contact inhibition, structural integrity of the gels and standard curves linear regression. We aim to ensure cell viability remains between 70-80% to ensure we have enough cells for testing and observation purposes, while limiting contact inhibition that Primary Uterine Smooth Muscle cells exhibit. We aim to model both the stiffness of uterine fibroids and the stiffness of the affected myometrium. Uterine fibroids have an average stiffness value of 2.12 kPa (Jondal et al., 2018). The stiffness of the myometrium of an affected individual has a value of 18.6 kPa (Rogers et al.,

2008). Additionally, we aim to ensure that the control conditions do not result in unexpected morphological changes and that cells maintain a certain sterility, without any contamination. Another constraint is to ensure that the gels are visibly intact after soaking in the various materials that are used in our design concepts. Along with, ensuring that all of the standard curves have an R value that is greater than 0.8 to ensure that the standard curve will provide accurate information for the experiments. Additionally, we compiled these criteria into the table below.

Table 6: Testing Criteria

Item	Criteria
Cell viability	**> 80%**
Cobalt chloride 100% diffusion	**< 24 hours**
Hydrogel stiffness values	**2kPa - 6kPa**
Maintain structural integrity of hydrogels	**Gel is visibly intact**
Standard curves match linear regression	**R Value > 0.8**

5.0 Testing Methods for Design Verification

5.1 2D Viability Testing

To assess the design concepts, three overarching tests were identified: viability, stiffness, and diffusion. These three experimental methods were chosen to determine which design concept most accurately meets the system's clinical and engineering needs. Viability testing was divided into 2D viability and 3D viability testing. 2D viability was used on the 2D hypoxia model and to determine how cobalt chloride ($CoCl_2$), used to induce hypoxia, affects cell viability. While it was relevant to the model to induce hypoxia, it was necessary not to induce cell death.

General materials needed for this method are 96 well plates suitable for tissue culture, micropipettes with corresponding tips, spectrophotometer and the Cell Titer 96 Aqueous One Solution Reagent (Cell Titer). All wells were seeded on the same day at a density of 1×10^4 cells per well and given 24 hours to adhere. $CoCl_2$ was added at different concentrations for both short and long-term exposure (2 hours and 24 hours), then read on a plate reader with the Cell Titer at an absorbance of 490 nm. These tests determined how the health and conditions of cells were

impacted through several factors and models. To fabricate a standard curve for both cell populations, the 96 well plates were set up as shown below.

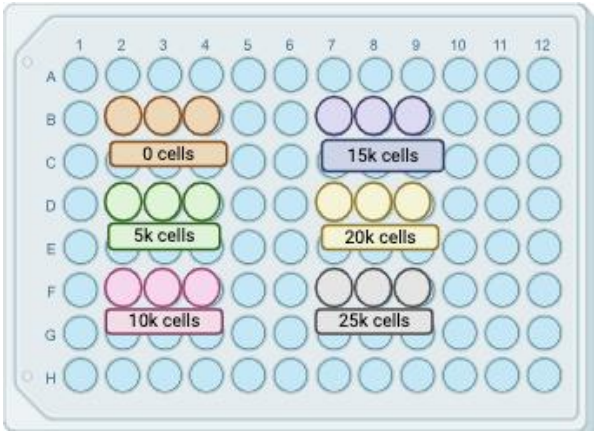


Figure 11. 96 well plate set up for standard curve fabrication. Image created in BioRender with WPI institutional license.

For experimental data, well plates were seeded at a density of 1 x 10^4 cells per well. Varying concentrations were placed in each well along with controls. These plates were set up as shown in Figure 12.

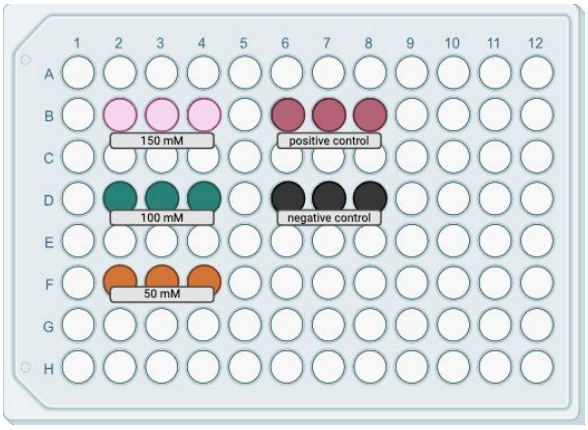


Figure 12. 96 well plate set up for 2D experimental tests. Image created in BioRender with WPI institutional license.

5.2 3D Viability Testing

3D viability was used on the top layer diffusion (figure 5) and 3D co-culture (figure 7) models to evaluate how the hydrogel polymerization affected the HUtSMC and 3T3 fibroblast viability int the 3D culture conditions. General materials needed for this method were the same as the 2D viability testing protocols. For top layer diffusion the 20mg/mL alginate hydrogel was

polymerized with the 150mM $CaCl_2$ with the HUtSMC inside the composite collagen/alginate hydrogel. The interactions of the Primary Uterine Smooth Muscle Cells and 3T3 fibroblast cells had to be evaluated with calcium chloride and hydrogel. The polymerized gels were allowed 24 hours to settle and seed before using the Cell Titer to evaluate the viability of the HUtSMCs and 3T3 fibroblasts. For the 3D co-culture model, the 3D viability assessed the viability of cells both inside and on top of the hydrogel. There was a standard curve created for the 3D viability of both top layer diffusion and 3D co-culture. These tests determined how the health and conditions of cells were impacted by the polymerizing of the hydrogel with $CaCl_2$. For experimental data, the 96-well plates were set up as shown in the left side of Figure 13. A side view of the well plate can be seen on the right side of Figure 13. The viability of the cells should be over 70% for all viability.

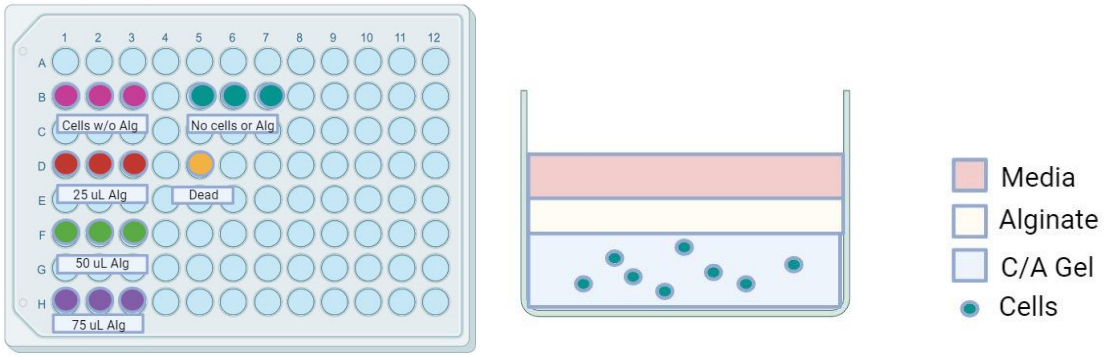


Figure 13. 96 well plate set up for 3D viability experimental tests (left). BioRender image of the side view for each well in the 96 well plate used for 3D viability experimental testing (right). Image created in BioRender with WPI institutional license.

5.3 3D Top Layer Diffusion Testing

Diffusion testing was used for the top layer diffusion experiment to evaluate how the fluorescein sodium salts diffused through the composite collagen/alginate hydrogel. The groups that were used have a layer of the composite collagen/alginate hydrogel with a layer of alginate to replicate the stiffness of myometrium. This set up attempted to understand how estrogen would diffuse through a myometrium to get to the cells within the hydrogel.

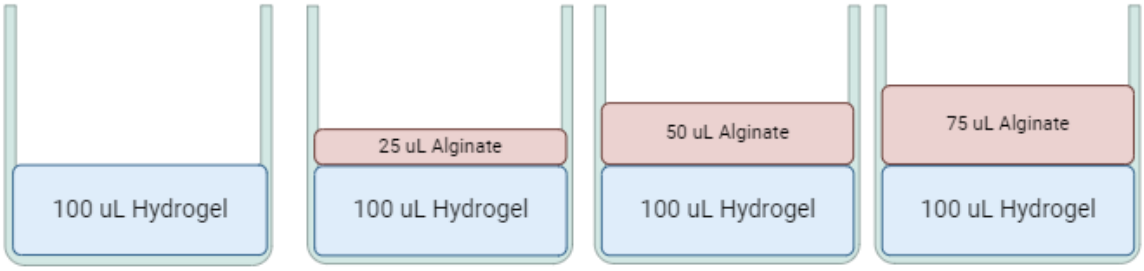


Figure 14. Representative diagram of the various set ups used for the 3D top layer diffusion experiment. Image created in BioRender with WPI institutional license.

The hydrogel was created using TeloCol®-10 (Advance BioMatrix) at a concentration of 5mg/mL with alginate at a 15 mg/mL concentration. The alginate was polymerized using 100 µM calcium chloride. After the hydrogel was polymerized for 50 minutes another layer of alginate only was added on top of the hydrogel at varying volumes of 25, 50 and 75 µL to vary transport by changing the height of the overlaid material. The added layer of alginate was polymerized for 10 minutes using 100 µM calcium chloride before 100 microliters of the 1mg/mL fluorescein sodium salt solution was placed into the wells. The plate was evaluated at 30 mins, 1 hr, 2hrs, and 3 hrs after the fluorescein sodium salt solution had been added. This 96-well plate experiement was initially attempted to be read on a Keyence BZ-800 All-in-One Fluoresence microscope. This initial testing was to see if Z-stack imaging could be used to evaluate where the leading edge of diffusion was located in the hydrogel at the time points. When the plate was viewed through the microscope it was found that there was too much signal to get a clear image of where the leading edge of the diffusion was located.

Analysis was readjusted to using transwells in order to accurately evaluate the absorbance of the fluorescein sodium salts diffused through the hydrogel set up. In the transwell set up there was the donor solution which was the fluorescein sodium salt solution above the composite hydrogel in the transwell. There was an acceptor solution which was the PBS solution beneath the composite hydrogel outside of the transwell. This allowed for the donor solution to diffuse into the acceptor solution through the hydrogel and the transwell before going into the acceptor solution. This second iteration of testing used 5 transwells with two having the hydrogel and the alginate top layer while the other three only had the hydrogel as shown in Figure 15. This experiment had a top layer of alginate that was 25 µL. At various time points between 30

minutes and 31 hours, 25 µL of the acceptor solution was taken out of the 24-well plate to be read on the VICTOR® Nivo™ multi-mode microplate reader at an absorbance of 492 nm.

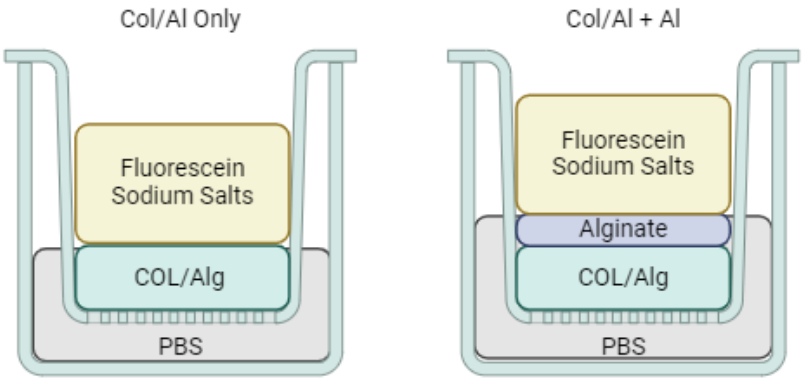


Figure 15. Representative diagram of the transwells set up with both the hydrogel only and hydrogel with an alginate layer. Image created in BioRender with WPI institutional license.

5.4 2D Hypoxia Diffusion Testing

For the 2D hypoxia design concept, the diffusion of cobalt chloride was used to create a hypoxic envirnoment. The groups used had a composite collagen/alginate hydrogel created using TeloCol®-10 (Advance BioMatrix) at a concentration of 5mg/mL with alginate at a 15 mg/mL concentration. The hydrogel is first polymerized and soaked in a solution of PBS and cobat chloride. Then moved to a PBS only solution where we can monitor the amount of the cobalt chloirde diffuses at various time points using a plate reader. After initial testing, it was found that the hydrogel would disintegrate when incubated at 37°C and 5% CO_2 for 24 hours.

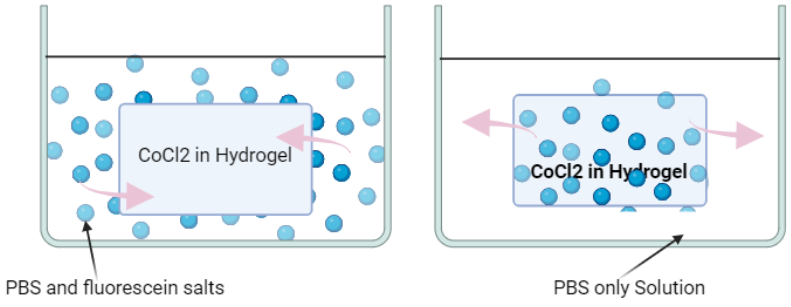


Figure 16. 2D hypoxia diffusion testing design concept. Image created in BioRender with WPI institutional license.

To figure out which part of the hydrogel was disintegrating, a new experiment was designed to evaluate both the 5mg/mL collagen and 15mg/mL alginate with the $CoCl_2$ in

individual wells. After separating the two parts of the hydrogel, experiments were set up to use an alginate only gel with a collagen top layer that would be used to adhere cells to. This new experimental set up had its own set up challenges due to the $CoCl_2$ and PBS solution that was taken from the alginate hydrogel would soak into the collagen in the second well as shown in Figure 17.

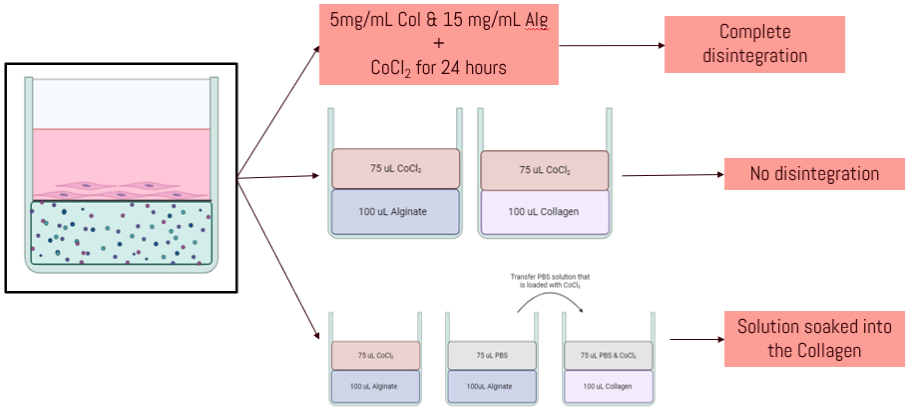


Figure 17. 2D hypoxia diffusion experimental set up for the three iteration of testing that were used. Image created in BioRender with WPI institutional license.

5.5 Stiffness Testing

To determine the stiffness of the composite collagen/alginate hydrogels, an AntonPaar 302e WESP rheometer was used in oscillatory shear to evaluate the shear storage modulus (G'). The storage modulus of the gel informs whether the gel accurately models the ECM stiffness relative to native myometrial and fibroid tissue. According to literature, a stiffness value of greater than 4kPa ensures that the composite hydrogel stiffness is greater than healthy myometrial tissue (Rogers et al., 2008). Literature also suggests the stiffness of some fibroids lies around 18kPa, though a stiffness that great is difficult to achieve using a composite collagen/alginate hydrogel. Prior to determining stiffness, an amplitude sweep at 1 rad/sec constant angular frequency and linear ramp of 0.1-1% strain was run for each gel to verify the linear viscoelastic region (LVR), in which the storage modulus is constant across a range of strain values. The storage modulus of each gel composition was then determined using an Anton Parr Rheometer, via a constant frequency, constant strain time sweep at constant 1 rad/sec and a strain value that fell within the LVR. The gels were fabricated prior to testing, at a 1-inch diameter and about 1mm height. The

measurement tool used was a 25mm parallel plate. Storage Modulus values were determined by taking the average storage modulus reading from time = 50s to the end of the run (times = 150s). To evaluate whether varying the alginate or collagen concentration had a significant effect on the stiffness, an unpaired t-test was performed between groups.

5.6 Novel Composite Gel Formation

Rheological analysis was performed on pre-polymerized hydrogel pucks, however initial attempts to fabricate these pucks resulted in significant cratering of the surface when the $CaCl_2$ polymerizing solution was applied with a micropipette. To fabricate composite collagen/alginate hydrogel pucks at the desired dimensions with a smooth surface topography, a novel fabrication method was developed. PDMS sheets were fabricated with a thickness of roughly 1mm, and circular punches were used to cut ring molds from the sheet with an inner diameter of 1 inch and an outer diameter of 1.25 inches. The ring molds were then placed on a large glass petri dish. Prior to gel fabrication, the glass petri dish was placed in the incubator at 37°C. To create the puck, a total volume of 900µL composite hydrogel was necessary, made up of equal volumes of collagen and alginate. 450µ L of TeloCol®-6 or TeloCol®-10 is neutralized according to the distributor's instructions, then an equal volume of pre-prepared alginate is added to the collagen. The collagen/alginate mixture was slowly pipetted up and down while gently swirling to fully combine both hydrogels without incorporating bubbles. The composite hydrogel is then loaded into the PDMS mold and incubated at 37° C for 50 minutes. After incubation, a small piece of dialysis tape is carefully layered over the gel, then a second ring mold is placed on top of the dialysis tape and first mold. 1000µ L of 150mM solution of $CaCl_2$ is pipetted on top of the dialysis tape and left to incubate at room temp for 10 minutes. Finally, the gel puck is washed with 1X PBS 3 times for 5 minutes each, then transferred carefully to the rheometer.

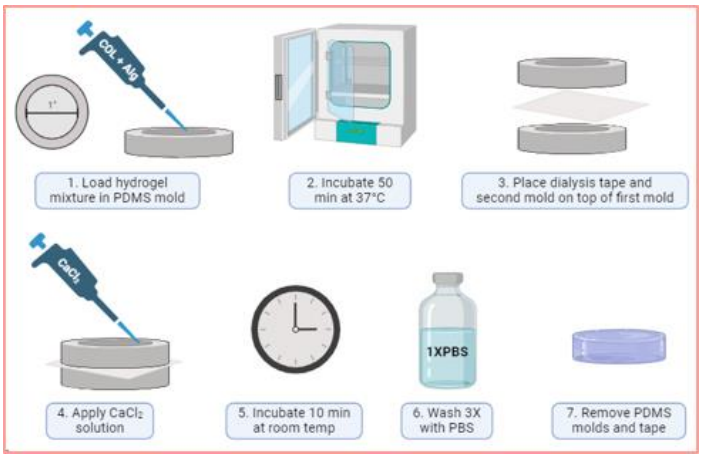


Figure 18. Schematic for novel hydrogel fabrication. Image created in BioRender with WPI institutional license.

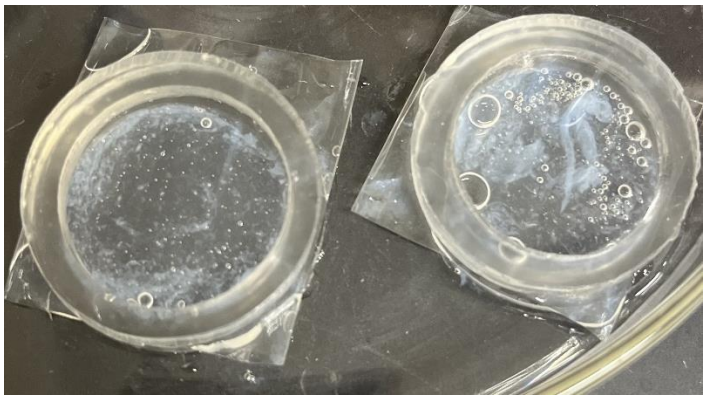


Figure 19. Photograph of novel composite collagen/alginate hydrogel fabrication setup.

5.7 Representative Cell Populations

3T3 fibroblast cells derived from mice (3T3) and primary uterine smooth muscle cells (HUtSMCs) were both utilized for viability studies. 3T3 cells were sourced from WPI's BME Cell Repository and HUtSMCs were sourced from ATCC. To maximize the time and ease of analysis for the model systems, NIH/3T3 fibroblast cells were utilized in testing and design concepts of 2D and 3D viability testing. This cell line is known to be robust and easily manipulated. As the HUtSMCs proved difficult to maintain over the course of this project, 3T3 fibroblasts proved to be an appropriate representative cell line for testing and design concepts. Use of this cell line allowed for better time management, ease of analysis, and reproducibility of results.

6.0 Results & Discussion

6.1 2D Viability Results

Testing for 2D viability was first accomplished through generating standard curves for both cell populations, 3T3 fibroblast and HUtSMC. Plating known cell counts was necessary as a baseline for further analysis of cell health and conditions. For standard curves, the plates were seeded at known densities ranging from 0-60,000 cells per well. Twenty µL of CellTiter reagent was placed into each well after plates were seeded for 10-24 hours at 37°C. These plates were incubated for another hour, then placed in a spectrophotometer at 490 nm to be read. A standard curve was generated from this data, which allowed for analysis of experimental plates. A few trials of generating a standard curve from the HUtSMC population had an R value significantly lower than acceptable. It was hypothesized that the HUtSMC population reacted poorly with the high seeding density within the small volume of the 96 wells. Standard curves were then readjusted to better accommodate these cells and were reevaluated for both cell populations from 0-25,000 cells per well. This allowed a more accurate standardization around the seeding density of both 3T3 fibroblasts and HUtSMCs, as well as limiting contact inhibition of HUtSMCs The standard curves used for 3T3 and HUtSMCs had R^2 values of 0.9265 and 0.8865 respectively, as shown in Figures 20 and 21, values deemed acceptable for future analysis.

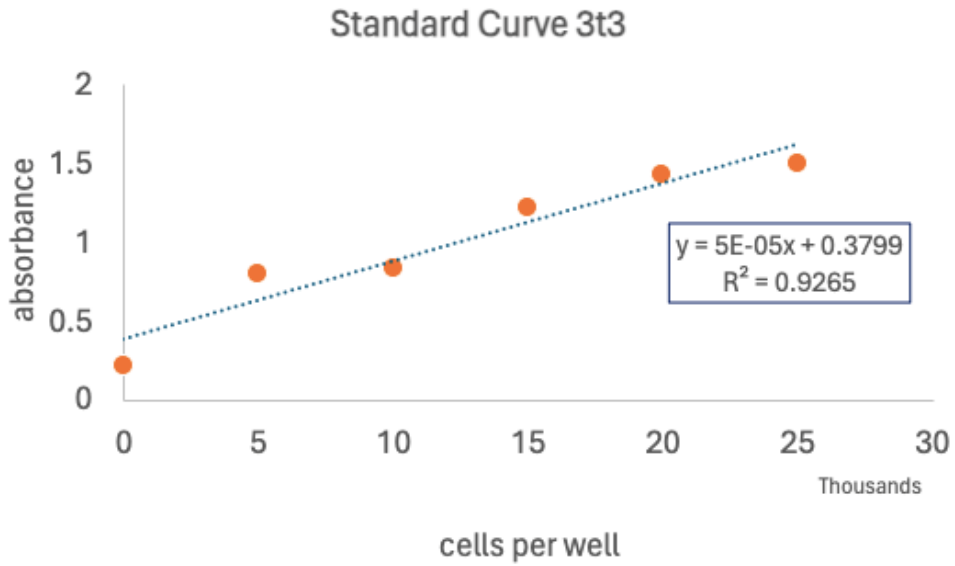


Figure 20. 3T3 Fibroblast standard curve generated over a range of 0-25,000 cells per well (n = 3).

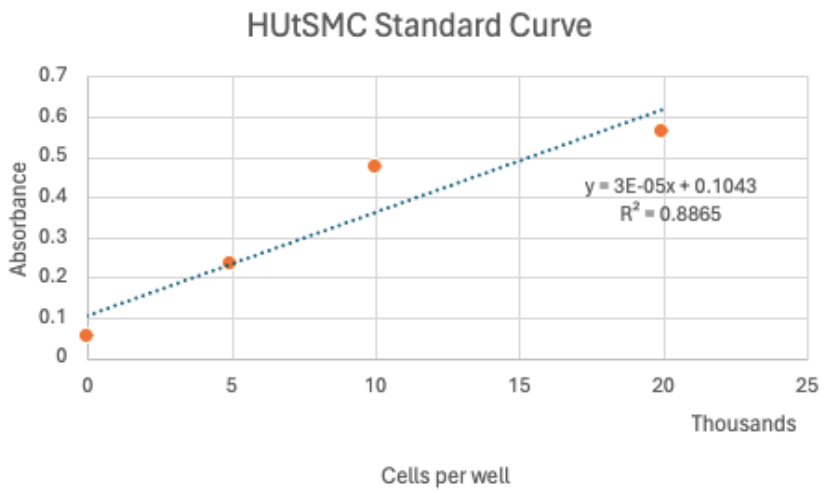


Figure 21. HUtSMC standard curve generated over range of 0-20,000 cells per well (n = 3).

Once standard curves were generated, experimental tests could proceed. Cells were seeded at 10,000 cells per well and with varying concentrations of $CoCl_2$ (50 mM, 100 mM, and 150 mM) to test the upper and lower limits of toxicity of this compound. A positive and negative control were present for every test, where positive controls contained cells in media with no $CoCl_2$, and negative controls contained dead cells.

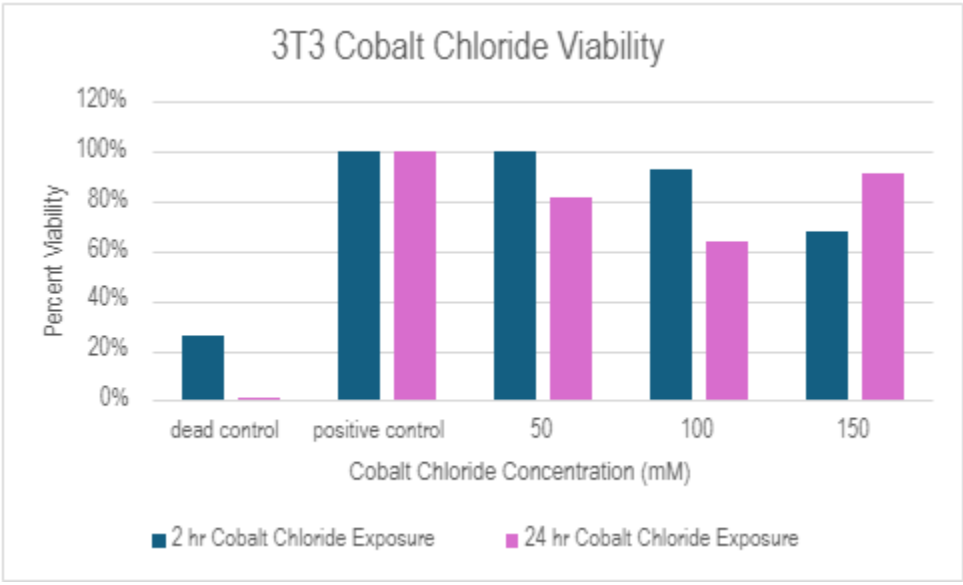


Figure 22. 3T3 viability (normalized to positive control) after short (2 hr) and long (24 hr) exposure to $CoCl_2$ (n = 3).

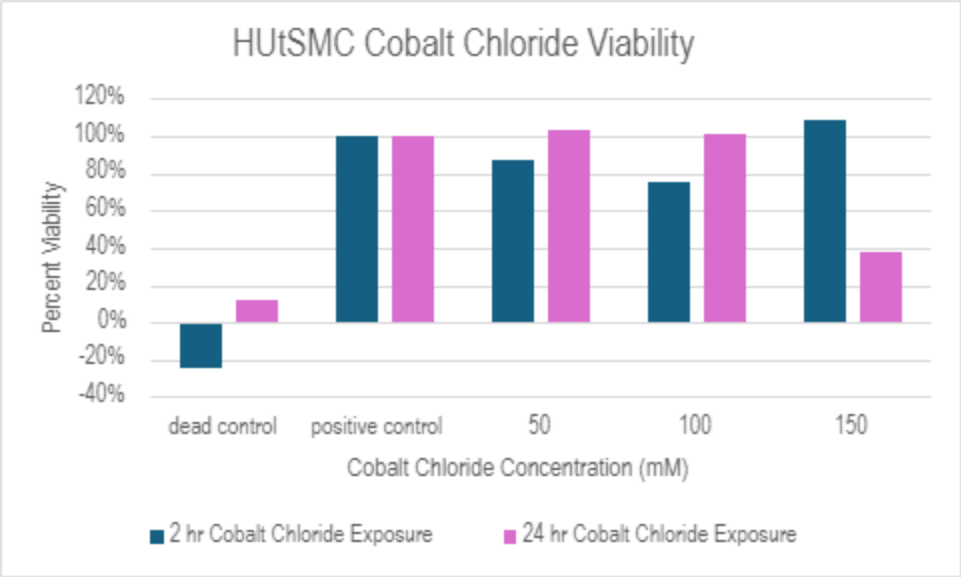


Figure 23. HUtSMC viability (normalized to positive control) after short (2 hr) and long (24 hr) exposure to $CoCl_2$ (n = 3).

Results from 2D viability testing determined that 3T3 exposure to a 150 mM $CoCl_2$ solution impacted viability after 2-hour exposure, but not for 24-hour exposure. The 100 mM $CoCl_2$ solution exhibited opposite effects, with viability impacted during 24-hour exposure, but not 2-hour exposure. Exposure at both time points to the 50 mM $CoCl_2$ solution did not impact viability at all as shown in Figures 22 and 23. $CoCl_2$ exposure inhibited cell proliferation and division at both 2 and 24-hours of exposure for HUtSMCs. Although the quantitative data for HUtSMCs looked healthy, when images were taken, it was shown that cells had clumped together and died. The CellTiter visibly reacted with the clumps of live cells, while the rest of the well did not emit any color change, as shown in Figure 25. This was indictive that these cells presented a negative reaction to the $CoCl_2$. Preliminary research indicates that $CoCl_2$ may induce apoptosis in certain primary cell lines. This has not yet been explored in HUtSMC populations but may explain discrepancies in data.

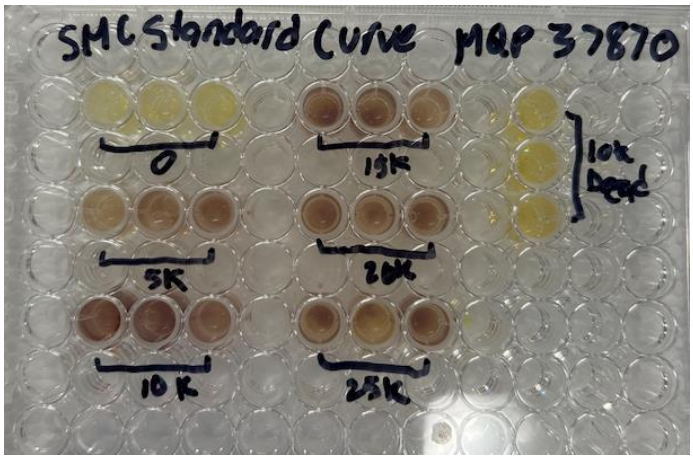


Figure 24. Standard curve generated for HUtSMCs, with dead controls. Figure serves to model the colorimetric changes observed when CellTiter reacts with viable cells.

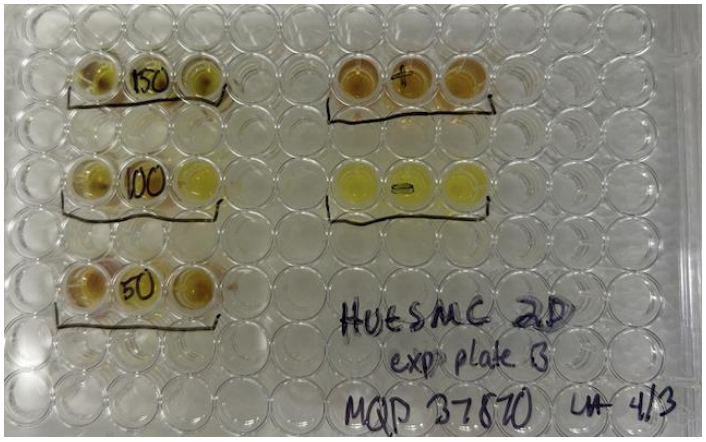


Figure 25. Experimental plate of HUtSMC population indicating cell clumping and death (indicated in yellow) due to 2-hr $CoCl_2$ exposure.

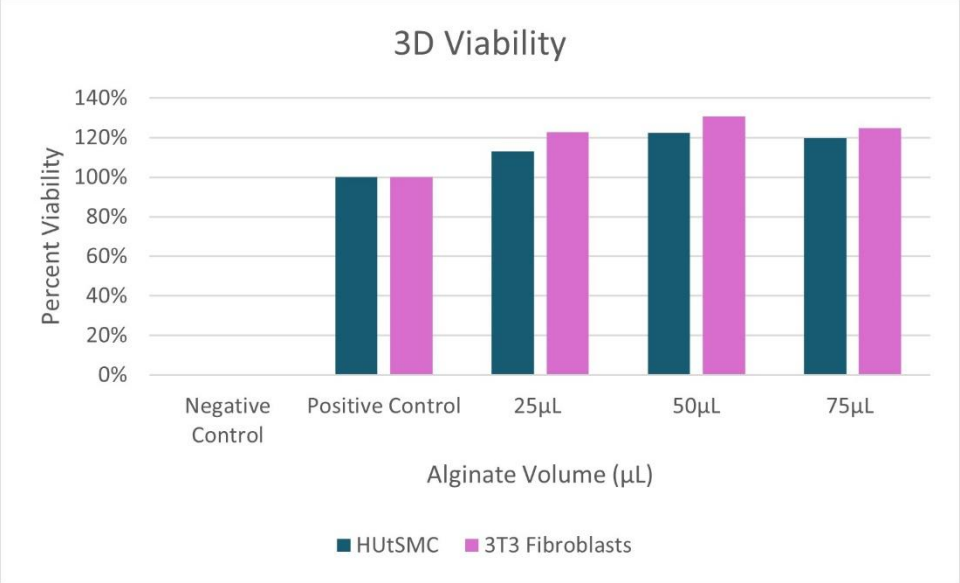
6.2 3D Viability Results

Testing for 3D viability was completed by combining 10mg/mL collagen, 20mg/mL, 150mM $CaCl_2$, and either 3T3 fibroblast or HUtSMC cells in a 96-well plate. Alginate layers were added on top in various volumes of 25μL, 50μL, and 75μL to change the height of the alginate later. Cells were seeded at a density of 10,000 cells per well. CellTiter was placed in the media after a 24–48-hour incubation period on top of the alginate and collagen hydrogel. It was then read with a plate reader.

The gel formation was not successful using a regular pipette, so a positive displacement pipette was used. The normal pipette caused the gel to not be uniform and contain many bubbles. We completed 2 trials of each of the cell types within the gels. As shown in Figure 26, the highest viability of the cells was the 50μL of alginate, but there was not a significant difference

across the different volumes. Cells appeared to be more viable compared to the positive control, meaning alginate may increase cell viability. The graphs are normalized to the live control. The positive control was live cells, and the negative control was dead cells.

Figure



26:HUtSMC and 3T3 Fibroblast 3D viability (normalized to positive control) (n = 2).

6.3 Stiffness Results

The strain values used for gels formulated with TeloCol®-6 and TeloCol®-10 bovine collagen were 0.5% and 0.9% strain respectively, as informed by the amplitude sweep to identify the LVR. As shown in Figure 27, the average stiffness values (storage modulus, G') for the composite gels was highly variable. The stiffness of the composite collagen/alginate hydrogels saw the greatest increase when altering the alginate concentration, whereas altering the collagen concentration had relatively little impact on the stiffness of the gels. However, the only statistically significant difference between groups was between the 5/10 and 5/15 collagen/alginate formulations. While the stiffness values we observed undershot our goal, this does confirm that altering the alginate concentration can effectively modulate the stiffness of the composite collagen/alginate gels. We attribute this error to high batch variability when producing the alginate stocks, as well as heterogeneity when creating the composite gel pucks used to perform rheological analysis. The fabrication method to create these pucks may have also

introduced elements of batch variability due to leakage of the $CaCl_2$ solution through gaps in the PDMS mold layers when polymerizing the gels.

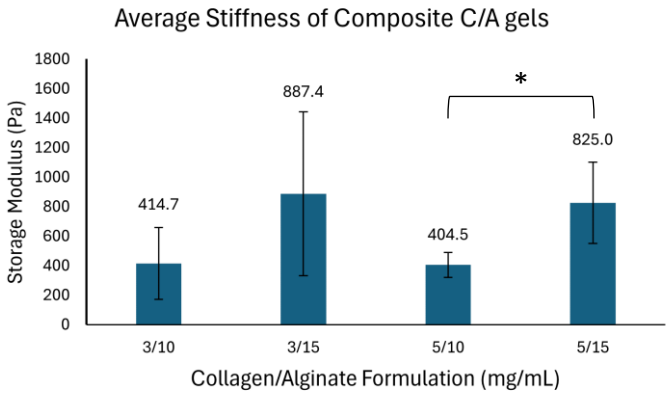


Figure 27. Average Stiffness values of composite collagen/alginate gels (n = 3-5, p < 0.05).

When investigating the stiffness of the stock collagen and alginate used to formulate the composite gels, we saw the highest variability in the TeloCol®-10 (10 Col) bovine collagen, and the 30mg/mL Alginate solution (30 Alg), as shown in Figure 28. The TeloCol®-6 bovine collagen (6 Col) exhibited less variability in the stiffness than the TeloCol®-10, which may be explained by the preparation differences between the two collagen types. For context, TeloCol®-6 is prepared with a neutralization solution provided by the manufacturer, using the same ratio for every batch, while TeloCol®-10 must be pH adjusted using a 0.5N NaOH solution every batch, leading to greater variability.

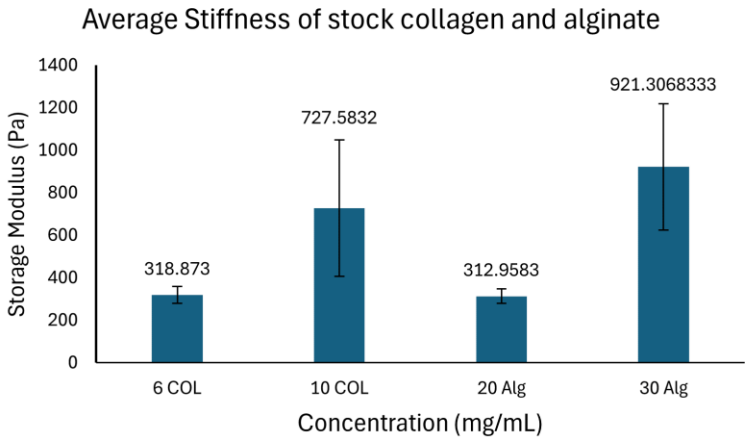


Figure 28. Average stiffness values of stock collagen and alginate gels (n=3).

6.4 Diffusion Results

6.4.1 Top Layer Diffusion

For the top layer diffusion, the hydrogels were created within both 96 and 24 well plates to see which sizing worked the best for our testing. The original testing did not provide quantifiable results. Images were taken at 1 hour increments as shown in Figure 29.

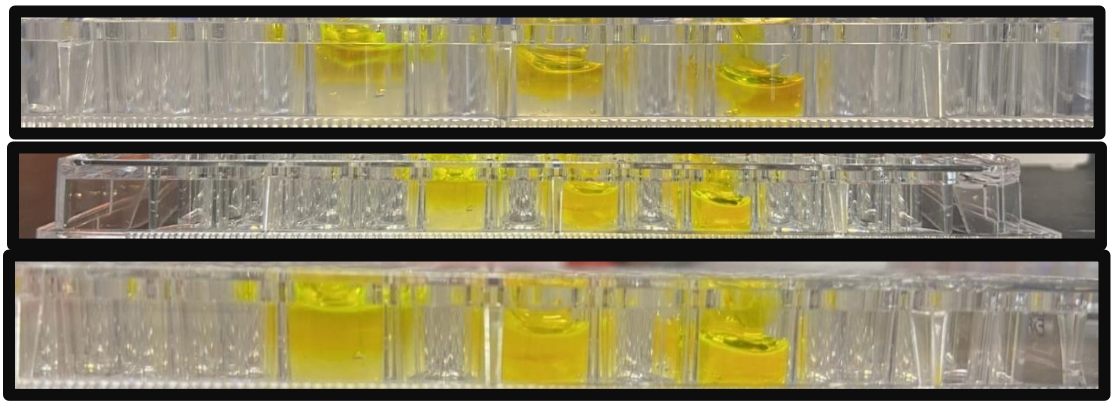


Figure 29. The top image is the diffusion of the fluorescein sodium salts at 1 hr. The middle image is at the two-hour time point. The bottom image is at the three-hour time point. The wells (from left to right) had a decreasing amount with the alginate layer.

A Keyence fluorescence microscope was used to assess the diffusion of the fluorescein sodium salts. When looking at the wells through the microscope, there was no distinguishable difference in the z-plane due to the brightness of the fluorescein sodium salts throughout the hydrogel. After this first iteration of testing, the design was readjusted to be analyzed using a different technique. With the transition to using transwell inserts, a standard curve to relate absorbance signal from fluorescein sodium salts to concentration was performed. A constraint of the experiment was that the R value needed to be greater than 0.8, the first iteration of the standard curve had an R value that was 0.98. The range of concentration that the standard curve was accurate for was from 0 micrograms/mL to 150 micrograms/mL.

With the standard curve created, the equation provided was used to insert the absorbance values for y and solve for the various concentrations of the solution that was removed from the acceptor solution at various time points. The concentration values that were found showed that the hydrogel on its own would begin to reach equilibrium before the hydrogel with the additional alginate layer would in Figure 31. More iterations of testing over a longer period to understand

when equilibrium will occur for both hydrogels set ups. This would definitively tell if the extra alginate layer slowed the rate of diffusion.

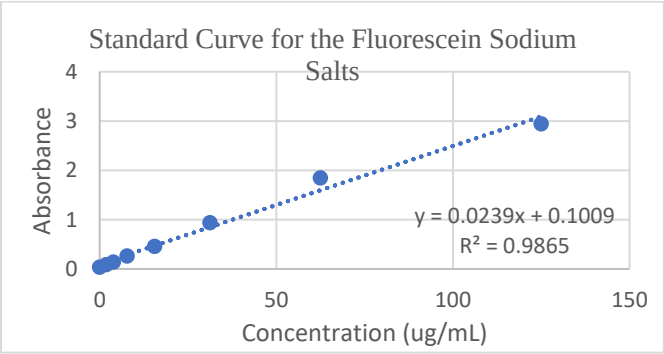


Figure 30. A standard curve created from the Fluorescein Sodium Salts (492nm absorbance).

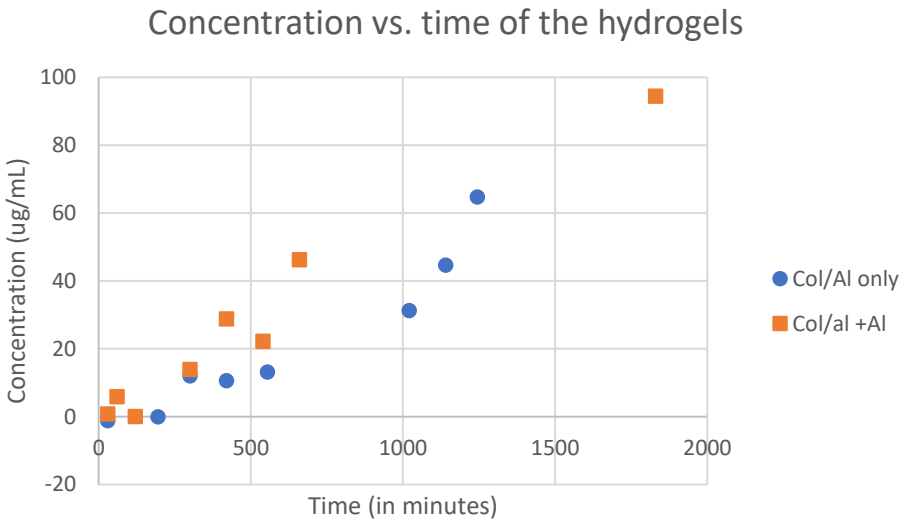


Figure 31. A comparison of the diffusion through a hydrogel only material and the hydrogel with the layer of alginate over a 31-hour period.

6.4.2 2D Hypoxia Diffusion

For the 2D hypoxia diffusion, a $CoCl_2$ solution is created using 0.0024g of $CoCl_2$ and10 mL of DI water was added for a solution of 0.001 M concentration. That solution was added to 55.67 mL of DI water to create a final concentration of 150 µM. In the first iteration of testing 5mg/mL of collagen and 15mg/mL alginate was polymerized for the hydrogel. The hydrogel was soaked in 100 µL of the $CoCl_2$ solution for 6 hours before the solution was removed and replaced with 100 µL of 1x PBS. Samples of the PBS solution was removed from the well at 3, 6-, 12-,

18-, and 24-hour marks. At the 24-hour mark, the hydrogel had completely dissolved in the well. Due to the hydrogel's disintegration a second iteration of the experiment was created to understand which part of the hydrogel was disintegrating. This second experiment used both the 5mg/mL collagen and the 15mg/mL alginate in two separate wells. The $CoCl_2$ was added again for 6 hours before being removed and replaced with PBS. The experiment found that both the collagen and alginate separately would not disintegrate when left alone with the $CoCl_2$ and PBS for 24 hours. Given that both materials did not disintegrate, a third iteration of testing was created to use an alginate only hydrogel with a collagen top layer so that the cells would still be able to adhere and survive on the collagen and the alginate can be loaded with the $CoCl_2$. Testing was completed to identify whether a thin layer of collagen could withstand the CoCl2 concentrations that would diffuse out of the fully loaded alginate layer below. The alginate and collagen layers were created in 2 separate wells so that it would be visually distinct whether the collagen layer maintained its structure. First, the alginate layer was incubated with 100uL of the CoCl2 solution for 6 hours to fully load it into the gel, then the excess solution was removed. In order to simulate the diffusion of CoCl2 into the collagen layer, 1xPBS was then incubated with the fully loaded alginate layer for 12 hrs., then removed from the alginate and transferred to the collagen well. However, the solution applied to the collagen was found to have soaked into the collagen layer and could not be removed for further analysis. To avoid this in the future, another iteration of testing should occur with a greater volume of 1xPBS after the alginate is fully loaded.

7.0 Final Design and Validation

Throughout the duration of this project, design objectives were utilized as a guideline for testing and model fabrication. Through our pairwise comparison chart, we determined the most important objectives were accurate to cell composition (Objective #3), accurate to ECM composition (Objective #2), and reproducible (Objective #5).

Objective #3 was only partially met through completion of this project. While we were able to utilize 3T3 fibroblast cells as a representative cell population, this cell line does not fully capture all the cell behavior of the HUtSMCs. Additionally, an immortalized cell line such as 3T3 did not replicate the same apoptotic behavior through exposure to $CoCl_2$ as the primary cells

did. Overall, health and viability aspects of this immortalized cell line do not fully represent that of HUtSMCs *in vitro*.

Objective #2 was also only partially met. Although a composite collagen/alginate hydrogel was fabricated that supported cell health and viability, the gels exhibited high variability and did not meet the target stiffness level of 2kPa - 6kPa . An important aspect of the uterine myometrium that contains fibroids is its excessive stiffness. Although a hydrogel was fabricated, additional iterations with different set ups need to be performed to meet this target stiffness so molecular transport through the uterine myometrium can be fully modeled.

Objective #5 was an important objective for the group to follow, however it was not met. We experienced lots of batch variability within alginate fabrication, leading to inconsistent stiffness values. Inconsistencies with cell maintenance also contributed, as the HUtSMCs exhibited increased clumping and cell death through exposure to $CoCl_2$. Modeling the fibroid/myometrium interface (Objective #1) was not met through this project. While we worked to incorporate a 3D modeling system that modeled the fibroid/myometrium interface, issues with $CoCl_2$ and coculturing of cells led us to choose top-layer diffusion as our final design concept. While this model represents molecular diffusion through the myometrium, the project goal shifted to accomplish this, not necessarily the fibroid/myometrium interface. Additionally, we were limited by the lack of fibroid cells, and high cost associated with them.

We were able to fully meet was ease of analysis (Objective #6). Viability assays were easily read on a spectrophotometer; fluorescein salt diffusion was analyzed with a Keyonce microscope; and rheological data was easily obtained and compiled through the rheometer. Readable, accurate data was collected and compiled through these methods.

Cost efficiency (Objective #4) was also met through this project. We had a budget of $1250 that was mostly adhered to through the purchase of materials for this study. We spent $1335 through this project, and only necessary materials were purchased.

Finally, this project was achievable within the timeframe (Objective #7). Testing methods were completed to determine cell health and viability, characterize stiffness of a composite collagen/alginate hydrogel, and analyze diffusion through the hydrogel. Although a comprehensive model was not fabricated, all individual components were tested to their best ability to determine the molecular transport through the uterine myometrial tissue.

In section 3.3, we outlined the ISO Standards necessary for completion of this project. Those standards were:

ISO 10993-1:2018-Biological evaluation of medical devices- Part 1: Evaluation and testing within a risk management process

ISO/AWI TR 4752- Inventory of methods for detection of microbiological contamination in mammalian cell culture

ISO/TC 150/SC 7- Tissue-engineered medical products

ASTM F813-20-Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices

The ISO Standards were closely met, as proper cell culture techniques were followed, and contamination was kept to a minimum. This model was properly evaluated and tested for risk management and for future applications. Proper laboratory techniques were maintained throughout the project.

On a larger scale, the overall impact that this project would have on different facets needed to be evaluated. These facets were economic impact, environmental impact, societal impact, political impact, ethical concerns, health and safety concerns, manufacturability, and sustainability.

7.1 Ethics statement

As part of our engineering approach, our team carefully considered the impact of our designs in a number of contexts listed below. We did our utmost to consider not just the ethical concerns that impact us as researchers, but especially those who could be impacted by our designs down the line, in the event that these designs are implemented on a larger scale. These considerations impacted not just the initial development of these design concepts, but also any iterations we made to our designs in the latter portion of the project.

7.1.1 Economic Impact

By focusing our designs on developing the *in vitro* research space of uterine fibroids, our team hopes to make a positive impact on the economics of fibroid research. Staying within a budget of roughly $1000, we have explored lines of research that are very accessible to labs with limited funding opportunities. By reducing the volume of collagen by 50% and replacing it with

the much more economically accessible sodium alginate hydrogel, we were able to significantly reduce the cost of the 3D hydrogel without compromising cellular viability. We also utilized cost-effective lab techniques such as adapting a commercial pressure cooker for autoclaving applications.

7.1.2 Environmental Impact

While maintaining sterility is an integral part of cellular research, our team aimed to reduce as much plastic waste as possible while completing our testing, for a minimal impact on the environment. When possible, our team utilized reusable glass dishes rather than disposable plastic well plates to manufacture the hydrogel pucks used in rheological testing. We also decided to maintain the minimum number of cell flasks necessary to complete testing in order to conserve materials necessary for passaging and media changes. Proper chemical disposal techniques were used to avoid contamination of reagents and biohazards into the water supply and the greater environment.

7.1.3 Societal Impact

Our team recognized the importance of 3D architecture to understanding the mechanisms and development of uterine fibroids, but also understood that sourcing patient explant material for organoid research is not trivial for the majority of researchers. By designing a platform to investigate myometrial cells in 3D without the need for hard to source explants, we hoped to improve the accessibility of uterine fibroid research and increase the collective knowledge on uterine fibroids. If more researchers are given the opportunity and means to focus their research on uterine fibroids, the research space becomes one step closer to an improved treatment for fibroids. Identifying an effective treatment plan for uterine fibroids would improve the quality of life for millions of women, and understanding the impact that fibroids have may help reduce stigma associated with reproductive diseases in women.

7.1.4 Political Impact

Currently, the issues of women's health are highly political in the United States of America. By bringing uterine fibroid research into the public eye, we hope to increase the understanding that the public and politicians have about women's reproductive diseases, so that

government funding of women's health research might increase. Understanding of uterine fibroids as well as potential treatments may also become increasingly politically relevant within the sphere of universal healthcare, where it may impact what types of treatments are covered by health insurance.

7.1.5 Ethical Concerns

The greatest ethical concern within our research we feel is the negative impact that misleading results may have on the research space. The relatively low sample size of experiments this research (generally n = 3) means our conclusions should be interpreted with caution, as the data may suggest otherwise with a greater sample size. Independent research should also be conducted so that erroneous assumptions are not made. Additionally, the team recognizes the ethical implications of using a very narrow range of human myometrial cells, in our case a single cell line. Human biology is diverse, and experiments using cells from a single individual might not be applicable to all patients, as there may be unknown differences in individuals with different sex characteristics or heritages.

7.1.6 Health and Safety Concerns

While using proper laboratory safety techniques our team does not anticipate our designs to pose any great health and safety risk, however we did utilize human and mouse cell lines that should be treated as a biohazard and disposed of correctly. We also used acid solutions to pH the TeloCol®-10 bovine collagen which should be handled carefully to minimize exposure. Proper biohazard disposal of reagents and other research materials is also important for maintaining the safety of lab personnel. Additionally, careful handling of dry ice used in transporting research materials between buildings and proper technique retrieving cells from liquid nitrogen cryo-storage should be used to avoid burns to the skin.

7.1.7 Manufacturability

Overall, our team believes our designs to be generally easy to manufacture in the average laboratory setting provided the correct stock materials. The techniques used to maintain the cell populations are fairly standard, and the protocols for creating the composite hydrogels are contained in the appendices of this report. However, we do believe that the production of the pre-

polymerized gels used in rheological experiments should be iterated on to reduce $CaCl_2$ solution loss during gel polymerization. We also believe that a potential obstacle in manufacturing these gels may be our current method of alginate production and sterilization, which seem to introduce significant batch variability.

7.1.8 Sustainability

While our team does not believe that our methods are less sustainable than the average research method, we do share common sustainability concerns, such as the sustainability of common materials like fetal bovine serum (FBS). Current debates on the ethical implications of using animals in research and animal-derived research materials may lead to a reduction of such products. In such an event it may be necessary to pursue other media formulations for maintaining our cell populations. Similar concerns may be voiced about the use of bovine-derived collagen in the hydrogel, which may be addressed by utilizing synthetic hydrogels such as polyethylene glycol (PEG) that have been altered for necessary cell adhesion.

8.0 Conclusions and Future Recommendations

Based on our testing and analysis of our objectives, our primary conclusion is that the 3D top loaded design model shows promise as a means of modulating molecular transport for study of uterine fibroids and the myometrium. While the stiffness testing performed on the collagen/alginate composite hydrogels seems to suggest a high degree of batch variability, preliminary results from the diffusion testing seem to suggest that a top layer of alginate may be an effective means to modulate molecular transport through a hydrogel. Also, 3D viability assays suggest the top-layered hydrogel format does not negatively impact cellular viability. While investigating the behavior of myometrial and fibroid cells under hypoxic conditions holds merit for improving understanding of the fibroid microenvironment, our findings suggest that further iterations should be made to the 2D hypoxia model to ensure structural integrity of the $CoCl_2$ loaded gel.

It is recommended that future work is done to investigate the performance of the alginate top layer as modulator of molecular transport. Experimentation should be performed to evaluate the modulation of diffusion when varying the concentration and volume of the alginate top layer, as well as further analysis of the data to identify key diffusion parameters such as diffusion

coefficient of permeability. Additionally, future testing should directly confirm the $CoCl_2$ treatment is producing hypoxic conditions in the cells. Potential strategies include using a western blot assay for hypoxia inducible factor 1 alpha (HIF-1 α) (Wu & Yotnda, 2011). Identifying the cause of composite gel disintegration when loaded with $CoCl_2$ would also help develop future iterations of the 2D hypoxia model.

To address the high batch variability in the stiffness of the composite gels, alternate methods of alginate preparation should be investigated. Potential strategies to mitigate volume loss from heating the alginate solution include the use of sonication for alginate preparation, and UV sterilization methods to replace autoclaving. When preparing pre-polymerized gel pucks for rheological studies, it is also recommended to prepare the unpolymerized composite gel for each puck individually, as we suspect batch preparation of the unpolymerized composite gel may have resulted in uneven distribution of alginate and collagen between pucks.

Future developments of *in vitro* uterine fibroid modeling involve combing multiple existing model systems. Where the TME is a complex system, a single model system fails to accurately address all the physiological aspects *in vitro*. Combining multiple model systems allows for the advantages of each one to be expressed. (Mu et al., 2023). At the molecular level, targeted nanoparticles, CRISPR Cas-9, and single cell sequencing are evolving technologies. Understanding the molecular basis of uterine fibroids is critical to developing a cohesive *in vitro* modeling system. (Machado-Lopez et al., 2021). A large gap within clinical and engineering needs lies in finding a multifaceted approach that addresses tissue interface, cell composition, fibrotic stiffening, 3D structures, and signaling all at once. Future *in vitro* models should aim to address all these needs within a single model. To improve upon the work of this project, iterations made to the 3D top loaded diffusion model would provide valuable insight into the mechanisms of molecular transport in the uterine myometrium.

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Appendix

Appendix A: Prepping the 6mg/mL COL

Note: It is recommended that the collagen and other working solutions be chilled and kept on ice during the preparation of the collagen.

1. Determine the desired volume of collagen required.
2. Transfer 1 part of chilled neutralization solution into a sterile mixing vessel or tube.
3. Transfer 9 parts of the TeloCol®-6 collagen into the sterile mixing vessel or tube for a total of 10 parts.
4. Gently agitate the mixture or pipet up and down to mix. Vortexing is not recommended.
5. Dispense the collagen mixture in the desired sterile plates or culture vessels.
6. Incubate at 37°C for 1 hour for gel formation.

Appendix B: Prepping the 10mg/mL COL

Note: It is recommended that the collagen and other working solutions be chilled and kept on ice during the preparation of the collagen.

Note: TeloCol®-10 is highly viscous and may be difficult to effectively mix.

1. Slowly add 1 part of chilled 10X PBS or 10X culture media to 8 parts of chilled collagen solution with gentle swirling.
2. Make sure to calibrate pH reader
3. Adjust pH of mixture to 7.2–7.6 using sterile 0.1 M NaOH. Monitor pH adjustment carefully (pH meter, phenol red, or pH paper). Mix with gentle swirling.
4. Adjust final volume to a total of 10 parts with sterile water and mix.
5. To prevent gelation, maintain temperature of mixture at 2–10°C.
6. Dispense the TeloCol®-10 collagen mixture in the desired sterile plates or culture vessels.
7. To form gel, warm to 37°C. Allow approximately 30 to 60 minutes for gel formation.

Appendix C: 3D Viability Testing Hydrogel Procedure

Collagen alginate hydrogel formation

1. 8 microL of neutralization solution was taken from ice pack and placed into a 2mL tube
2. 72 microL of TeloCol was added to the neutralization solution on ice
3. Added 80 microL of alginate 20 mg/ml into the same 2mL tube
4. Add cells to the mixture incorporating the cells by moving the solution in and out of the pipettor.
    a. Cell count per well will be 10k. Get this concentration by getting the total cell count and divide by how many mL media it is suspended in. Convert to cells/uL. Add some number of that is 10k cells.
    b. Example calculations
        i. 97,77,70,103 total cells
        ii. 1,735.000 cells/mL
        iii. 1735 cells/uL
        iv. 10k = 5.8uL

5. Took the 80 microL and placed into two wells of a 96 well
6. Place in incubator for 60 minutes
7. Remove from incubator
8. Add 80 microL of $CaCl_2$ into each well
9. Allow to sit for 10 minutes
10. Remove the $CaCl_2$
11. Add 75 microL of 10x PBS to rinse twice in each well
12. Remove the PBS from the well
13. Add 80µLculture medium to the top of the gel
14. Let sit 24-48 hours
15. Add 20uL of CellTiter
16. Incubate the plate at 37°C for 1–4 hours in a humidified, 5% CO2 atmosphere.
17. Record the absorbance at 490nm using a 96-well plate reader.

Appendix D: **Top Layer Diffusion Testing Protocol**

1. Pipette total of 5µLof Neutralization solution and 45µLTeloCol into each well of a 96 well plate while it is sitting on ice.
2. Get a 24 well plate and place PDMS mold into two wells.
3. Pipette 5µLof Neutralization solution and 45µLTeloCol into each PDMS mold
4. Pipette 50uL 30 mg/mL Alginate into PDMS mold along with into the wells of the 96 well plate.
5. Incubate for 60 minutes at 37 C and 5% CO2.
6. Remove from incubator.
7. Add 100µLof 150 µM $CaCl_2$ into both molds and the 96 well plate.
8. Allow to sit for 10 minutes
9. Add 75µLof 10x PBS and rinse wells and molds twice.
10. Remove PBS from wells and molds.
11. Place 100µLof 10% fluorescein salt solution into each well and mold.
12. Image on a fluorescent microscope at 15, 30, 45, 60-minute time marks.

Appendix E: Preparing the composite hydrogel

1. Clean the glass plate and place in incubator at 37C to warm up
2. Prep 450uL of ~~teloCOL~~TeloCol in a 2mL tube according to manufacturer's instructions
3. Add 450uL of pre-prepared Alginate and carefully pipette up and down to mix
4. Load into thin circular PDMS mold (~1mm thickness) so that the gel mixture is even and flush with the top
5. Incubate at 37C for 60 min
6. Place dialysis tape over the top of the pdms mold carefully, avoiding any air bubbles
    a. To improve dialysis tape application, you may wet the tape briefly to make it flexible, then remove excess moisture before applying it to the mold
7. Place the second pdms mold over the dialysis tape and press gently around the edge to secure
8. Add 1000µLof 150mM $CaCl_2$ and lit sit at room temp for 10 min

9. Aspirate the $CaCl_2$ solution
10. Wash 3X with 1XPBS , letting each rinse sit for 5 min
11. For storage, place the pre-polymerized gel in a 6 well plate with 3mL of 1X PBS and refrigerate.

Appendix F: Generating 2D Viability Standard Curve

1. Seed 96 well plates (as shown above) 10-24 hours in advance and place in incubator
2. Thaw the CellTiter 96® AQueous One Solution Reagent. It should take approximately 90 minutes at room temperature, or 10 minutes in a water bath at 37°C, to completely thaw the 20ml size.
3. Pipet 20µl of CellTiter 96® AQueous One Solution Reagent into each well of the 96-well assay plate containing the samples in 100µl of culture medium. Note: We recommend repeating pipettes, digital pipettes or multichannel pipettes for convenient delivery of uniform volumes of CellTiter 96® AQueous One Solution Reagent to the 96-well plate.
4. Incubate the plate at 37°C for 1–4 hours in a humidified, 5% CO2 atmosphere.
5. Record the absorbance at 490nm using a 96-well plate reader.

	0					15k					
	5k					20k					
	10k					25k					

Appendix G: 2D Viability Experimental Protocol

1. Seed 96 well plates (as shown above) 10-24 hours in advance and place in incubator
2. Pipet5 50µl of each $CaCl_2$ solution into well plate shown below and shake lightly to mix
    a. Prepare 3 concentrations of $CaCl_2$ solutions using DI water

	150uM				Pos Control						
	100uM				Dead Ctrl						

	50uM										

3. Thaw the CellTiter 96® AQueous One Solution Reagent. It should take approximately 90 minutes at room temperature, or 10 minutes in a water bath at 37°C, to completely thaw the 20ml size.
4. Pipet 20µl of CellTiter 96® AQueous One Solution Reagent into each well of the 96-well assay plate containing the samples in 100µl of culture medium. Note: We recommend repeating pipettes, digital pipettes or multichannel pipettes for convenient delivery of uniform volumes of CellTiter 96® AQueous One Solution Reagent to the 96-well plate.
5. Incubate the plate at 37°C for 1–4 hours in a humidified, 5% CO2 atmosphere.
6. Record the absorbance at 490nm using a 96-well plate reader.

WPI

An Evaluation of Abnormal Growth Within the Female Reproductive Track in Clinical and Research Studies

A Major Qualifying Project Submitted to the Faculty of Worcester Polytechnic Institute In partial fulfillment for the Degree of Bachelor of Science

Submitted by: Tiffany Foote
Advisors: Dr. Brenton Faber

This report represents the work of one or more WPI undergraduate students submitted to the faculty as evidence of completion of a degree requirement. WPI routinely publishes these reports on the web without editorial or peer review.

Submitted on 04/30/2024

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1.0 Anatomy and Physiology

1.1 Overview

Three features in the female reproductive track that are affected by abnormal growths in the area are the ovaries, the oviduct (uterine tube) and the uterus. The uterus has two important tissues within the female reproductive track are the endometrium and the myometrium. The three main types of abnormal growths found within the female reproductive track are benign tumors, cysts and tissue. The endometrial tissue can grow into the myometrium of the uterus or on the outside of the uterus depending on the type of growth occurring (Taran et al., 2013; Tsamantioti & Mahdy, 2023). The uterus and surrounding area are supported by the round ligament, transverse cervical ligament, uterosacral ligament and the broad ligament (Hoare). These ligaments provide structural support for the uterus and keep it in the correct location.

1.2 The Uterus

1.2.1 Anatomy of the Uterus

The uterus consists of 3 main components the body, the cervix and the two uterine cornuas (Hafez & Hafez, 2000). It is a muscular organ which is an important environment for developing a fetus (*Genital Tract | SEER Training*, n.d.). Adult females primary pelvic organ is the uterus which is about 3 x 2 x 1 inches before the experience of childbirth (Hoare & Khan, 2023). The inner lining of the uterus is called the endometrium which is an important tissue in the menstrual cycle. The main function of the endometrium is to “metabolize carbohydrates, lipids and proteins” which is directed in part due to the hormones that are produced from the ovaries (Hafez & Hafez, 2000). The endometrium is shed off during menstruation typically every month for women (*Genital Tract | SEER Training*, n.d.).

1.2.2 Function of the Uterus

The main function of the uterus is to support the reproductive process by completing sperm transport, regulates the function of a corpus luteum, and initiation implantation during the early stages of pregnancy (Hafez & Hafez, 2000). A corpus luteum is a temporary organ that is important for fertility (Oliver & Pillarisetty, 2023). The uterus is the location where a fetus will develop during pregnancy. It will expand during a female's first pregnancy before decreasing in size during menopause (*Genital Tract | SEER Training*, n.d.).

1.3 The Ovaries

1.3.1 Anatomy of the Ovaries

The ovaries are paired intraperitoneal endocrine organs that are found in the lower right and left of the abdomen (Gibson & Mahdy, 2023). Each ovary is a solid structure whose size is approximately 3.5 x 2 x 1 centimeters which is comparable to the size of a golf ball (Gibson & Mahdy, 2023; *Ovaries | SEER Training*, n.d.). The ovaries are typically close to the oviducts along with being surrounded by various ligaments and arteries such as the medical umbilical ligament and the internal iliac artery (Gibson & Mahdy, 2023). The main ligament that holds the ovary in its correct location is the suspensory ligament (Gibson & Mahdy, 2023). The volume of the ovaries will change as a female age from starting off very small with <1 milliliter when the female is two years old and going to a max of about 7.7 milliliters for female's around the age of twenty years old (Gibson & Mahdy, 2023). There are multiple layers that make up each ovary. Starting with the outside layer that is the outer epithelium, next is the tunica albuginea, the cortex and finally the medulla (Gibson). Each layer has specific properties that help the layer achieve its primary functions.

1.3.2 Function of the Ovaries

The main function of the ovaries is to produce hormone which in turn affect the uterus and in turn the rest of the body (Gibson & Mahdy, 2023). Some hormones that are released by the ovaries are estrogen and progesterone which are important factors in the survival of abnormal growths within the area of the uterus. The second function of the ovaries is to house oocytes which are important for the first steps of producing a fetus (Gibson & Mahdy, 2023).

1.4 The Oviducts

1.4.1 Anatomy of the Oviducts

The oviducts are more commonly known as the fallopian or uterine tubes. Oviducts are hollow seromuscular organs that begin at the uterine cornua and end at the ovary (Han & Sadiq, 2023). The oviducts are typically 11-12 centimeters long and have diameter of only about 1 millimeter maximum (Han & Sadiq, 2023). The oviducts have multiple components similarly to the ovaries with a fimbriated infundibulum, ampulla, isthmus and intramural parts (Hoare & Khan, 2023). The part of the oviduct that is most important for their functionally is the ampulla because that is the site that is most commonly fertilized (Han & Sadiq, 2023).

1.4.2 Function of the Oviduct

The oviducts connect the uterus to the ovaries and is the main pathway that hormones such as estrogen and progesterone travel through (Hoare & Khan, 2023). Their main function is to be a channel for the movement of oocytes and has the location of where they are fertilized (Han & Sadiq, 2023).

2.0 Pathophysiology

2.1 Uterine Fibroids

Uterine fibroids are benign tumors that are made of uterine smooth muscle cells, and they can grow in various locations around the uterus (McWilliams & Chennathukuzhi, 2017). The tumors form through the congregation of the smooth muscle layer of the uterus which is the myometrium (McWilliams & Chennathukuzhi, 2017). The size of uterine fibroids varies from <5 millimeters to >10 centimeters (Bérczi et al., 2015). Uterine fibroids grow in the myometrium tissue of the uterus and grow in a disordered ball shape (Sefah et al., 2023). The three main classifications of uterine fibroids are subserosal, intramural and submucosal (Sefah et al., 2023). The main distinction between the three is their locations within the uterus as seen in Figure 2. Within the subserosal and submucosal classifications there are pedunculated fibroids which can grow within or outside of the uterine cavity (Sefah et al., 2023).

Symptoms of uterine fibroid include abnormal uterine bleeding, pelvic compression, anemia and consistent sensation of urination (Bérczi et al., 2015). Symptoms vary depending on which classification of uterine fibroids a patient has.

2.2 Polycystic Ovary Syndrome

Polycystic ovary syndrome is developed during the early pubertal years, yet the majority of the research has been done with adult women in clinical studies (Witchel et al., 2019). It is the most common endocrine disorder for women of reproductive age (Ibáñez et al., 2017). Polycystic ovary syndrome is characterized by irregular menstrual cycles, anovulation and acne (Witchel et al., 2019). There are different criteria for diagnosis between adult women and adolescent girls (Witchel et al., 2019). For adult women diagnosis, Rotterdam criteria is followed which requires a patient to have biochemical hyperandrogenism, clinical hyperandrogenism, oligo-anovulation

and polycystic ovarian morphology of 20 follicles per ovary (Christ & Cedars, 2023). Adolescent girls are similar but do not require pelvic ultrasounds and even if they have the correct criteria they can still be regarded as “at risk” and not actually having polycystic ovary syndrome (Witchel et al., 2019). About 60%-80% of patients with polycystic ovary syndrome have an excess of androgen which is important because the hirsutism and hyperandrogenism are products of the excess (Ibáñez et al., 2017). Polycystic ovary syndrome prevents the selecting of dominant follicles during the early stages of ovulation so chronic anovulation occurs for women (Ibáñez et al., 2017).

2.3 Endometriosis

Endometriosis is characterized by the development of endometrial tissues “in anatomical positions and organs outside of the uterine cavity” (Tsamantioti & Mahdy, 2023). It is an inflammatory condition that is estrogen dependent (Burney & Giudice, 2012). There are three pelvic cavity categories that endometriosis cases can be diagnosed with currently. The three types are superficial peritoneal, ovarian and deep (Saunders & Horne, 2021). The main symptoms that occur are chronic pelvic pain and impaired fertility (Tsamantioti & Mahdy, 2023). These growths typically affect the outside of the uterine cavity, the surrounding ligaments and can even affect the intestinal tract and urinary system (Tsamantioti & Mahdy, 2023). There is not a solidified pathophysiology of endometriosis because there are theories of various types of endometrioses that have not been properly researched. These types would be based off factors like genetics, environmental and immunological information (Tsamantioti & Mahdy, 2023). There is research into seeing how retrograde menstruation may be another factor that can cause endometriosis along with oxidative stress within the area (Tsamantioti & Mahdy, 2023). Other aspects that have been researched as hallmarks for the disease and being genetically predisposed,

endometriosis being estrogen dependent and progesterone resistant along with causing chronic inflammation in various locations (Burney & Giudice, 2012). All these areas are currently on-going research in order to fully understand how they cause and affect endometriosis (Burney & Giudice, 2012).

2.4 Adenomyosis

Adenomyosis is when there is endometrial tissue in the form of glands and stroma that are located within the myometrium (Ferenczy, 1998). Adenomyosis was first described in 1860 and yet there is very limited knowledge about how it is caused and how it should be treated (Upson & Missmer, 2020). Adenomyosis is very similar to endometriosis the largest difference is that adenomyosis occurs within the uterine cavity (Zhai et al., 2020). There are multiple contributing factors into adenomyosis including physical trauma and physiologic trauma of the endometrial-myometrial interface can contribute to the progression of adenomyosis (Zhai et al., 2020). Another factor that contributes is various growth factors promoting adenomyotic nodules that may allow for the invasion of the endometrial tissue into the myometrium (Zhai et al., 2020). Some genetic factors that result in hypoestrogenism and progesterone resistance support the endometrial tissue migration and proliferation within the myometrium (Zhai et al., 2020).

3.0 Epidemiology

3.1 Uterine Fibroids

Approximately 80% of premenopausal women will have uterine fibroids at some point although only 25% will show clinical symptoms that would allow for a diagnosis (Sefah et al., 2023). Some groups that are at a higher risk of developing uterine fibroids are women over 50 years old and women of African descent have >80% likelihood to have uterine fibroids that have

clinical symptoms (Sefah et al., 2023). Current treatments lack accessibility to all the women who may be struggling with uterine fibroids (Sefah et al., 2023).

3.2 Polycystic Ovary Syndrome

About 6%-20% of reproductive age women are affect by polycystic ovary syndrome although diagnosis is complicated because normal female pubertal development and polycystic ovary syndrome are characterized the same (Witchel et al., 2019). In 2012, it was estimated that about 5 million reproductive aged women were affected by polycystic ovary syndrome (Rasquin et al., 2023). Various studies have shown that women of Mexican descent are at a high risk when compared to Caucasian and African descended people (Rasquin et al., 2023). Insulin resistance affects 50-70% of the women who struggle with polycystic ovary syndrome (Sirmans & Pate, 2013). Women who have higher amounts of testosterone can also face a higher risk of developing polycystic ovary syndrome. Polycystic ovary syndrome is a genetic disease that can be passed down throughout families (Sirmans & Pate, 2013).

3.3 Endometriosis

Endometriosis only affects about 6-10% of women at reproductive age although it is underdiagnosed due to the requirement of a surgery diagnosis (Burney & Giudice, 2012). When surgery occurs the doctors need to find endometrial tissue outside of the uterus in what is called lesions (Saunders & Horne, 2021). Women on average are approximately 25-29 years old when they receive a diagnosis for endometriosis (Wellbery, 1999). There are about 50% of women who are asymptomatic but find out they have endometriosis due to fertility issues (Saunders & Horne, 2021). Studies have shown that women with endometriosis lesions are at a higher risk for "developing ovarian and breast cancer, melanoma, asthma, rheumatoid arthritis and cardiovascular disease" (Saunders & Horne, 2021).

3.4 Adenomyosis

There is a lack of research into the epidemiology of adenomyosis when compared to other the other non-cancerous reproductive health conditions (Upson & Missmer, 2020). One reason for the lack of epidemiological information about adenomyosis is that most research was completed after a hysterectomy had an occurred so that limited the information that could be collected (Upson & Missmer, 2020). Another factor that led to the lack of epidemiological information is due to underreporting symptoms and doctors underdiagnosing patients who were reporting (Gunther & Walker, 2023). Due to hysterectomies being the choice of treatment and research leads most researchers to believe that there were severe symptoms of heavy menstrual bleeding and pelvic pain (Upson & Missmer, 2020). The estimated definition of the affected population is pre-menopausal women around 30-40 years old (Gunther & Walker, 2023). If women are diagnosed younger than 30 years old, they are classified as “juvenile cystic adenomyosis which is characterized by more extensive hemorrhage within myometrial cysts” and typically requires either a myomectomy or hysterectomy (Gunther & Walker, 2023). There are some studies that show that adenomyosis can lead to an increased risk of having a preterm birth, a miscarry or preeclampsia (Upson & Missmer, 2020).

4.0 Etiology

4.1 Uterine Fibroids

There is no known cause of uterine fibroids but there are many aspects that affect the growth of uterine fibroids such as age, race, and vitamin D deficiencies (Sefah et al., 2023; Tanos & Berry, 2018). Uterine fibroids “are derived from a single cline of a smooth muscle cell” (Tanos & Berry, 2018). Due to the lack of information about the etiology of uterine fibroids, the

field is struggling with creating treatments that work as long-term solutions to uterine fibroids (McWilliams & Chennathukuzhi, 2017).

4.2 Polycystic Ovary Syndrome

There are multiple factors that are contributors to the development of the disease, most of which are genes that affect the "levels of steroidogenesis and androgenic pathways" (Rasquin et al., 2023). Other factors that cause the disease is that polycystic ovary syndrome is genetic about 70% of the time and environmental factors such as obesity and insulin resistance increase the risk of developing polycystic ovary syndrome (Rasquin et al., 2023). Some other factors that affect women with polycystic ovary syndrome are diet, lifestyle choices and gut dysbiosis (Singh et al., 2023). All these factors cause excessive androgen secretion from the ovaries along with other instigators of polycystic ovary syndrome (Singh et al., 2023).

4.3 Endometriosis

There are theories that women of reproductive age that suffer from retrograde menstruation could possibly be more at risk of developing endometriosis. There is a large portion of women who have retrograde menstruation, so research is continuing into which other factors with the retrograde menstruation cause endometriosis (Tsamantioti & Mahdy, 2023). There is also research going into how endometrial tissue can diffuse into lymphatics and vasculature to spread into the other areas that it affects. There are some questions into what factors of a female would affect or promote this diffusion capability (Tsamantioti & Mahdy, 2023). Other research has occurred into oxidative stress along with genetic factors that may be affecting the endometrial tissue that is moving to other locations (Tsamantioti & Mahdy, 2023).

4.4 Adenomyosis

Similarly, to the other abnormal growth diseases there is very limited information about the etiology of adenomyosis in research (Gunther & Walker, 2023). Some of the current theories is that there is a disrupted boundary between the endometrium and myometrium which allowed for cell proliferation and hyperplasia (Gunther & Walker, 2023). Another theory is that pluripotent stem cells differentiate incorrectly leading to ectopic endometrial tissue in locations that were not expected (Gunther & Walker, 2023).

5.0 Treatments and Prognosis

5.1 Overview

There are currently very limited treatments for uterine fibroids, endometriosis, adenomyosis and polycystic ovarian syndrome. Four of the "best" treatments for these conditions are a hysterectomy, myomectomy, uterine artery embolization or lifestyle changes. A hysterectomy is a surgery that removes the uterus and the cervix (*Definition of Total Hysterectomy - NCI Dictionary of Cancer Terms - NCI*, 2011). This procedure can also remove the ovaries and the fallopian tube depending on the reason for the surgery. When the uterus, cervix, ovaries and fallopian tubes are removed it is called a "total hysterectomy with a bilateral salpingo-oophorectomy" (*Definition of Total Hysterectomy - NCI Dictionary of Cancer Terms - NCI*, 2011). This surgery typically occurs through the vagina or through an incision in the abdomen. A myomectomy is another surgery option for people who struggle with any of these conditions. A myomectomy can typically be done as either an open myomectomy or a laparoscopic myomectomy which is minimally invasive. This surgery type requires three small incisions on the abdomen rather than the larger one that is required for a hysterectomy (Andou et al., 2020). Uterine artery embolization is another surgery procedure which does spare the uterus

but “involves the complete occlusion of both uterine arteries with particulate emboli” in order to induce necrosis for only the uterine fibroids and no the surrounding tissues. Uterine artery embolization has been found to both allow for women to safely carry a child to full term and cause ruptures during childbirth. Some of the lifestyle changes that are recommended overall for treatments are increasing intakes of fruits, vegetable and vitamin D (*Uterine Fibroids and Diet - PMC*, n.d.).

5.2 Uterine Fibroids

There is currently no treatment option for uterine fibroids that is long term and maintains fertility (McWilliams & Chennathukuzhi, 2017). The current clinical treatment options are a hysterectomy, myomectomy, uterine artery embolization or hormonal therapies (Bérczi et al., 2015; Sefah et al., 2023). All the current treatments are to manage the symptoms of uterine fibroids and does not actually prevent or effect the growth and survival of uterine fibroids (Bérczi et al., 2015).

There has been some research into various types of surgeries that can occur as treatment options for uterine fibroids. One Cochrane Library review found nine studies with 808 women who struggle with uterine fibroids and its treatment options. The rational of this report was that when endoscopic techniques would be used for the surgery types then women will recover faster. This review found that women who underwent laparoscopic myomectomies had significantly less pain report at the 6- and 48-hour mark compared to the women who had an open myomectomy (an incision along the abdomen like those done with hysterectomies). These women rated their pain at 1-3 points lower at these two-time marks using a visual analogue (VAS) zero to 10 scale. This report concluded that women who received the laparoscopic myomectomy “reported less postoperative pain, lower postoperative fever and short hospital

stays when compared to those who received an open myomectomy." A big limitation of this review is that it was only containing premenopausal women. Uterine fibroids can grow anywhere there is smooth muscle cells and are not clinically relevant to only premenopausal women.

Another treatment for uterine fibroids is commonly used in a uterine artery embolization. This treatment has been found to improve the symptoms of the women who receive the procedure but also has an increased risk for other postoperative problems like post-embolization syndrome and post-procedure pain. A Cochran review investigated seven studies with 793 women who received either a uterine artery embolization or hysterectomies or myomectomies specifically for uterine fibroid treatments. While there was not substantial evidence to show that fertility outcomes may be better for myomectomies than uterine artery embolization, the report did find that the risk for minor complications along with requiring follow up surgeries was higher for uterine artery embolization.

Another treatment for uterine fibroids is acupuncture. There has not been a lot of clinical studies into if this treatment truly does work for treatment of uterine fibroids, but many believe it may help due to its assistance for other diseases. Traditional Chinese Medicine theory "suggests that acupuncture can stimulate the body and improve and rectify the disturbed and dysfunctional organs when used in specific areas of the body. Because uterine fibroids have been theorized to be a factor of improper feedback loops between sex hormones and growth factors, some believe that it can be possible to target this problem through "acupuncture on the pituitary gland, the thyroid gland system and the central nervous system. More research into this field would be helpful so it could possibly become another "lifestyle" change for women to take to try to manage either their symptoms or the uterine fibroids growth.

5.3 Polycystic Ovary Syndrome

The current best treatment for polycystic ovary syndrome for those who have been diagnosis and classifies as “at risk” is management of the symptoms through “education, lifestyle interventions and therapeutic interventions” (Witchel et al., 2019). There is no treatment, other than surgeries, that fully removes polycystic ovary syndrome, it can only be managed through actions of weight loss or increased physical activity (Ibáñez et al., 2017). Pharmaceuticals are typically added along with lifestyle changes but can be used separately (Ibáñez et al., 2017). Oral contraceptive pills are one option of medications that can used to treat adolescent females with polycystic ovary syndrome but need to be separately evaluated on the damage its own side effects can cause in the long term (Ibáñez et al., 2017).

A Cochrane review investigated how safe laparoscopic ovarian drilling is for treating polycystic ovary syndrome. This review included 38 trials with 3326 women who are struggling with polycystic ovary syndrome. Laparoscopic ovarian drilling was found to possibly decrease live birth after the procedure from about 42% chance following medical ovulation induction to 28-40% chances. It also reduces the rate of a women being able to successfully have multiple pregnancies in her lifetime. There is some uncertainty around these claims due to the quality of the evidence in these reports.

Acupuncture has been clinically tested for how it affects polycystic ovary syndrome of premenopausal women who sought fertility. A Cochrane review evaluated eight randomized control trials containing a total of 1546 women. The results from these tests are not dependable due to the low or very low quality of their evidence. Overall, it is assumed from the sources that acupuncture can actually have adverse effects for women who struggle with polycystic ovary syndrome.

There is a Cochrane review that investigates the affect diet and exercise changes can have on women who struggle with polycystic ovary syndrome. There were fifteen studies found who contained 498 women total who fit the correct criteria. Due to low quality evidence, it is assumed that a healthier lifestyle may result in weight loss and possible reduction in male hormone levels. There were no studies that gave information on how diet and exercise could possibly affect live births, miscarriage or regulating menstrual cycles which are all problems that women with polycystic ovary syndrome struggle with.

5.4 Endometriosis

There is no cure for endometriosis. The current best treatments, other than surgery, are oral contraceptives in order to relieve some of the pain that the patient feels (Wellbery, 1999). There are some medical treatments that doctors will prescribe after failure with pharmaceuticals such as a synthetic androgen that inhibits various factors and creates a hypoestrogenic state where the endometrial atrophy occurs (Wellbery, 1999). Problems with this treatment is the side effects that are common enough that it is not typically recommended as a best source of treatment (Wellbery, 1999). There is more research going into similar treatments that are tolerated better by patients (Wellbery, 1999). Surgery is most common in patients who are infertile and have an advanced version of endometriosis because of the “mechanical clearance of adhesions and obstructive lesions” (Wellbery, 1999). Occasionally hysterectomies or oophorectomies will occur for women who are in extreme pain and do not want children (Wellbery, 1999).

A Cochrane review was conducted to evaluate one study that had 67 patients that fit the inclusion criteria for women with a laparoscopically confirmed diagnosis of endometriosis. This review was looking for comparisons of acupuncture to either a placebo or no treatment. Because

it is only one clinical trial that is being evaluated there needs to be more research done to confirm their results of acupuncture lowering the pain for the women who were struggling with endometriosis. The women in this study were between the ages of 22 and 47. They were all located around the Guangdong Provincial Hospital of Traditional Chinese Medicine, China. They all had dysmenorrhea that was patient grades as mild, moderate, or severe pain.

Laparoscopic surgery is a common treatment for endometriosis as an attempt to lower pain and infertility. A Cochrane review that includes fourteen randomized control trials with 1563 women found that laparoscopic "surgery may increase intrauterine pregnancy rates compared to diagnostic laparoscopies". There is limited information about the effects of laparoscopic surgery due to the low-quality evidence or bias that was found in the randomized control trials presented.

5.5 Adenomyosis

The only cure for adenomyosis is currently a hysterectomy which is not preferred (Gunther & Walker, 2023). The treatment that is prescribed before that is hormonal therapies like oral contraceptives, nonsteroidal anti-inflammatory drugs, uterine artery embolization or myomectomies (Gunther & Walker, 2023). For juvenile cystic adenomyosis that normal treatments are to complete medical therapy and a surgery that is either a myomectomy or a hysterectomy (Gunther & Walker, 2023).

6.0 Evaluation

6.1 Introduction

Cochrane Reviews has a total of 9224 study reviews within that total there is 353 reviews that are relevant to abnormal growth within the female reproductive track. Overall, that is about 3% of the reviews that Cochrane evaluates. The quality of the overall research was very low to

moderate based on the Cochrane values. The research that was evaluated in this review is not able to provide strong clinical recommendations for patients who struggle with PCOS, endometriosis, uterine fibroids or adenomyosis.

6.2 Methods

Cochrane Library has a collection of systematic reviews of research. There are multiple types of reviews. The main type of review in this evaluation is an intervention reviews which assess the "effectiveness/safety of a treatment, vaccine, device, preventative measure, procedure or policy" (*About Cochrane Reviews | Cochrane Library*, n.d.). When searching through the Cochrane Reviews, a review was chosen to be included if its main research goal was to assess uterine fibroids, PCOS, endometriosis or adenomyosis. A factor used to exclude reviews was if they were drug trials as a treatment option. Through this process, nine various reviews were chosen to be included in this evaluation. These nine reviews included a total of 105 studies with a total of 8,879 patients (Figure 1). All patients are affected by uterine fibroids, PCOS, endometriosis or adenomyosis. The patients in these studies were included if they were of reproductive age and have a diagnosis of uterine fibroids, PCOS, endometriosis or adenomyosis. Diagnosis must be confirmed laparoscopically, with ultrasound or through clinical signs and symptoms. For patients with PCOS were required to have diagnosis by Rotterdam criteria.

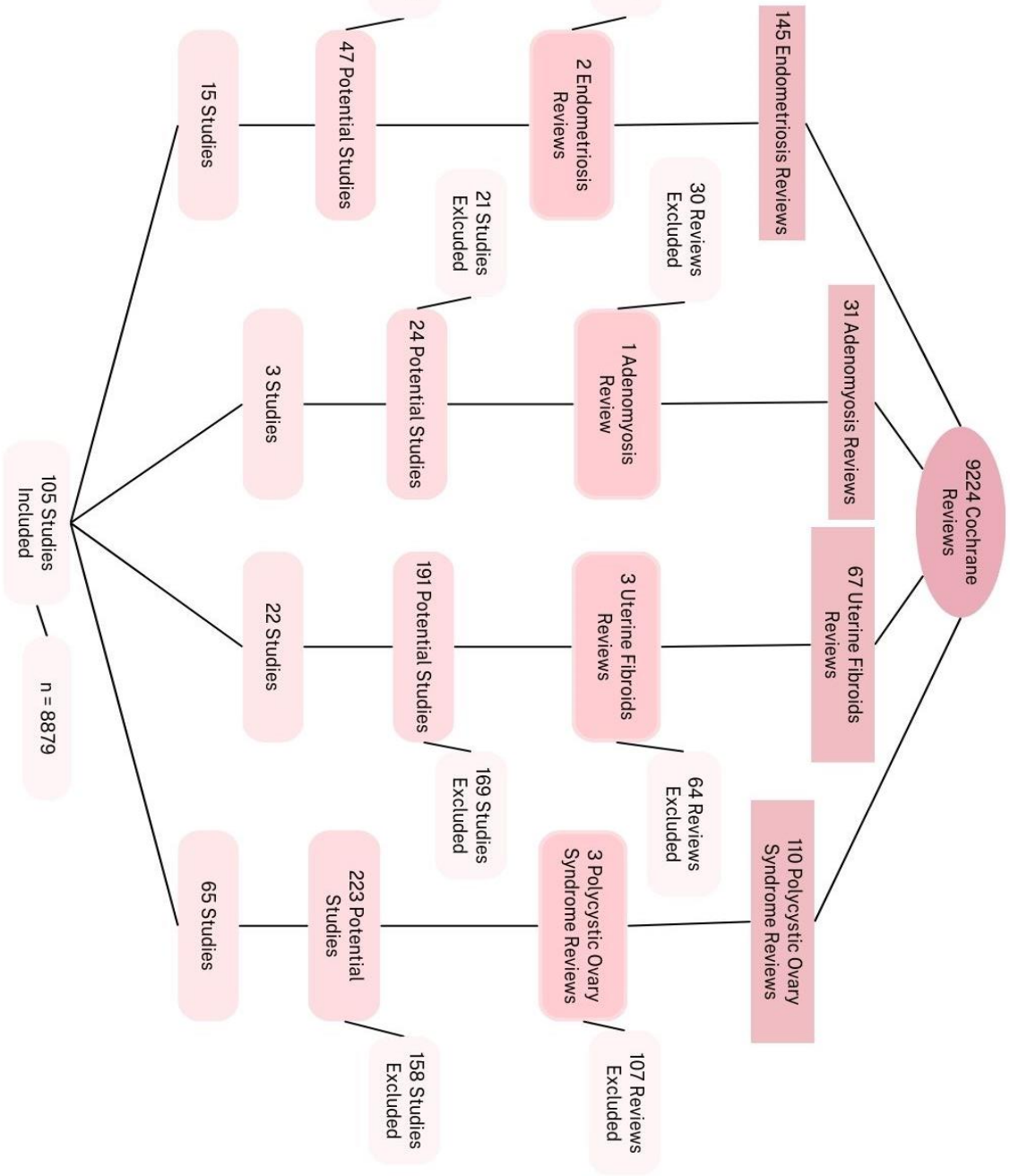

Figure 1. Study Flow Diagram representing the process taken to acquire which Cochrane Review articles to evaluate.

6.3 Results

Overall, there is a limited number of patients who were included in these review articles with a total patient count of only 8879 patients. 72% of the total patients evaluated were found within 3 review articles. The majority of that research occurred within the polycystic ovarian syndrome and there was only one review that covered adenyomsis. There was a large disparity found in the amount of current research for each disease.

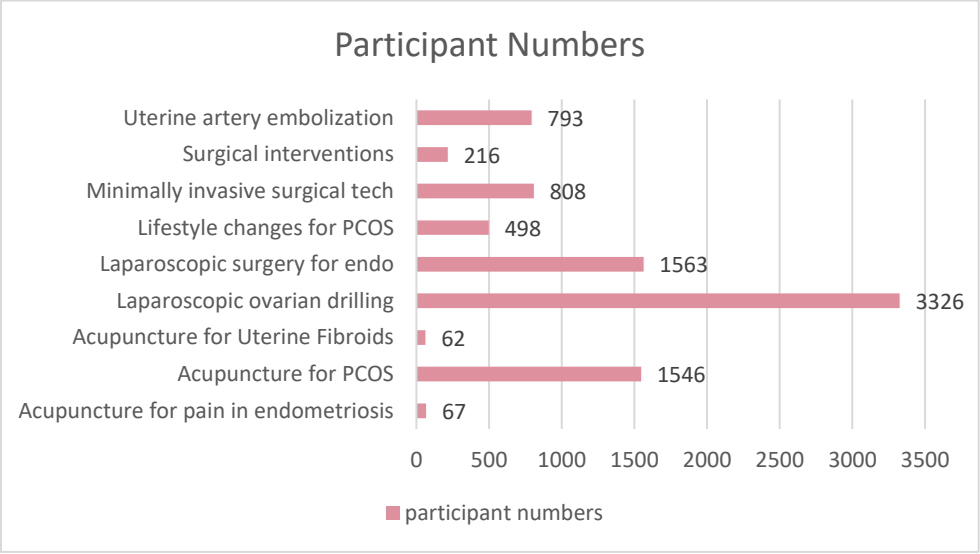

Figure 2. A comparison of all the participants within each of the nine review articles that were found through Cochrane Review Articles.

When evaluating the 9 review articles from Cochrane Review presented that for all the research that is occurring within the field there is very limited information. Six out of the nine review articles used in this evaluation had less than a 20% inclusion rate for all their possible studies due to review. There are various reasons that the studies that were excluded, some including that they were review articles, editorials, incorrect patient populations, not randomized trials, and no control group to compare to (Figure 3). All these exclusion criteria show that overall, the collected data in the field is not dependable.

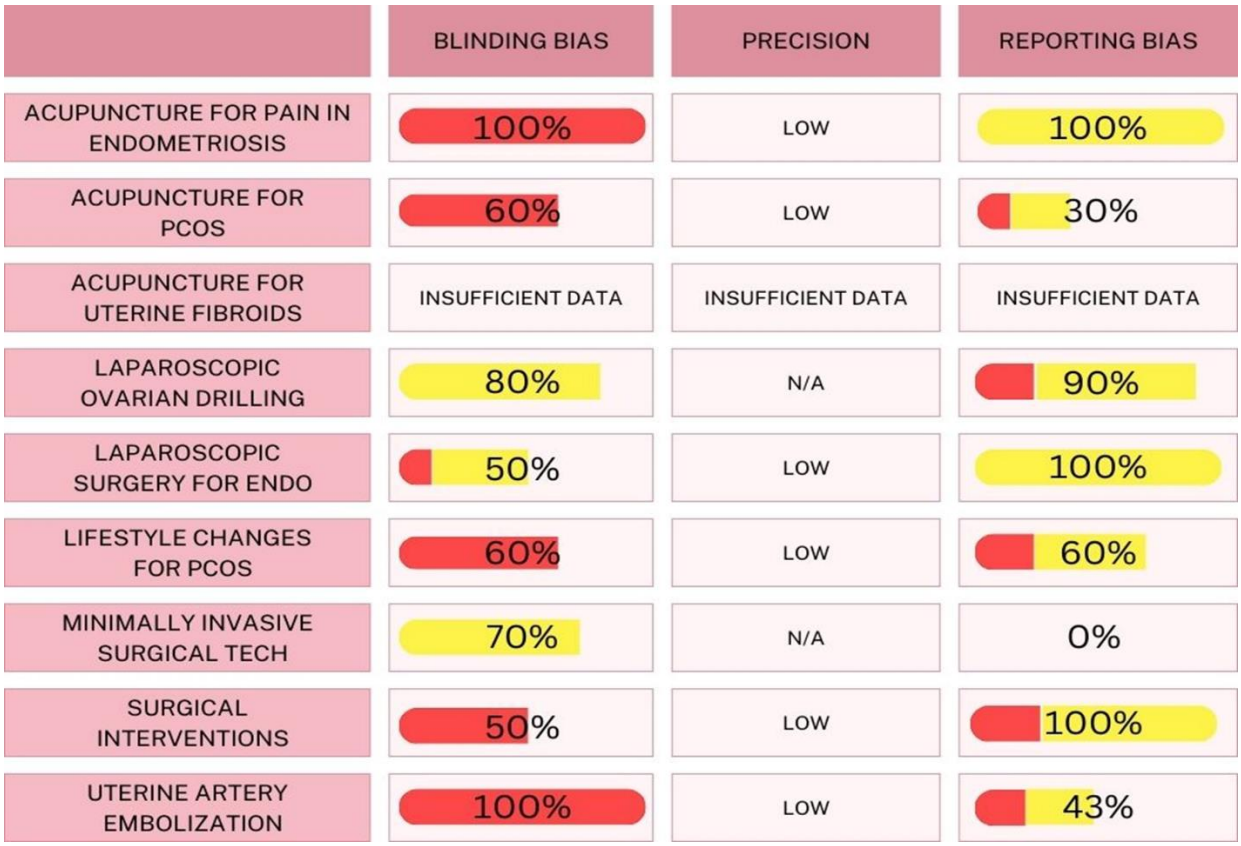

Figure 3. Evaluation of the two major biases that in turn lead to the low-quality research. Red presents the reports with a high level of bias found while yellow represents an unclear amount of bias.

Further research was completed to understand the amount of research going into each of the four fields evaluated. It was found that in the National Institute of Health in the United States of America, there was only a recent increase in funding for all the diseases. Although adenomyosis currently does not have any funding from the National Institute of Health for research despite being acknowledged as a disease.

	UTERINE FIBROIDS	POLYCYSTIC OVARY SYNDROME	ENDOMETRIOSIS	ADENOMYOSIS
TYPE OF GROWTH	TUMOR	CYST	TISSUE	TISSUE
NIH FUNDING ESTIMATE IN MILLIONS (2022)	$15	$9	$27	N/A
MORTALITY RATE IN 2021	95	49	38	N/A
PATIENTS EVALUATED	1,663	5,370	1,630	216

Figure 3. A comparison of PCOS, uterine fibroids, endometriosis, and adenomyosis types of growth, mortality rate and National Institute of Health funding.

The funding from the National Institute of Health varied from 0 to 27 million dollars in 2022 and each disease is getting more funding every year since 2010 (*RePORT*, n.d.). Before the early 2000s there was very little funding for each disease. These are low mortality rates for all the diseases, but they are chronic illnesses which affect the patient's quality of life.

More research needs to be completed to achieve a greater understanding of how PCOS, endometriosis, adenomyosis, and uterine fibroids develop and can be treated. The is currently a lack in quality research that is not clinical drug trials. Further research can be completed in order to understand the differences between the diseases and which treatments could possibly work for each one.

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