DIETARY INTERVENTION WITH WHOLE FLAXSEED OR VITAMIN D HAS BENEFICIAL EFFECTS ON LEIOMYOMAS IN THE OVIDUCT OF THE LAYING HEN

BY

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THESIS

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ABSTRACT

Uterine leiomyomas, or fibroids, have a symptomatic incidence of around 40% and are a leading cause of reproductive morbidity in reproductively-aged women. Symptoms include menorrhagia, pelvic pain, and infertility. They are characterized by excessive production of extracellular matrix components, such as collagen. Leiomyomas are responsive to estrogen and progesterone and occur more frequently as women age. Risk factors for fibroids include obesity, race, localized injury, and stress, while parity has been shown to be protective against fibroids. Inflammation has been hypothesized to play a key role in their development. Traditionally, hysterectomy or myomectomy has been the treatment for fibroids. However, hysterectomy does not preserve a woman's fertility. Currently, there are no effective long-term, non-surgical treatments available for fibroids. Hormone therapies, such as GnRH analogs cannot be used for long-term treatment because they induce a state of hypoestrogenism which leads to post-menopausal symptoms. Researchers have turned to dietary intervention as a possible therapy for fibroids because it is non-invasive, safe for long-term use, and relatively inexpensive. Two potential candidates for dietary intervention are flaxseed and vitamin D_3. Flaxseed consists of two parts: lignan and oil. The lignan portion of flaxseed contains phytoestrogens which may inhibit fibroid growth through the antagonism of estrogen. Flaxseed oil is rich in α-linolenic acid, which has known anti-inflammatory properties. Vitamin D_3 is a hormone involved in calcium and bone homeostasis that also possesses anti-inflammatory and anti-fibrotic properties. Because the laying hen is the only animal to develop leiomyomas spontaneously as they age, they are an ideal animal model for the study of fibroids. Hen leiomyomas occur as polyps on the smooth muscle layer of the oviduct with increasing abundance as hens age and have the same

histological and molecular markers as human uterine fibroids including desmin, smooth muscle actin, increased collagen, and steroid hormone receptors.

Two studies were conducted to assess the efficacy of dietary intervention with whole flaxseed and vitamin D_3. The first study involved feeding White Leghorn hens an isocaloric control (basic layer) or 15% whole flaxseed diet *ad libitum* from the end of their second year of lay to the completion of their third. At the end of the study, hens were euthanized and fibroids were counted, measured, and collected. Immunohistochemical analyses of fibrosis (Gomori's Trichrome Stain), proliferation (Proliferating Cell Nuclear Antigen), angiogenesis (von Willebrand Factor), NF-κB p65, oxidative stress (nitrotyrosine), cellular senescence (Sudan Black B), and prevalence of mast cells (Toluidine Blue) were performed on fibroid tissue sections. Quantitative RT-PCR was performed to determine changes in expression levels of mRNA for collagen I, transforming growth factor-β3 (TGF-β3), and prostaglandin-endoperoxide synthase 2 (*PTGS2*). Western blots were carried out to determine changes in protein levels for COX-2. There was a slight decrease in the incidence of leiomyomas in the flaxseed group versus the controls. There was no significant difference in the volume of leiomyomas ($p=0.3$), although previous work by our group demonstrated a significant decrease in the size of leiomyomas from hens fed a similar diet of 10% whole flaxseed diet for 24 months. Fibroids from hens fed the flaxseed-supplemented diet for 12 months showed a modest decrease in proliferation when compared to controls and a significant decrease in numbers of blood vessels per unit area of fibroid. There were no differences in p65 translocation to the nucleus, a measure of Nuclear Factor kappa B (NF-κB) activation, or tissue levels of nitrotyrosine, a measure of oxidative stress, between treatment groups. Sudan Black B staining showed that leiomyomas from both treatment groups were not undergoing cellular senescence while Toluidine Blue staining did

show mast cell infiltration into fibroids from both groups. Fibroids from hens fed the whole flaxseed diet had slightly decreased collagen I (4-fold change, p=0.06) and TGFβ-3 expression (6-fold change, p=0.06), while *PTGS2* (6-fold change, p=0.04) expression was significantly downregulated when compared to fibroids from hens fed the control diet. COX-2 analysis through western blot showed a non-significant decrease in relative COX-2 expression in leiomyomas from flaxseed-fed hens compared to those from control hens.

In the second study, White Leghorn hens having completed their second year of lay were fed either an isocaloric control (basic layer), 10% whole flaxseed, or vitamin D_3 (69 μg/kg/day) diet for 15 months. Twenty-five hens were sacrificed at the start of the trial to determine the baseline incidence of leiomyomas. Blood was drawn from the same subset of hens every three months until the end of the trial. After 15 months on their respective diets, hens were euthanized. Oviducts were collected, and fibroids were counted, measured, and either flash frozen in liquid nitrogen or fixed in 10% neutral buffered formalin for further analysis. Leiomyoma incidence increased from 44% at the start of the trial to 78%, 74%, and 71% in the control, whole flaxseed, and vitamin D groups, respectively after 15 months. The number of leiomyomas per hen in all three groups increased significantly after fifteen months when compared to the baseline; there were no differences between groups after fifteen months. Plasma carbonylated protein levels decreased significantly in the whole flaxseed and vitamin D_3-treated hens after 6 months on their respective diets; these levels were significantly lower than those of the control hens after 9 months in both groups. qRT-PCR analysis showed a downregulation of collagen I (50-fold change, p=0.02), TGF-β3 (50-fold change, p=0.003), and *PTGS2* (100-fold change, p=0.001) gene expression in leiomyomas from hens fed the vitamin D_3-supplemented diet after 15 months

when compared to those from control hens; there were no differences between the whole flaxseed group when compared to the control.

We conclude that dietary intervention with whole flaxseed or vitamin D_3 may have beneficial, therapeutic effects on leiomyomas found in the oviduct of the laying hen. Our results support further investigation into the use of these supplements in women with fibroids as they could provide a safe, effective, long-term, and non-invasive treatment option that currently is not available.

To my husband: through all of the laughter and tears, you have been there for me—comforting me through my darkest hours and celebrating each and every victory, no matter how small. I cannot thank you enough for your patience as I pursue my goals. You truly bring joy to every aspect of my life. I love you.

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INTRODUCTION

Uterine leiomyomas, or fibroids, are benign tumors formed from the smooth muscle cells of the myometrium. They are characterized by excessive deposition of extracellular matrix (ECM) components, such as collagen. Currently, there is little known about the etiology of fibroids. Chronic inflammation is hypothesized to play a key role in their development due to its interplay with fibrosis. As the most common gynecological tumors in reproductively-aged women and the leading cause for hysterectomy in the United States, symptomatic fibroids cause pelvic pain, menorrhagia, recurrent pregnancy loss, and infertility. Several risk factors have been associated with fibroids including obesity, stress, race, lack of parity, and other genetic and environmental factors. Studies have shown the incidence of fibroids to increase with age in pre-menopausal women while menopause tends to decrease the size of fibroids. These studies collectively support the dependence of fibroids on the ovarian steroid hormones estrogen and progesterone. Historically, treatment options for fibroids have been very limited and compromise a woman's fertility with hysterectomy and myomectomy being the two most common treatments. More recently, gonadotropin-releasing hormone (GnRH) analogs have been used as a pharmaceutical treatment for fibroids. However, while successful at decreasing fibroid size, GnRH analogs cause hypo-estrogenism and thus, are not approved for long-term use. The effectiveness of GnRH analogs is also reversed upon discontinuation of treatment. Because of the limited treatments available for fibroids that allow women to preserve their fertility, research into dietary intervention therapies has become more popular. The purpose of our studies was to determine whether the anti-inflammatory properties of whole flaxseed or vitamin D could be exploited as a potential therapy for the treatment of leiomyomas found on the oviduct of the laying hen, our validated animal model for uterine fibroids.

CHAPTER 1

Literature Review

Introduction to Uterine Leiomyomas:

Uterine leiomyomas are the leading cause of hysterectomy in the United States[1] and are responsible for up to an estimated 34.37 billion dollars spent annually, both directly and indirectly, on women's healthcare[2]. Fibroids are benign tumors that form from the smooth muscle cells of the myometrium and are dependent upon the ovarian steroid hormones estrogen and progesterone[3]. In order to measure fibroid incidence, Cramer and Patel serially sectioned 100 uteri at 2-millimeter intervals following total hysterectomy and found that 77 of them contained at least one fibroid; this was an incidence significantly higher than fibroids identified upon routine pathological examination alone[4]. Fuldeore and Soliman reported that women with symptomatic fibroids suffer from pelvic pain (menstrual and non-menstrual), menorrhagia, painful intercourse, and infertility[5]. They also reported African American women to have an increased prevalence of fibroids when compared to Caucasian women, and that prevalence increases with age regardless of race[5]. In fact, by their late forties, 50% of African American women have clinically significant fibroids compared with only 35% of Caucasian women[6]. Environmental and genetic factors such as injury, stress, obesity, and race have been shown to increase the risk of developing symptomatic fibroids[1,7]. Despite knowing the incidence, symptoms, and risk factors of fibroids, their etiology remains largely unknown although chronic local inflammation is hypothesized to play a vital role in their development[8]. Currently, very few treatments for fibroids are available. These range from surgical options, such as hysterectomy,myomectomy, and uterine artery embolization, to limited pharmaceutical options, such as gonadotropin releasing hormone (GnRH) analogs[9,10]. These will be discussed in more detail later.

Risk Factors and Treatment of Uterine Leiomyomas:

Several environmental and genetic risk factors exist for the development of uterine leiomyomas, including local injury or infection, stress, obesity, hypertension, race, and parity—the latter having a protective effect[1,7,8,11]. Ganesa Wegienka proposed a hypothesis regarding how inflammation plays a role in the development of uterine leiomyomas. Briefly, factors such as localized injury, infection, stress, and obesity cause the body to be in a chronic, low-grade inflammatory state[8]. If the uterus suffers an insult or multiple insults (i.e., menses), the immune response will become pro-inflammatory, leading to smooth muscle cell proliferation and fibrous tissue formation[8].

Obesity, a component of metabolic syndrome, has been linked to uterine leiomyomas[1,7,12–16]. Takeda et al. established the relationship between metabolic syndrome and fibroids by showing that patients with increased body mass index (BMI), blood pressure, fasting plasma glucose, and hypertriglyceridemia had a higher incidence of symptomatic leiomyomas[12]. A more recent study looking at women with both symptomatic and asymptomatic fibroids had similar findings. Tak et al. found that pre-menopausal, parous women with metabolic syndrome (increased body fat, waist circumference, blood pressure, and low-density lipoprotein cholesterol levels) also had a higher prevalence of leiomyomas[13]. They also found that metabolic syndrome prevalence was greater in women with multiple fibroids than women with just one[13].

In addition to obesity, hypertension has also been associated with increased risk of fibroids[11,12,17,18]. As mentioned in the previous paragraph, Takeda et al. associated metabolic syndrome with an increased risk for leiomyomas[12]. Another case-control study conducted several years earlier studied women with at least one fibroid confirmed by uterine sonogram or histologically. They found that women with hypertension had a 1.7-fold increased risk for

fibroids and that risk increased from 1.7- to 2.1-fold if the patient was taking anti-hypertensive medication[11]. Luoto et al. performed a cross-sectional study of women undergoing hysterectomy and found that 42% of hypertensive women had uterine fibroids compared to only 37% of non-hypertensive women[17]. Finally, a prospective study of hypertension and fibroid risk found that for every 10-mmHg increase in diastolic blood pressure, the risk for uterine leiomyomas increased by 8% for patients not taking anti-hypertensive medication and 10% for those on anti-hypertensive medication[18]. The main hypothesis for the association between hypertension and increased fibroid risk is that high blood pressure could lead to injury of smooth muscle cells and increase cytokine activity, leading to uterine fibroid development in a process similar to atheromatous plaque development[11,12,18]. Furthermore, atherosclerotic plaque cells and uterine leiomyoma cells are both monoclonal in origin[19–21] and behave similarly in culture[22].

Race has also been shown to be a risk factor for uterine leiomyomas. African American women have a 2-3 times greater risk of developing fibroids when compared to Caucasian women[5,6,23–26]. Baird et al. found 30-40% of African American women in their mid- to late-thirties and 50% in their late forties to have clinically significant leiomyomas compared with only 10-15% and 35% of Caucasian women, respectively[6]. Another study demonstrated the heavier burden uterine fibroids pose on African Americans, and their results show that they are two times more likely to have abdominal bloating and pressure compared with Caucasian women[26]. Studies also suggest that African Americans are more likely to have other fibrosis-related diseases, such as keloids, that have an incidence of 15-20%[27,28]. While the reasons for these skewed risks are unclear, data gathered between 2005-2014 show that African American women were more likely to be obese than white women[29]. One study showed a link between BMI, ancestry, and fibroid risk. Specifically, they showed that African American women are

more likely to have uterine leiomyomas and that the correlation is stronger with a higher BMI suggesting that BMI modifies uterine leiomyoma risk in African American women[23]. It has also been shown that serum levels of vitamin D tend to be lower in African Americans than Caucasians[30,31]. Because of the increased likelihood of an African American woman to be both obese and deficient in vitamin D, it is possible that more African American women are in a chronic pro-inflammatory state and thus, at greater risk for developing fibroids compared to white women. In fact, Ciebiera et al. found that patients with higher BMI and serum transforming growth factor β3 (TGF-β3) levels, but lower serum vitamin D levels were more likely to have uterine leiomyomas than patients with normal BMI, serum TGF-β3, and serum vitamin D levels, although this study was conducted with Caucasian women[32].

Parity, on the other hand, has been shown to lower the risk of uterine leiomyoma development[33–38]. Walker et al. found a significant decrease in leiomyoma incidence in Eker rats that had multiple litters compared to those with just one litter[34]. Another study demonstrated significantly decreased relative risk of fibroids with increased number of term pregnancies[35]. Baird and Dunson hypothesize that parity could be protective due to postpartum uterine involution[38]. It is also possible that since a woman is not cycling during pregnancy, there is less of a chance for local inflammation at the uterus, thus making parity protective.

Some genetic factors linked to uterine fibroid development include mutations in the *mediator complex subunit 12* (*MED12*) and *high mobility group AT-hook 2* (*HMGA2*) genes. *MED12* is one of the twenty-six subunits of the Mediator complex[39]. The Mediator complex interacts with RNA polymerase II to regulate the expression of protein-coding genes[39]. Mäkinen et al. examined a number of uterine leiomyomas and identified that *MED12* was mutated in 70% of them[40]. Since then, Al-Hendy et al. found that knockdown of *MED12* reduces proliferation of

human leiomyoma cells *in vitro* by suppressing Wnt/β-catenin signaling in addition to downregulating the expression of cell cycle- and fibrosis-associated proteins[41]. *HMGA2* (also known as *HMGI-C*) is a member of the high mobility group (HMG) family consisting of DNA-binding proteins which are components of chromatin[42]. Schoenmakers et al. identified the *HMGI-C* gene on chromosome 12q15 as being consistently altered in various benign mesenchymal tumors, including uterine fibroids, suggesting a link between the development of benign tumors and the HMG family[43]. A subsequent study confirmed the role of *HMGA2* in the development of leiomyomas using the Eker rat model[44]. They also found that atypical *HMGA2* expression could result from inactivation of the *Tsc2* gene (which gives rise to leiomyomas in the Eker rat , discussed later), thus providing a link between fibroids found in humans and in the Eker rat model[44]. Targeting the downstream effects of these genetic mutations could provide novel, long-term therapies for women suffering from symptomatic fibroids.

The standard treatment for fibroids used to only include surgical procedures such as hysterectomy or open myomectomy[9,45–47]. Now, a variety of minimally-invasive and non-surgical treatment options exist that are more advantageous than surgical procedures because they preserve women's fertility. Examples of minimally invasive procedures include vaginal myomectomy, fibroid myolysis, magnetic-resonance-guided focused ultrasound surgery (MRgFUS), uterine artery embolization (UAE), and laparoscopic uterine artery occlusion (LUAO)[45]. Non-surgical treatment options include hormonal therapies such as GnRH analogs and selective progesterone receptor modulators (ulipristal acetate)[9,46,48,49]. In addition to fertility preservation, other advantages of these procedures include less time spent in the hospital, faster recovery times, and less time spent off of work. Disadvantages include recurrence of or no resolution of symptoms resulting in need for surgery (hysterectomy) and additional side effects,

such as menopause-like symptoms and bone loss. These disadvantages decrease the quality of life for women with fibroids.

Despite the increasing use of these minimally invasive and non-surgical treatment options, complications are a common occurrence[8,22]. When evaluating the efficacy of MRgFUS five years post-procedure, Quinn et al. found that while MRgFUS is a safe treatment for leiomyomas, it has a high re-intervention rate of approximately sixty percent[51]. The authors hypothesize that this could be due to the percentage of non-perfused volume (NPV) of fibroid tissue as they saw a re-intervention rate of fifty percent with MRgFUS treatments that achieved at least a fifty percent NPV[51]. A meta-analysis comparing UAE and LUAO to surgical options (hysterectomy or myomectomy) concluded that UAE had increased minor complications, mainly persistent symptoms such as menorraghia, when compared to surgical procedures as well as an increased chance of surgical re-intervention two to five years later[10]. They also found that LUAO resulted in more cases of persistent symptoms (menorrhagia) along with a higher chance of surgical re-intervention within two years of the original procedure[10]. GnRH analogs, on the other hand, are not approved for long-term use in the treatment of leiomyomas because of their tendency to induce hypo-estrogenism and postmenopausal symptoms in women who take it[9,48,50]. Because of the lack of effective long-term, non-surgical treatment options, dietary intervention therapies might prove to be more successful in treating uterine leiomyomas in the long run.

Dietary Intervention as a Therapy for Uterine Leiomyomas:

Recently, researchers have begun to study the effectiveness of dietary intervention for therapeutical treatment of uterine leiomyomas due to the lack of available and effective long-term treatment options. *In vitro* studies have shown promising effects for using curcumin in the treatment of leiomyomas[52,53]. Malik et al. found that curcumin inhibited proliferation, increased

apoptosis, and decreased expression of fibronectin in cultured human leiomyoma cells in a dose-dependent manner[52]. The effects of curcumin on proliferation and apoptosis of leiomyoma cells were also confirmed by Tsuiji et al. using ELT-3 cells derived from the Eker rat[53]. Tsuiji et al. also found that curcumin exerted its effects on leiomyoma cells through the activation of peroxisome proliferator-activated receptor-gamma (PPARγ)[53].

Genistein, a phytoestrogen and the major isoflavone found in soybeans, has been found to inhibit proliferation of leiomyoma cells *in vitro*[54] as well as dysregulate the cell cycle and down-regulate the TGF-β signaling pathway by decreasing expression of activin A and Smad3[55]. *In vivo*, Sahin et al. found that genistein reduced the size and incidence of spontaneous leiomyomas in the oviducts of Japanese quail[56]. Soy products are becoming popular in many diets, however, studies suggest that genistein may have serious effects on women's reproductive health[57–59]. For example, while genistein has an anti-proliferative effect on leiomyomas, it may also have significant negative effects on other parts of the reproductive system. A study conducted by Patel et al. used an *in vitro* follicle culture system to show that genistein inhibits the growth of antral follicles through cell cycle inhibition, decreases estradiol levels, alters estradiol precursor hormone levels, and dysregulates expression of steroidogenic enzymes[57].

Other phytochemicals have been identified that have been shown to have anti-inflammatory, anti-fibrotic, anti-proliferative, and anti-angiogenic properties in various biological systems and could potentially be used as a therapy for fibroids[60]. For example, delphinidin, an anthocyanidin found in grapes, pomegranates, and other berries, has been shown to suppress proliferation and migration of ovarian clear cell carcinoma cells by downregulating the Protein Kinase B (Akt) and Mitogen-Activated Protein Kinases (MAPK) signaling pathways[61]. Cho et al. demonstrated that delphinidin can inhibit TGF-β1-induced α-smooth

muscle actin, fibronectin, and collagen I expression in nasal polyp-derived fibroblasts through inhibition of MAPK and NF-κB[62]. Although not reviewed in this paper by Islam et al., flaxseed has been shown to possess anti-inflammatory properties and researchers have begun studying its effects as a therapeutical agent for various morbidities such as ovarian cancer and cardiovascular disease[63–69]. With respect to uterine leiomyomas, a case-control study by Atkinson et al. found a modest inverse association between uterine fibroid development and urinary excretion of enterodiol and enterolactone—two lignan metabolites found in flaxseed[70]. While more research is needed to confirm these findings, flaxseed could prove to be a beneficial dietary intervention therapy for uterine fibroids.

A lot of promise has been shown in the use of vitamin D as an anti-fibrotic agent[71–73]. Vitamin D is a fat-soluble vitamin that is typically involved in the regulation of calcium absorption and bone homeostasis[74]. It is also known to be involved in the regulation of various inflammatory cytokines such as interleukin (IL)-10, IL -1, IL-6, TNF-α, and IFN-γ[75–78]. Vitamin D_3, or cholecalciferol, is the form of vitamin D found in most dietary supplements. Cholecalciferol (biologically inactive) must be converted to 25-dihydroxyvitamin D in the liver prior to its final conversion to 1,25-hydroxyvitamin D, the biologically active form of vitamin D, in the kidney[74]. Dr. Ayman Al-Hendy's group has contributed much to the study of vitamin D and uterine leiomyomas[79–86]. They first showed that vitamin D inhibited proliferation of human leiomyoma cells *in vitro* suggesting lack of vitamin D to be a risk factor for developing fibroids[79]. Since then, they have also demonstrated that 1,25-hydroxyvitamin D_3 reduces expression levels of matrix metalloproteinases 2 (MMP-2) and 9 (MMP-9)[80], increases the expression of vitamin D receptor (VDR)[81], and decreases the expression of extracellular matrix (ECM)-associated proteins[81] in human uterine leiomyoma cells suggesting its potential to be

used as a long-term treatment for fibroids. Finally, an *in vivo* study conducted by Dr. Al-Hendy (using a mouse model injected with ELT-3 cells derived from the Eker rat) supported using paricalcitol, a vitamin D receptor (VDR) activator, given orally, as a new approach in treating symptomatic fibroids since there was significant shrinkage of leiomyomas after treatment with paricalcitol[82]. As can be seen by the numerous studies by Dr. Al-Hendy and colleagues, vitamin D possesses great potential to be used as a treatment for uterine fibroids and more *in vivo* dietary intervention studies are needed.

Animal Models of Uterine Leiomyomas:

Animal models have provided a plethora of advancements in the field of medicine, largely due to their smaller size and relatively cheap cost. Several animal models for leiomyomas are available including the Eker rat, a mouse human leiomyoma-xenograft model, and the laying hen. The most widely used animal model is the Eker rat. The Eker rat carries a mutation in the *Tsc2* (tuberous sclerosis complex 2) gene that makes it susceptible to tumor formation[87]. Eker and Mossige first observed that this rat strain was predisposed to the development of renal adenomas with some tumors resembling carcinomas[88]. Further characterization of the Eker rat mutation has shown a predisposition of these rats to renal cell carcinomas and leiomyosarcomas in addition to leiomyomas[89]. Because of the rarity of leiomyosarcomas in women and their high incidence in Eker rats (and renal cell carcinomas), these have been considered to not be truly representative of leiomyomas in women.

Studying human leiomyomas *in vivo* is a difficult task to accomplish in women. Mouse xenograft models provide a way to perform extensive research on these tumors *in vivo* without the need for establishing clinical trials. (This is not to say that clinical trials are not necessary as they are required to test the efficacy of proposed therapies in patients.) The development of

xenograft models involves taking human leiomyoma cells or fragments of leiomyoma tumors and transplanting them into immunodeficient mice under the kidney capsule[90,91]. The grafted leiomyomas retain characteristics of the original tumors when supplemented with estrogen and progesterone[90]. These characteristics include the typical disorganized, whorled appearance of smooth muscle cell bundles, collagen deposition, and expression of smooth muscle actin, estrogen receptor alpha (ERα), and progesterone receptor (PR) that are found in human uterine leiomyomas[90,91]. Unfortunately, this model also has several limitations. First: it is expensive, second: it depends on having access to fresh human leiomyoma tissues, and third: the success of the model depends on the ability of the transplanted human leiomyoma cells to attach, grow, and form the tumors.

Our proposed animal model, the laying hen, develops benign smooth muscle tumors on her oviduct without predisposition to malignancies, such as the leiomyosarcoma—unlike the Eker rat. Because the laying hen develops these tumors spontaneously, there is no need for surgery to implant human leiomyoma cells; thus making the hen a much more cost effective model than the mouse xenograft model. As mentioned previously, the laying hen develops leiomyomas spontaneously on her oviduct as she ages[92,93]. Berry et al. demonstrated that like those found in women, fibroids from the laying hen express ERα, PR, proliferating cell nuclear antigen (PCNA), and the anti-apoptotic protein Bcl-2[92]. Our lab further characterized the laying hen as an animal model for human leiomyomas. We found that the incidence of leiomyomas increases significantly in the hen after the second year of lay with an overall incidence of 77%, similar to women[93]. The number of fibroids per hen also significantly increases after the second year of lay[93]. Furthermore, leiomyomas in the laying hen were confirmed to possess a smooth muscle phenotype, increased collagen deposition, and increased expression of estrogen and

progesterone receptor and Bcl-2 when compared to the normal smooth muscle cells of the oviduct[93]. In addition, specific-pathogen-free (SPF) hens had virtually no leiomyomas when compared to hens housed at the University of Illinois Poultry Research Farm[93]. This supports the idea that infection and inflammation play a role in the development of fibroids. Thus, the laying hen as an animal model for human uterine leiomyomas may prove to be a useful tool when used in conjunction with *in vitro* studies and other animal models such as the Eker rat and mouse xenograft models.

Inflammation and Fibrosis:

Inflammation is defined as a physiologic immune response of tissue to injury that occurs in two phases: the acute phase and the chronic phase[94]. The acute phase of inflammation is characterized by increased blood flow and vascular permeability to the area of tissue damage[94]. Interstitital fluid, leukocytes, and other inflammatory mediators (i.e., cytokines) accumulate in the area during the acute phase[94]. During the chronic phase of inflammation, humoral and cellular responses are mounted that are unique to the pathogens that are present at the site of tissue damage[94]. Chronic inflammatory diseases, such as rheumatoid arthritis, lupus, and metabolic syndrome differ from acute infections (i.e., wounds, influenza, etc.) because unlike acute infections, the stimuli causing the inflammatory response are never fully removed[95]. Two pro-inflammatory pathways, and potential targets for uterine fibroid therapy include the prostaglandin and nuclear factor kappa B (NF-κB) pathways[96–98].

Prostaglandin production by cells is regulated by cyclooxygenase enzymes. The cyclooxygenase family is made up of two enzymes: cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2)[99,100]. These enzymes are part of the prostaglandin pathway and are responsible for converting arachidonic acid to prostaglandins **(Figure 1)**[100]. COX-1 is

constitutively expressed in most tissues at a constant level whereas COX-2 is an inducible enzyme that is not present in cells until a specific stimulus occurs[100]. Most stimuli that induce COX-2 expression are pro-inflammatory cytokines while anti-inflammatory cytokines decrease COX-2 expression[100].

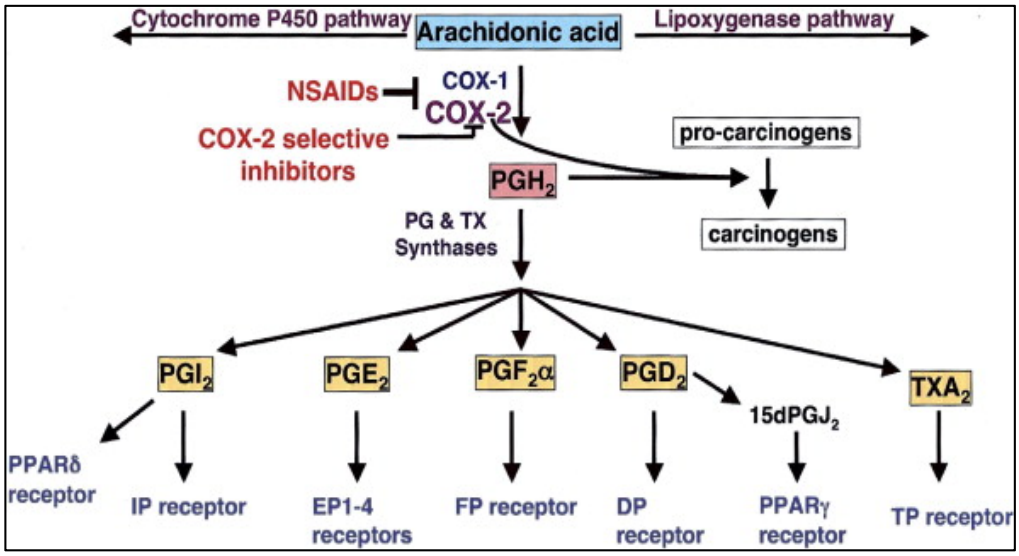

Figure 1: Overview of prostaglandin synthesis. *Wang et al., Gastroenterology, 2005*.

Several studies have shown success in down-regulating COX-2 expression as a potential treatment for breast, colon, and ovarian cancers both *in vitro* and *in vivo*[63,101–112]. For example, it was found that COX-2 expression was inducible in breast cancer cells *in vitro* and that treatment with American ginseng blocked that expression[101]. Another breast cancer study investigated the potential metastatic effects of sphingosine 1-phosphate (S1P) *in vitro* and *in vivo* and found that overexpression of the receptor, $S1P_3$, is a potential marker of metastatic breast cancer and that S1P upregulates the COX-2/PGE_2 pathway in breast cancer[105]. Using colon cancer as another example, one group found that treatment with various culinary herbs and spices (individually and in combination) reduced COX-2 expression levels, inhibited colorectal cancer cell growth, and promoted anti-inflammatory effects *in vitro*[107]. Jiang et al. examined the effects of retinoic acid

chalcone (RAC) both *in vitro* and *in vivo* on COX-2 expression in colon cancer cells. They found that RAC inhibits COX-2 and PGE_2 expression and suggested that PGE_2 receptors play an important role in this inhibition[111]. A final example of down-regulating COX-2 in cancer is found in the treatment of ovarian cancer. One group found that combining calcitriol and the COX-2 inhibitor, celecoxib, had an additive effect on the inhibition of COX-2 expression in an ovarian cancer cell line suggesting a correlation between vitamin D and prostaglandin metabolism in ovarian cancer[103]. Another study reported that celecoxib inhibits ovarian cancer cell growth *in vitro* and *in vivo* under different metabolic conditions through a variety of mechanisms related to the inhibition of COX-2[104].

In addition to the prostaglandin pathway, NF-κB is recognized as another major pro-inflammatory pathway with two distinct mechanisms for activation **(Figure 2)**[113]. In the canonical activation of NF-κB, pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) or interleukin (IL)-1 bind to their receptors on the cell membrane and activate the IκB-kinase (IKK) complex consisting of IKKβ, IKKγ, and IKKα. This complex then phosphorylates IκB, targeting it for ubiquitination and proteasome degradation, which causes it to release the p50/p65 dimer. This dimer then translocates into the nucleus and initiates transcription of downstream genes that regulate inflammation and cell survival[113]. The alternative pathway, on the other hand, is activated by cytokines of the TNF superfamily—excluding TNF-α. Ligands, such as CD40L and lymphotoxin β, bind to their receptors and activates NF-κB-inducing kinase (NIK), thus activating IKKα. IKKα phosphorylates p100, which is broken down into p52 while the rest of it is ubiquitinated and degraded by the proteasome. The RelB/p52 dimer is then

translocated into the nucleus where it binds DNA and facilitates transcription of genes that regulate B-cell activation[113].

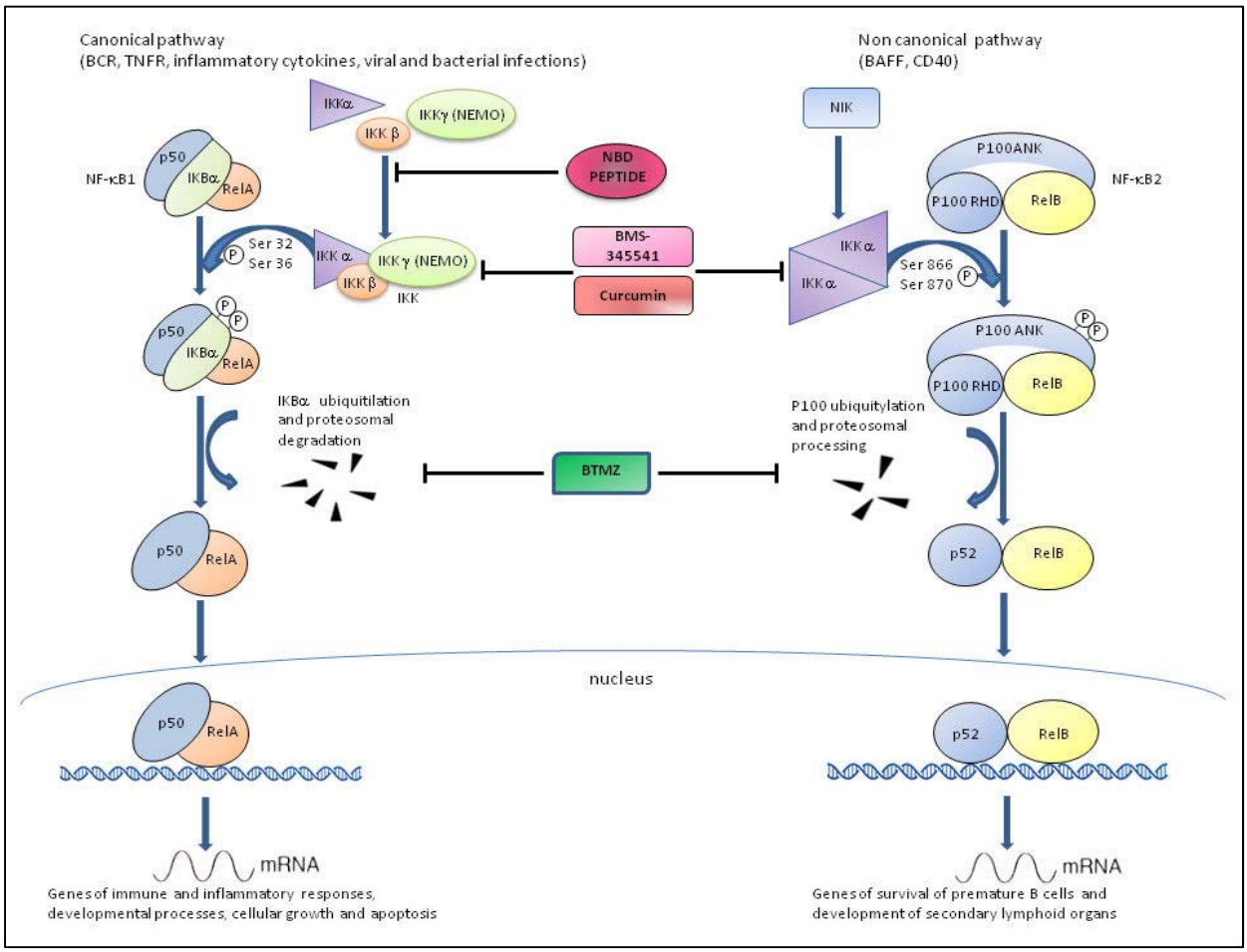

Figure 2: Canonical vs non-canonical activation of the nuclear factor kappa B signaling pathway. *Madonna et al., Journal of Translational Medicine, 2012.*

Down-regulation of the NF-κB signaling pathway has also been shown to be beneficial in breast, colon, and ovarian cancer prevention and treatment[98,101,112,114–117]. In breast cancer, one group found that pomegranate emulsion may prevent DMBA-initiated mammary tumorigenesis through down-regulation of the NF-κB pathway and up-regulation of the nuclear factor erythroid 2p45-related factor 2 (Nrf2) pathway (protects against oxidative stress caused by inflammation) *in vitro* and *in vivo*[114]. Another study, examining the effects of omega-3-polyunsaturated fatty

acids on colitis-associated cancer, found that docosahexaenoic acid (DHA) induced apoptosis of human colorectal cancer cells through the inhibition of COX-2 and NF-κB *in vitro*. This same study also found that *fat*-1 transgenic mice (which possess a gene that encodes for an enzyme allowing omega-6 fatty acid to omega-3 fatty acid conversion and therefore contain higher levels of omega-3 fatty acids and reduced omega-6 fatty acid levels) had significant protection from colitis-associated carcinogenesis through the blocking of β-catenin dissociation, induction of 15-prostaglandin dehydrogenase, and the repression of COX-2 and NF-κB[112]. Finally, it was shown that melatonin therapy reduced the size and incidence of ovarian cancer tumors *in vivo* through inhibition of the toll-like receptor 4-mediated signaling pathway due to the dampening of MyD88 and TRIF signaling pathways, in which NF-κB plays an important role[117].

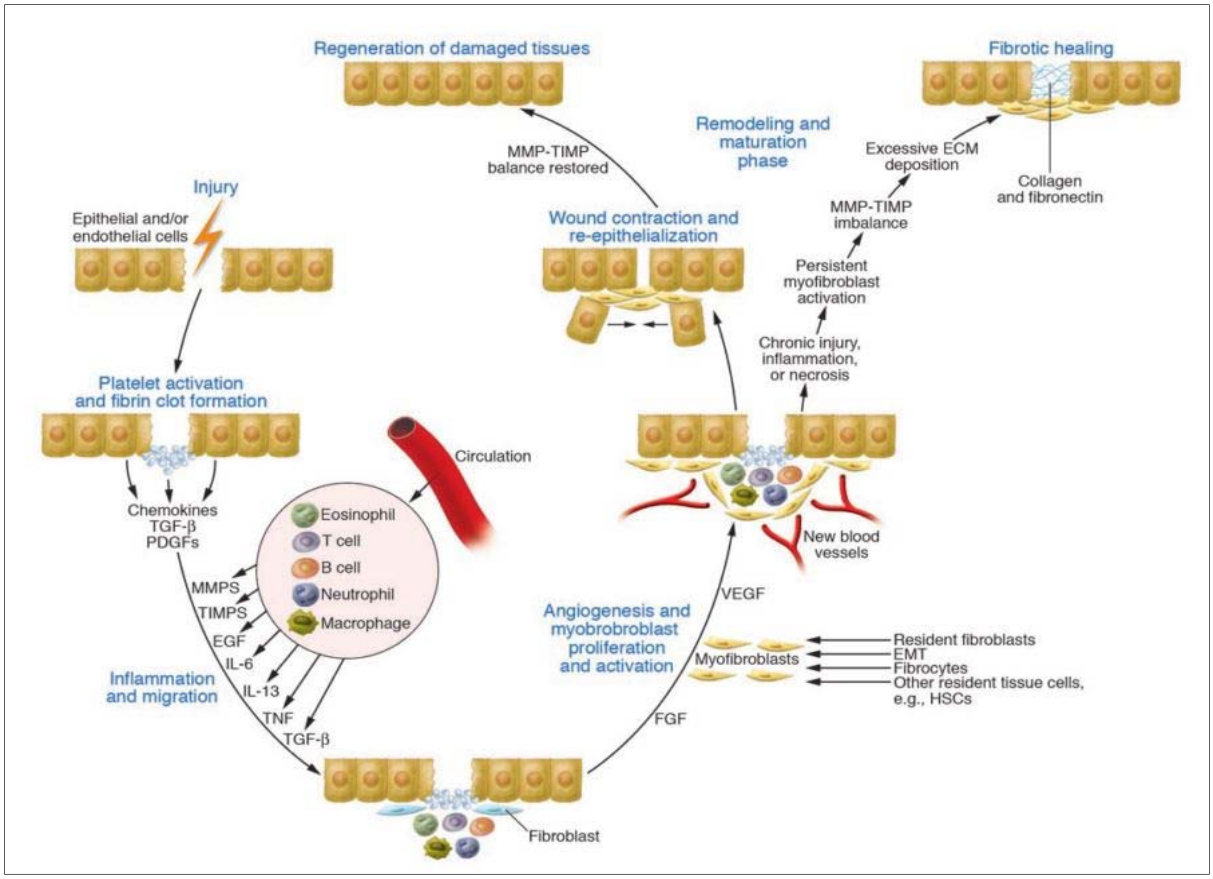

Figure 3: Wound healing vs. fibrosis. Inflammatory mediators, when unchecked, promote fibrosis through chronic myofibroblast activation and excessive deposition of ECM components. *Wynn, T.A., Journal of Clinical. Investigation, 2007.*

In contrast to the various cancers discussed above, leiomyomas are not malignant tumors. However, it has been well established that chronic inflammation leads to fibrosis—the central event to fibroid development[118–121]**. Figure 3** demonstrates how inflammatory mediators, when left unchecked, result in the formation of fibrotic tissue. Normally, after tissue injury, inflammatory mediators released from damaged cells initiate rapid healing responses, such as blood clot formation, vasodilation, basement membrane disruption, and recruitment of additional inflammatory cytokines. Eventually, new cells take the place of damaged ones, and the healing process is completed[118]. When repeated injuries cause chronic inflammation, this healing process is disrupted by constant activation of myofibroblasts which ultimately leads to the synthesis of excessive ECM[118]. Myofibroblasts are cells that express smooth muscle proteins, such as actin, and are thought to be derived from two sources: differentiation from fibroblasts and through epithelial-to-mesenchymal transition (EMT) from epithelial cells[118,120]. Several cytokines have been implicated in myofibroblast activation, including TNF-α, IL-1β, IL-4, IL-6, the IL-10 family, IL-13, and TGF-β.

TGF-β is a key, pro-fibrotic cytokine involved in the development of fibroids and has three different isoforms (TGF-β1, TGF-β2, and TGF-β3)[122–124]. Arici and Sozen found that TGF-β3 is expressed 3.5 times more in leiomyomas than in the myometrium[122]. Furthermore, fibronectin expression was also increased in leiomyomas compared to the myometrium[122]. They also found that in culture, treatment of leiomyoma cells with TGF-β3 induces fibronectin expression in addition to proliferation; TGF-β3 also increased proliferation of myometrial cells[122]. Another study treated myometrial and leiomyoma cells with TGF-β3 and found increased mRNA and protein expression of collagen 1A1, fibronectin, and connective tissue growth factor—all of which are proteins found in ECM[124]. Several signaling pathways are

implicated in the mechanism of action behind TGF-β's fibrosis-inducing capabilities, however, the Smad signaling pathway is arguably one of the most studied pathways in relation to TGF-β. Activation of Smad involves TGF-β binding to Smad-coupled receptors. Following ligand binding, a heterodimer (Smad2/Smad3) is phosphorylated and becomes activated. These R-Smads then form a heterotrimer with Smad4. This heterotrimer then translocates to the nucleus where it can regulate the transcription of pro-fibrotic genes.

Anti-Inflammatory Properties of Flaxseed:

Flaxseed (*Linum usitatissimum*) is a cultivated plant that consists of two components: the lignan portion and the oil portion. The lignan portion (mainly secoisolariciresinol diglycoside, or SDG) acts as a phytoestrogen and may play a role in the prevention and treatment of estrogen-associated diseases[67]. Flaxseed oil is rich in α-linolenic acid, an omega-3-fatty acid. Flaxseed has been associated with reducing inflammation in a variety of diseases such as type-2 diabetes and cardiovascular disease. Jangale et al. showed reduced oxidative stress and down-regulation of NF-κB in the kidney of type-2-diabetic mice through dietary intervention with flaxseed oil[68]. In assessing cardiovascular disease risk, α-linolenic acid was found to have a protective effect by significantly decreasing C-reactive protein (pro-inflammatory marker) levels as well as decreasing lipid and lipoprotein levels[69]. In addition to its beneficial health effects on type-2 diabetes and cardiovascular disease, flaxseed has been shown to be beneficial in the treatment of several cancers, including ovarian cancer.

Dr. D.B. Hales' group at Southern Illinois University School of Medicine has demonstrated the protective effects of flaxseed on ovarian cancer in the laying hen model as well as elucidated some of the mechanisms by which flaxseed may be exerting its protective effects[63,64,66]. Flaxseed appears to reduce ovarian cancer severity in the laying hen through the

reduction of PGE_2 primarily by inhibition of COX-2[63,64]. Hens fed a flaxseed diet also were found to have a down-regulation in Akt and NF-κB signaling pathways as well as altered estrogen metabolism. Taken together, these studies have established great potential for the use of flaxseed as a therapeutic agent in combating inflammation in various diseases and cancers.

Anti-Inflammatory/Anti-Fibrotic Properties of Vitamin D:

Vitamin D is a fat-soluble vitamin that is important in the regulation of calcium and bone homeostasis[74]. In addition to the aforementioned regulatory functions, vitamin D has also been shown to regulate various inflammatory cytokines[71,75–78] as well as fibrosis[72,73,86]. Khoo et al. found that vitamin D_3 was able to shift the immune response to *M. tuberculosis* from pro-inflammatory to anti-inflammatory by reducing TNF-α and IFN-γ production and increasing IL-10 production[75]. Another study demonstrated vitamin D's ability to indirectly inhibit IL-6 production through the inhibition of TNF-α production[125]. With regard to vitamin D's receptor (VDR), Zerr et al. found that activation of VDR downregulates TGF-β/Smad signaling and concluded that decreased levels of VDR may cause hyperactive TGF-β signaling and fibroblast activation in systemic sclerosis[72]. Vitamin D_3 has also been shown to suppress the expression of pro-fibrotic genes such as fibronectin, collagen type 1, and PAI-1 through the downregulation of TGF-β3 and downstream Smad signaling[86]. The aforementioned studies provide strong evidence to support vitamin D's anti-inflammatory and anti-fibrotic potential. Therefore, dietary intervention with vitamin D should be pursued as a potential therapy for inflammatory and fibrotic diseases.

Summary

Uterine leiomyomas, or fibroids, are the most common benign, gynecological tumors affecting women of reproductive-age. While their etiology remains unknown, inflammation and the steroid hormones estrogen and progesterone are hypothesized to play a key role in fibroid development and maintenance. Risk factors for leiomyomas include local injury, stress, obesity, hypertension, race, and lack of parity. The laying hen has been validated as an animal model for fibroids as they develop them spontaneously on their oviduct as they age. Whole flaxseed and vitamin D both have been shown to possess anti-inflammatory properties and thus might be useful in the prevention and treatment of uterine leiomyomas. Based on the information provided in this literature review, the purpose of our studies were to investigate the effects of whole flaxseed and vitamin D supplementation as a dietary intervention strategy in the treatment of uterine leiomyomas in the laying hen (*Gallus gallus domesticus*).

CHAPTER 2

Whole Flaxseed Supplementation has a Positive, Anti-Inflammatory Effect on Leiomyomas in the Oviduct of the Laying Hen

Abstract:

Uterine leiomyomas, or fibroids, are the leading cause of hysterectomies in the United States. Fibroids are the most common benign, gynecological tumors in women with the incidence of symptomatic fibroids in reproductive-aged women being around 40%. These tumors are known to be a major cause of menorrhagia and also can cause pelvic pain and infertility. Leiomyomas are characterized by an overgrowth of extracellular matrix and fibrous tissue and are dependent on ovarian steroid hormones for growth and maintenance. Laying hens spontaneously develop leiomyomas in their reproductive tract, similar to women. Leiomyomas in hens occur as polyps on the smooth muscle layer of the oviduct with increasing abundance as hens age and have the same histological and molecular markers as human uterine fibroids including expression of desmin, smooth muscle actin, increased collagen, and steroid hormone receptors. Flaxseed has two major biologically active components, oil in the germ and phytoestrogen lignans in the hull, thus making it an ideal natural supplement for dietary intervention. Flaxseed oil is high in omega-3 fatty acids (alpha-linolenic acid) and these are known to act as inhibitors of prostaglandin pathways and as potent anti-inflammatories. Because of this, the purpose of our study was to determine whether diet supplementation with flax would have a beneficial therapeutic effect on leiomyomas. To assess the effects of whole flaxseed-supplemented diet versus a control diet, 2.5-year-old hens were fed either an isocaloric control or 15% whole flaxseed-supplemented diet *ad libitum* for 12 months. Hens were then euthanized, and fibroids were measured and collected. Immunohistochemical analyses of fibrosis (Gomori's

Trichrome Stain), proliferation (Proliferating Cell Nuclear Antigen), angiogenesis (von Willebrand Factor), NF-κB p65, oxidative stress (nitrotyrosine), cellular senescence (Sudan Black B), and presence of mast cells (Toluidine Blue) were performed on fibroid tissue sections. qRT-PCR was performed to determine changes in expression levels of mRNA for collagen I, TGF-β3, and *PTGS2*. Western blot was performed to determine changes in protein levels for COX-2. We found that there was a slight decrease in the incidence of leiomyomas in the flaxseed group versus the controls. There was no significant difference in the volume of leiomyomas. Morphologically, we did not observe any differences between fibroids from the two treatment groups. Fibroids from hens fed the flaxseed-supplemented diet for 12 months showed a modest decrease in proliferation when compared to controls and a significant decrease in numbers of blood vessels per unit area of fibroid. There were no differences in p65 expression or in tissue nitrotyrosine levels between treatment groups. Sudan Black B staining showed that leiomyomas from both groups were not undergoing cellular senescence while Toluidine Blue staining confirmed mast cell infiltration into fibroids from both groups. Fibroids from hens fed the whole flaxseed diet had modestly decreased collagen I and TGFβ-3 expression, and *PTGS2* expression was significantly downregulated when compared to fibroids from hens fed the control diet. The observed decreases in the number of blood vessels present and mRNA expression level of *PTGS2* support dietary supplementation with whole flaxseed may have a beneficial impact on leiomyomas through decreased angiogenesis and downregulation of the prostaglandin signaling pathway.

Introduction:

Uterine leiomyomas, or fibroids, are the most common benign tumors found in women of reproductive age. Formed from smooth muscle cells of the myometrium, fibroids are sensitive to estrogen and progesterone[3]. Approximately 77% of reproductively-aged women have fibroids[4], and 10-50% of fibroids (depending on race) are clinically significant[6]. Symptoms of fibroids include pelvic pain, menorrhagia, and infertility[5]. Currently, there are no effective long-term treatment options available for leiomyomas. Hysterectomy or myomectomy have traditionally been the routine treatment for fibroids causing them to be the leading indication for hysterectomy in the United States[1]. Minimally invasive techniques, such as MRgFUS, provide some relief, but have a high surgical re-intervention rate of approximately 60%[51]. Non-surgical techniques, such as the use of GnRH analog therapy reduces the size of fibroids, but induce a state of hypo-estrogenism, causing women to experience menopausal symptoms[48,50]. Furthermore, upon discontinuation of this therapy, fibroids typically re-grow[48,50]. Risk factors for development of leiomyomas include race, local injury, stress, obesity, and a chronic pro-inflammatory state[1,7,8]. Because there is no effective long-term, non-invasive treatment available for fibroids, the purpose of our study is to test the effects of dietary intervention with whole flaxseed as a therapy for leiomyomas. Flaxseed consists of two components: the lignan portion, made up of phytoestrogens that can potentially antagonize the action of estrogen in fibroid development, and the oil portion, primarily consisting of α-linolenic acid that has known anti-inflammatory properties[67]. Our previously validated animal model for fibroids, the White Leghorn hen, develops leiomyomas spontaneously on the oviduct with increasing age[93]. These benign tumors express higher levels of ER, PR, and α-SMA when compared to normal oviduct tissue, just as

one would see in women[93]. We hypothesize that dietary intervention with 15% whole flaxseed will have beneficial, therapeutic effects on leiomyomas found in the oviduct of the laying hen.

Materials & Methods:

Animals:

White Leghorn hens, aged 2 years and 9 months were randomly divided into groups (n=200/group) and fed either a control, 5% corn oil, 5% flaxseed oil, 5% fish oil, 10% defatted flaxmeal, or 15% whole flaxseed diet *ad libidum* for one year. Since corn oil is consists almost purely of omega-6-fatty acids, it was used as a negative control against flaxseed oil, which is rich in omega-3-fatty acids (specifically, α-linolenic acid). Fish oil served as a control for the type of omega-3-fatty acid since it is rich in eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA**). Table 1** contains the ingredient list and calculated analysis for each of the diets fed. Hens were group-housed at the University of Illinois Poultry Research Facility. After one year, hens were sacrificed via CO_2 asphyxiation and ovaries and oviducts were collected. All animal use was approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

Dietary Composition						
Ingredient	**Control**	**5% Corn Oil**	**5% Fish Oil**	**5% Flaxseed Oil**	**10% Defatted Flax Meal**	**15% Whole Flaxseed**
Corn, %	67.40	52.00	52.00	52.60	54.90	47.58
SBM, %	18.30	18.30	18.30	18.30	18.30	18.30
Corn Gluten Meal, %	3.00	5.00	5.00	5.00	0.00	0.00
Flaxseed (whole), %	0.00	0.00	0.00	0.00	0.00	15.00
Defatted Flax Meal, %	0.00	0.00	0.00	0.00	10.00	0.00
Corn Oil, %	0.00	5.00	0.00	0.00	0.00	0.00
Fish Oil, %	0.00	0.00	5.00	0.00	0.00	0.00
Flaxseed Oil, %	0.00	0.00	0.00	5.00	0.00	0.00
Qual Fat, %	0.00	0.00	0.00	0.00	3.80	2.50
Solka Floc, %	0.30	8.70	8.70	8.70	1.99	5.62
Limestone, %	8.75	8.75	8.75	8.75	8.75	8.75
Dical, %	1.50	1.50	1.50	1.50	1.50	1.50
Salt, %	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin Mix, %	0.20	0.20	0.20	0.20	0.20	0.20
Mineral Mix, %	0.15	0.15	0.15	0.15	0.15	0.15
DL-Met, %	0.10	0.10	0.10	0.10	0.10	0.10
Calculated Analysis	**Control**	**5% Corn Oil**	**5% Fish Oil**	**5% Flaxseed Oil**	**10% Defatted Flax Meal**	**15% Whole Flaxseed**
CP, %	16.56	16.49	16.49	16.49	17.04	16.50
TME, kcal/kg	2,816	2,815	2,815	2,815	2,816	2,815
Calcium, %	3.73	3.73	3.73	3.73	3.77	3.75
aPhosphorus, %	0.38	0.37	0.37	0.37	0.40	0.38
Met + Cys, %	0.67	0.67	0.67	0.67	0.72	0.64

Table 1: Dietary Composition. Ingredients listed as % inclusion.

Tissue Collection:

Upon completion of the study, hens were euthanized, and ovaries and oviducts were collected. Leiomyomas found on the oviduct were measured before either being flash frozen in liquid nitrogen or fixed in formalin and embedded in paraffin. Because our lab was only interested in the effects of whole flaxseed versus a control diet, we only used leiomyomas from those two diets for subsequent analyses. Flash frozen leiomyomas were stored at -80ºC. Formalin-fixed, paraffin-embedded leiomyomas were serially sectioned to a thickness of 5µm. Sections were floated on lukewarm deionized water prior to being placed on Fisherbrand™ Superfrost™ Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Slides were allowed to dry overnight on a slide warmer before being used for histological analyses.

Leiomyoma Incidence and Size:

Leiomyoma incidence was calculated by taking the number of hens per treatment group that had at least one fibroid, dividing that by the total number of hens per treatment group, and multiplying by 100. Data are presented as a percentage. Leiomyoma size was determined by measuring fibroids in three dimensions using millimeters as the unit. Volume was calculated using the formula $V=l \times w \times h \times 0.523$, which is the formula for the volume of an ellipsoid and has been used to calculate fibroid volume previously[127]. Data are presented as an average of fibroid volume per treatment group.

Gomori's Trichrome Stain for Fibrosis:

Gomori's one-step trichrome stain was performed in order to evaluate any differences in matrix deposition between leiomyomas in the control and whole flaxseed groups. Slides were deparaffinized through three washes of xylene before being rehydrated through graded ethanol

solutions of decreasing concentrations and tap water. Once the deparaffinization and rehydration steps were complete, slides were incubated in Bouin's solution (Sigma-Aldrich, St. Louis, MO, USA). Running deionized water was used to wash slides after incubation in Bouin's solution. Slides were then placed in MilliQ water for prior to being placed into Meyer's hematoxylin. After staining in hematoxylin, slides were washed in running tap water and stained in Gomori's trichrome stain (reagents from Sigma-Aldrich, St. Louis, MO, USA and Fisher Scientific, Pittsburgh, PA, USA). Following the trichrome stain, slides were dipped in acetic acid prior to the dehydration and clearing steps. In order to dehydrate and clear the sections, slides were passed through graded ethanol solutions of increasing concentrations and three washes of xylene. Finally, slides were mounted with ClearMount™ mounting medium (American MasterTech, Lodi, CA, USA).

Immunohistochemical Analyses for Proliferation, Angiogenesis, NF-κB, and Tissue-Level Oxidative Stress:

Antibody	Host Species/Dilution	Company
PCNA (NB500-106)	Mouse/1:1000	Novus Biologicals, Littletown, CO, USA
Von Willebrand Factor (A008229-5)	Rabbit/1:400	Agilent Pathology Solutions, Santa Clara, CA, USA
NF-κB p65 (AB16502)	Rabbit/1:1000	Abcam, Cambridge, MA, USA
Nitrotyrosine (06-284)	Rabbit/1:500	Millipore Sigma, St. Louis, MO, USA
Biotinylated Anti-Mouse IgG	Horse/1:100	Vector Laboratories, Burlingame, CA, USA
Biotinylated Anti-Rabbit IgG	Goat/1:100	Vector Laboratories, Burlingame, CA, USA

Table 2: Provides information on antibodies used along with the catalogue number, host species, dilution used, and company where the antibody was purchased.

To determine differences in proliferation, angiogenesis, and oxidative stress at the tissue level between fibroids from laying hens fed a control diet versus those fed a diet supplemented with 15% whole flaxseed, immunohistochemistry was performed for proliferating cell nuclear antigen (PCNA), von Willebrand Factor (VWF), NF-κB p65, and nitrotyrosine. Briefly, slides were deparaffinized through three washes of xylene and rehydrated through graded ethanol solutions of decreasing concentrations and tap water. Next heat-mediated antigen retrieval was performed using DAKO Target Retrieval Solution, pH 9 (Agilent Pathology Solutions, Santa Clara, CA, USA). Inactivation of exogenous peroxidases was performed by placing slides in 0.3% hydrogen peroxide/methanol. Blocking of non-specific binding was achieved by incubating the slides in 5% normal serum using the host-specific IgG kits from Vector Laboratories (Burlingame, CA, USA) diluted in PBST. Primary antibodies were diluted in 1% bovine serum albumin (BSA)/PBST **(Table 2)**. Slides were incubated in the primary antibody overnight at 4°C. Non-specific IgG (Vector Laboratories, Burlingame, CA, USA) for the appropriate host species was used as a negative control on one section of each slide instead of the primary antibody.

On the following day, slides were incubated in 1:100 dilution of biotinylated secondary antibodies **(Table 2)** appropriate for each primary antibody's host species in 1% BSA/PBST. Slides were incubated in ABC solution (Vector Laboratories, Burlingame, CA, USA). For PCNA, VWF, and NF-κB p65 slides, 3,3'-Diaminobenzidine/peroxidase (DAB) (Vector Laboratories, Burlingame, CA, USA) solution was used as a chromogen and then counterstained in Mayer's hematoxylin. Slides were dehydrated through graded ethanol solutions of increasing concentrations and cleared through three washes of xylene. Finally, slides were mounted with ClearMount™ mounting medium (American MasterTech, Lodi, CA, USA). For nitrotyrosine slides, AEC Solution (Thermo Fisher, Waltham, MA, USA) was used as a chromogen after ABC

solution incubation. Slides were counterstained in Mayer's hematoxylin. Because the AEC chromogen is soluble in ethanol, slides were mounted with VectaMount™ AQ Aqueous Mounting Medium (Vector Laboratories, Burlingame, CA, USA).

Sudan Black B Stain for Cellular Senescence:

When a cell is neither undergoing hypertrophy nor hyperplasia, it is considered senescent. Senescent cells are thought to be at the end of their lifecycle, thus, it would be helpful to assess this endpoint in testing treatments for fibroids[128]. In order to assess possible differences in cellular senescence between leiomyomas from hens fed a diet supplemented with 15% whole flaxseed versus a control diet, Sudan Black B staining was performed. Sudan Black B stains lipofuscin granules which have been shown to accumulate in senescent cells[129]. Briefly, slides were deparaffinized and rehydrated through xylene and graded ethanol solutions of decreasing concentration. Slides were then incubated in Sudan Black B (Sigma-Aldrich, St. Louis, MO, USA) solution at room temperature overnight. The following day, slides were rinsed in 70% ethanol until sections were pale grey before being transferred into two washes of ethanol. Slides were then cleared and mounted with ClearMount™ mounting medium (American MasterTech, Lodi, CA, USA).

Toluidine Blue Stain to Assess Presence of Mast Cells:

To determine any differences in mast cell infiltration in leiomyoma tissue between treatment groups, leiomyomas were sectioned to a thickness of 5 micrometers and then stained with Toluidine Blue. The Core Histology Lab in the Department of Comparative Biosciences at the University of Illinois kindly performed this staining. Their protocol is summarized below.

Sections were deparaffinized and hydrated through graded alcohol solutions to water. Slides were stained in 0.1% Aqueous Toluidine Blue solution for 10 minutes followed by rinsing in distilled water. Dehydration of sections occurred quickly through 95% and 100% alcohol. As this step differentiates the stain, slides were checked carefully throughout this process. Finally, slides were cleared through xylene and mounted.

RNA Isolation and cDNA synthesis:

RNA was isolated from fibroids using the Trizol method. Briefly, 50-100 mg of tissue were homogenized in 1 mL of Trizol using 2mm Tungsten Carbide beads and the TissueLyserII both from Qiagen. Fibroids were macerated for 25 second intervals with 5 seconds on ice between intervals. Following homogenization, 200 µL of chloroform were added to sample tubes. Tubes were vortexed and incubated at room temperature for 10 minutes. Following incubation, tubes were centrifuged at 12,000xg for 15 minutes at 4ºC in a microcentrifuge. After centrifugation, the aqueous layer was extracted. 500 µL of isopropanol and 5 µL of glycogen (for pellet visualization) were added to the aqueous layer and samples were vortexed and incubated overnight at -20ºC. The following day, samples were centrifuged at 12,000xg for 15 minutes at 4ºC in a microcentrifuge and the supernatants were discarded. Pellets were washed with 75% ethanol and centrifuged at 12,000xg for 5 minutes at 4ºC. Supernatants were again discarded and pellets were allowed to air dry. Finally, pellets were re-suspended in 22 µL of nuclease free water. A260/280, A260/230, and RNA concentration were measured using the Nanodrop. cDNA was synthesized from RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) per the manufacturer's instructions.

qRT-PCR:

Quantitative reverse transcription PCR was performed using a TaqMan ABI 7900 real time PCR machine at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. Changes in mRNA levels of collagen I (Gg03325897_m1), TGF-β3 (Gg03371523_m1), and *PTGS2* (Gg03320004_m1) from leiomyomas found in hens fed either the control or whole flaxseed-supplemented diet were measured. 18S (Hs99999901_s1) was used as the housekeeping gene. All primer probe sets were purchased from Applied Biosystems (TaqMan™, Foster City, CA, USA). Statistical analysis was performed on delta Ct values. Data are presented as relative fold change.

It is important to note that due to high variation in our housekeeping gene between samples, our relative fold change average of our control samples was not equal to 1. Therefore, we divided our control and flaxseed fold change averages by the average fold change value of the controls to accurately depict relative fold change in our graphs. Statistical analyses were not performed on these values as they induced artificial significance.

Protein Isolation:

NP-40 lysis buffer was used to isolate proteins from fibroids using the following protocol: approximately 50 mg of tissue were placed into 2 mL of lysis buffer. Using the TissueLyserII and 2mm Tungsten Carbide beads from Qiagen, fibroids were homogenized for 25 seconds followed by 5 seconds on wet ice. This step was repeated as necessary until fibroids were completely disrupted. Following homogenization, samples were centrifuged at 12,000xg for 10 minutes at 4ºC. The supernatant was divided into aliquots and stored at -80ºC until needed for future applications.

Western Blot:

Antibody	Host Species/Dilution	Company
COX-2 (160126)	Rabbit/1:1000	Cayman Chemicals, Ann Arbor, MI, USA
B-Actin (A5060)	Rabbit/1:1000	Sigma-Aldrich, St. Louis, MO, USA
HRP-linked Anti-Rabbit IgG (7074S)	Goat/1:5000	Cell Signaling Technology, Danvers, MA, USA

Table 3: Antibodies used for western blot analysis along with their catalogue number, host species, dilution used, and company purchased from.

Protein samples were thawed on wet ice and concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) prior to performing the western blot protocol. Gels were loaded with 15 mg of protein along with 4xLSB (3 parts protein to 1 part 4xLSB). BioRad protein ladder was used for the standards. The gels were run at 120V for 1 hour and then transferred to PVDF membranes (100V for 1 hour). Membranes were blocked in 3% BSA/TBST overnight at 4ºC on a shaker. The following day, the blocking solution was discarded and primary antibody for COX-2 was applied. Membranes were incubated in the primary antibody overnight at 4ºC on a shaker **(Table 3)**. On the third day, membranes were washed three times in TBST for 10 minutes prior to being incubated in the secondary antibody for 1 hour at room temperature on a plate shaker **(Table 3)**. Following another three washes in TBST, membranes were incubated in SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, MA, USA) for 5 minutes in the dark and visualized using an ImageQuant machine. Once imaging was complete, membranes were stripped for 15 minutes at room temperature on a plate shaker with Restore™ Western Blot Stripping Buffer (ThermoFisher Scientific, Waltham, MA, USA). Membranes were again blocked in 3% BSA/TBST overnight at 4°C on a plate shaker. The following day, the blocking

solution was discarded and primary antibody for the loading control (β-actin) was applied. Membranes were incubated in the primary antibody overnight at 4ºC on a shaker **(Table 3)**. On the final day, membranes were washed three times in TBST for 10 minutes prior to being incubated in the secondary antibody for 1 hour at room temperature on a plate shaker **(Table 3)**. Following another three washes in TBST, membranes were incubated in SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, MA, USA) for 5 minutes in the dark and visualized using an ImageQuant machine.

Nanozoomer Analyses:

All slides were scanned on the Nanozoomer Slide Scanner at the Institute for Genomic Biology Core Facilities at the University of Illinois at Urbana-Champaign and analyzed using the Nanozoomer software. For PCNA quantification, positive cells were counted in 10 high-power fields from 3 sections per sample and averaged together. Using the Nanozoomer software, the area of each high-power field was calculated and data are represented as number of positive cells per millimeter squared. Quantification of Von Willebrand Factor was similar, except all blood vessels were counted and the area of each fibroid section was calculated. Data are represented as number of blood vessels per millimeter squared. Finally, NF-κB p65 was quantified using the same method as for PCNA. Only cells with positive nuclear staining for p65 were counted as they were indicative of active NF-κB signaling.

Statistical Analyses:

All statistical analyses were computed using GraphPad Prism 7.0. The D'Agonstino and Pearson normality test and the student's t test were used. A p value less than 0.05 was considered significant.

Results:

Leiomyoma Incidence, Number of Leiomyomas per Hen, and Size:

After one year on either the control or 15% whole flaxseed-supplemented diet, we found that hens had an incidence of leiomyomas of 44.44% and 33.02%, respectively **(Figure 4A)**. The average number of fibroids per hen were 1.25 for those fed the control diet and 1.2 for those fed the 15% whole flaxseed diet **(Figure 4B)**. To calculate volume, leiomyomas were measured in 3 dimensions, and the formula $V = l \times w \times h \times 0.523$ was used. The average size of leiomyomas from the control group was 148.3 mm^3 (n=227) and the average for the flaxseed group was 96.62 mm^3 (n=197). There were no statistically significant changes in the average volume of leiomyomas in either group (p=0.31), although there appears to be a modest decrease in those from hens fed the whole flaxseed diet **(Figure 4C)**.

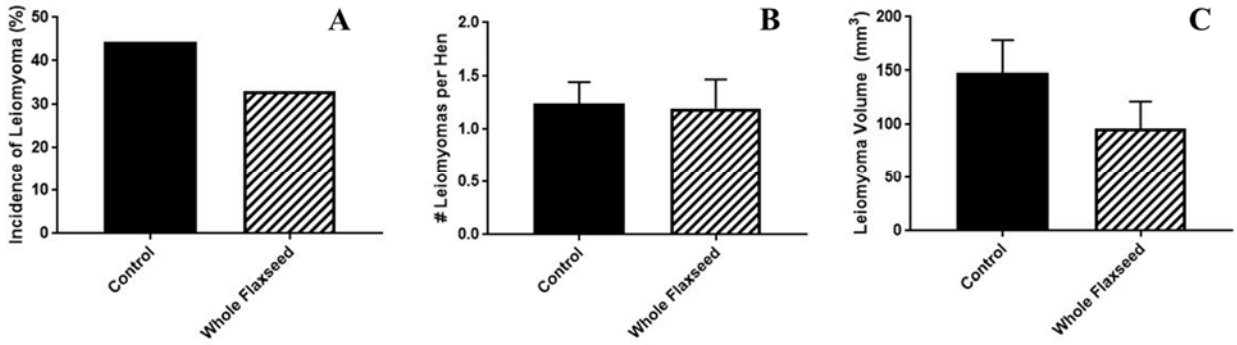

Figure 4: Incidence, number, and size of leiomyomas. A) After one year on their respective diets, incidences of fibroids were ~44% for control hens and ~33% for those fed the flaxseed diet. B) There were no differences in the number of fibroids per hen in either treatment group (control n=126, flaxseed n=106, p=0.10). C) There were no differences in fibroid size in either treatment group (control n=227, flaxseed n=197, p=0.31).

Morphology and Fibrosis:

We were interested in determining whether fibroids from hens fed a whole flaxseed-supplemented diet contained a lower amount of collagen than those from control hens. Gomori's trichrome stain was performed to assess collagen deposition. **Figure 5** shows a representative example of a fibroid from a hen fed a control diet and one from a hen fed a diet supplemented with whole flaxseed. The pink depicts smooth muscle cells while the blue represents collagen. We did not detect any differences in ECM deposition between control (n=18) and whole flaxseed (n=16) fibroids.

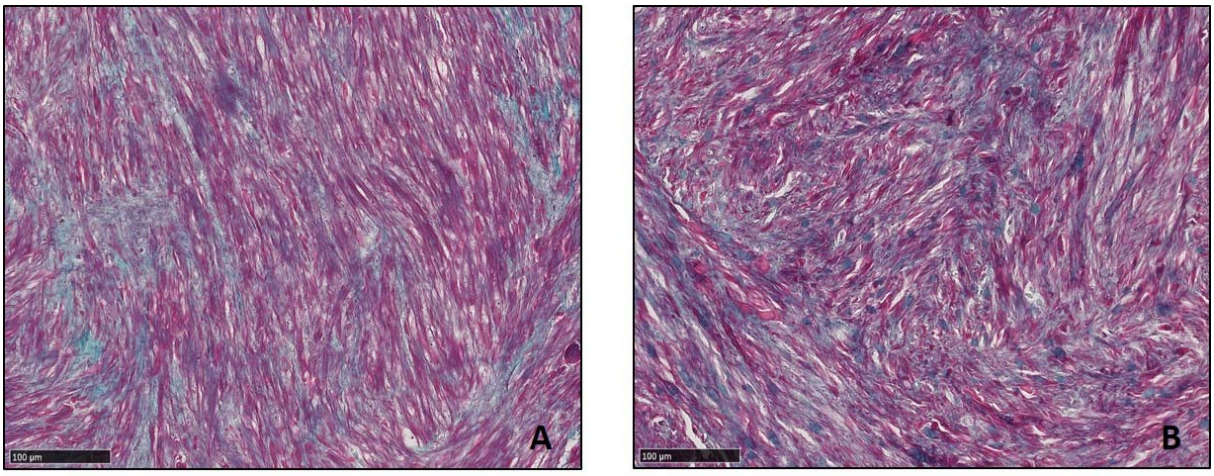

Figure 5: Representative Gomori's Trichrome Stain. Pink represents smooth muscle cells. Blue depicts collagen deposition. A) Leiomyoma from hen fed the control diet (n=18). B) Leiomyoma from hen fed the 15% whole flaxseed diet (n=16).

Proliferation:

Cellular proliferation plays an important role in tumor growth and survival. Thus, we next wanted to investigate whether whole flaxseed fibroids had decreased proliferation compared to the controls. Immunohistochemistry was performed and an antibody for PCNA was used. Fibroids from hens fed the whole flaxseed diet (n=22) had a trending decrease in the number of proliferating cells when compared to fibroids from control (n=21) hens (p=0.06, **Figure 6**).

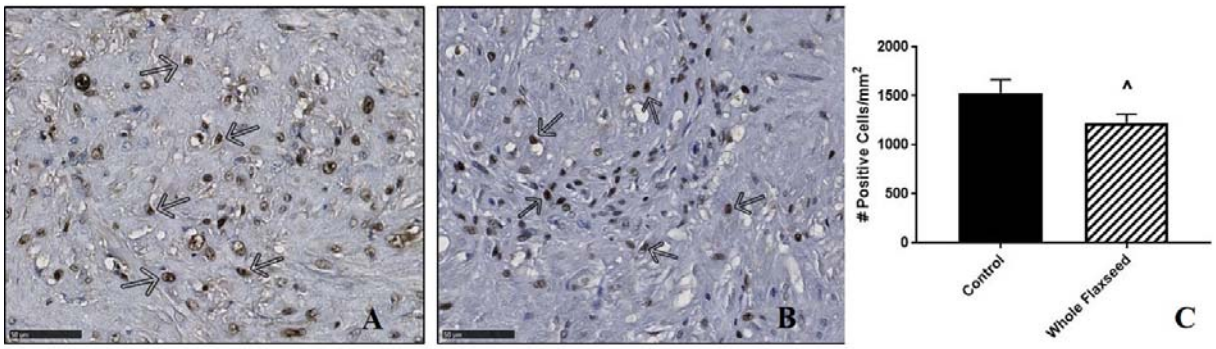

Figure 6: Proliferating Cell Nuclear Antigen. Examples of positive cells in leiomyomas from hens fed A) the control diet (n=21) and B) the 15% whole flaxseed diet (n=22). C) Quantification of the average number of positive cells per square millimeter in leiomyomas. There was a modest decrease in the number of proliferating cells from hens fed the 15% whole flaxseed diet (p=0.06).

Angiogenesis:

To assess differences in blood vessel formation in fibroids from hens fed either the control or flaxseed-supplemented diet, we immune-stained fibroid sections with an antibody against von Willebrand factor—a known marker of endothelial cells. We found that fibroids from hens fed a diet supplemented with whole flaxseed (n=14) had significantly fewer blood vessels when compared with those from hens on the control diet (n=14, p=0.02, **Figure 7**).

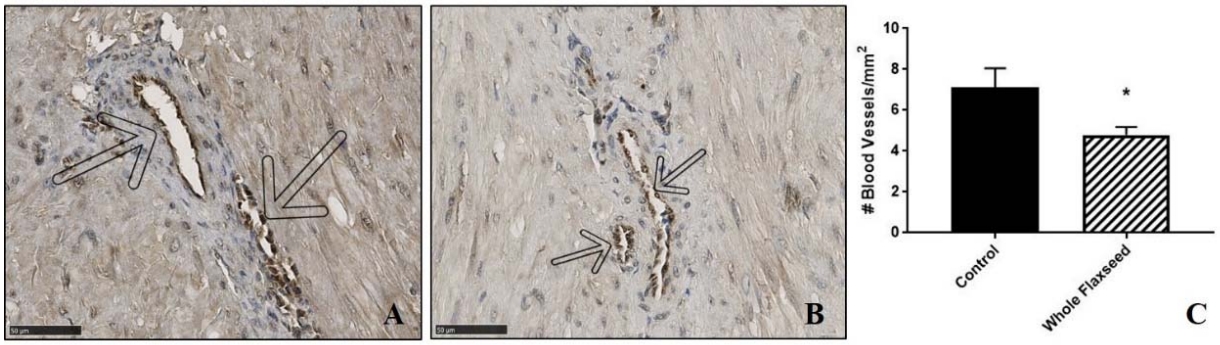

Figure 7: Von Willebrand Factor. Representative examples of blood vessels in leiomyomas from hens fed A) the control diet (n=14) and B) the 15% whole flaxseed diet (n=14). C) Quantification of number of blood vessels per square millimeter in leiomyomas**.** Fibroids from hens fed the flaxseed diet had significantly fewer blood vessels per square millimeter (p=0.02).

Nuclear Factor kappa B:

NF-κB is a well-known pro-inflammatory signaling pathway. In the classical activation of this pathway, IκB is phosphorylated causing release of the p50/p65 dimer and its subsequent translocation into the nucleus, thus rendering the pathway active. Immunohistochemical staining against p65 was performed to determine the localization of p65 in fibroids from hens fed either the control (n=12) or whole flaxseed (n=15) diets. Cells with active NF-κB signaling express p65 in the nucleus and were counted. 10 high-power-fields from each fibroid were averaged together to determine the average number of nuclear-positive p65 cells per square millimeter. We found no differences in cells expressing active NF-κB signaling between groups **(**p=0.86, **Figure 8)**.

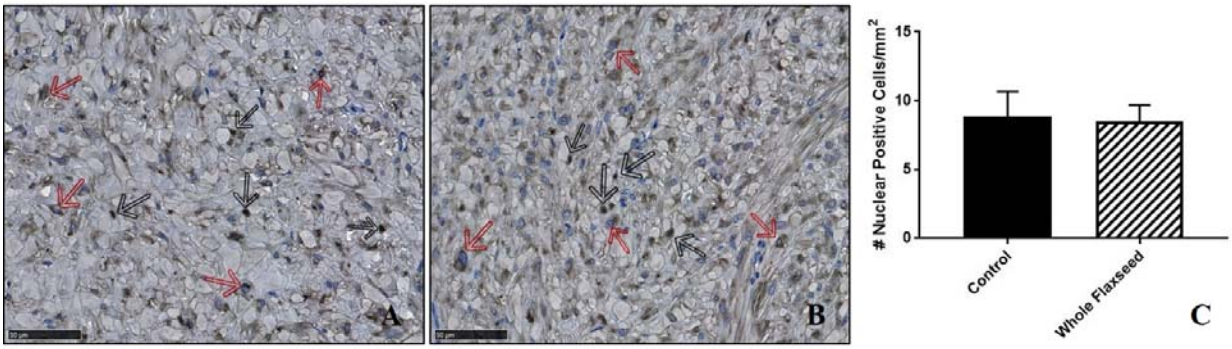

Figure 8: NF-κB p65 localization. Black arrows depict nuclear staining of p65 and active NF-κB signaling. Red arrows depict cytoplasmic staining of p65 and no active NF-κB signaling. A) Example of a fibroid from a hen fed the control diet (n=12). B) Example of a fibroid from a hen fed the 15% whole flaxseed diet (n=15). C) Quantification of nuclear p65 staining (p=0.86).

Measure of Oxidative Stress in Tissue:

Immunohistochemical staining for nitrotyrosine was performed to assess oxidative stress at the tissue level. Fibroids from hens in both the control (n=12) and whole flaxseed (n=17) groups showed a generalized staining for nitrotyrosine throughout the entire tumor sections (**Figure 9A-B, D-E**). However, we noticed that in samples that had some oviduct still attached to the fibroid, the oviduct showed virtually no nitrotyrosine staining **(Figure 9C,F)**. Therefore, we

decided to stain control oviduct samples in addition to the fibroids. As can be seen in **Figure 9G-I**, nitrotyrosine was localized to the smooth muscle cells and glandular epithelium in the oviduct.

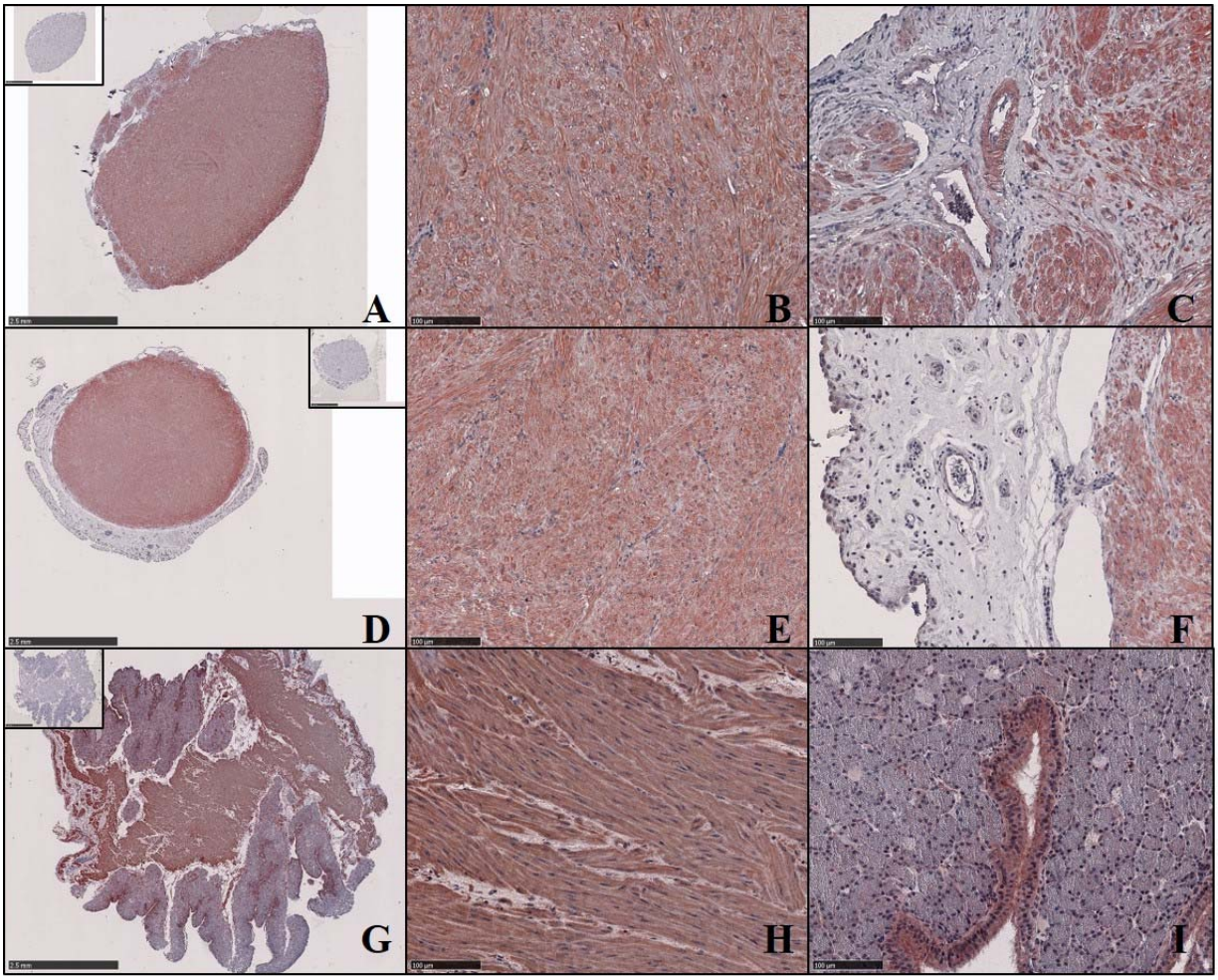

Figure 9: Nitrotyrosine. A) Overview of a fibroid from a hen fed the control diet (n=12). B) 20X view of the control fibroid (n=17). C) 20X view of the oviduct tissue attached to the control fibroid. D) Overview of a fibroids from a hen fed the whole flaxseed diet. E) 20X view of the flax fibroid. F) 20X view of the oviduct tissue attached to the flax fibroid. G) Overview of an oviduct section taken from a control hen. H) 20X view of smooth muscle cells of the oviduct. I) 20X view of glandular epithelium and surrounding stroma of the oviduct. The red staining depicts nitrotyrosine and the blue depicts the hematoxylin counterstain.

Cellular Senescence:

Sudan Black B staining was performed to determine levels of senescence, if any, in our fibroid samples. We found that neither leiomyomas from hens fed the control diet (n=14) nor those from hens fed the whole flaxseed diet (n=15) contained any senescent cells, as can be seen in **Figure 10B-C**. Human endometrium was stained as a positive control **(Figure 10A)**.

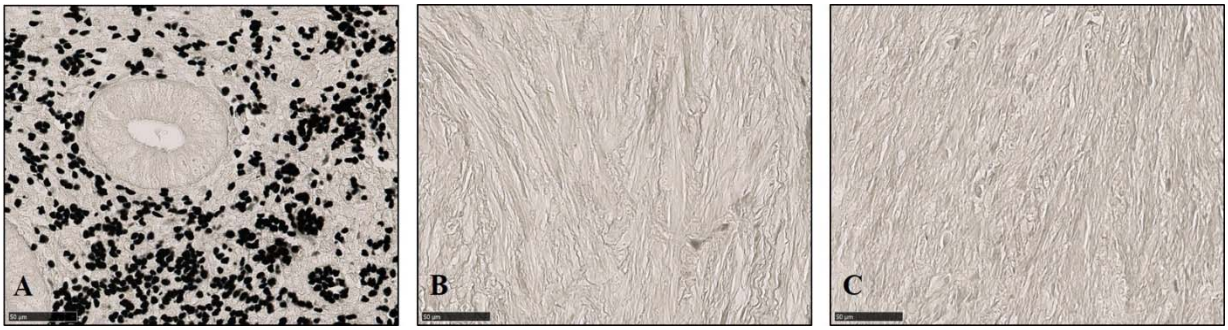

Figure 10: Sudan Black B Stain for Cellular Senescence. A) Human endometrium used as a positive control. The black stain represents the lipofuscin granules. B) Fibroid from hen fed the control diet (n=14). C) Fibroid from hen fed the diet supplemented with whole flaxseed (n=15).

Presence of Mast Cells:

Infiltration of immune cells, such as mast cells, is an important part of the inflammatory response. To determine whether mast cells were infiltrating into leiomyomas, toluidine blue staining was performed. We found mast cells present in fibroids from both control (n=19) and whole flaxseed treated hens (n=23). The majority of mast cells were found near the edges of leiomyomas as well as near blood vessels **(Figure 11)**.

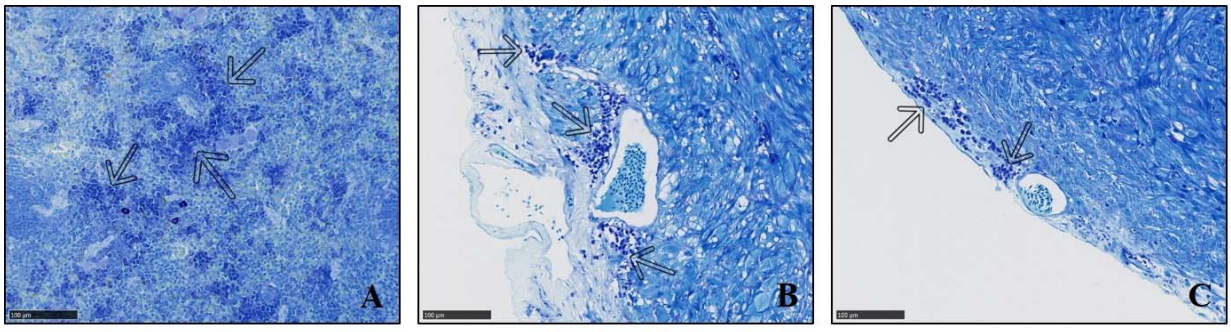

Figure 11: Toluidine Blue stain. Mast cells are stained deep blue (depicted by arrows). A) Mouse spleen used as a positive control. B) Fibroid from hen fed the control diet. C) Fibroid from hen fed the whole flaxseed diet. While mast cells appear throughout the fibroids, they were most commonly seen around the edges of fibroids and around blood vessels.

qRT-PCR:

Collagen I, TGFβ-3, and *PTGS2* expression levels were measured relative to the housekeeping gene, 18S, in leiomyomas from hens in each treatment group (control n=15, whole flaxseed n=14). We found that both collagen I and TGFβ-3 were downregulated in fibroids from hens fed the whole flaxseed diet, but this downregulation was not significant (**Figure 12A-B**). Leiomyomas from whole flaxseed-fed hens, on the other hand, did have a significant downregulation of the *PTGS2* gene (p=0.04, **Figure 12C**).

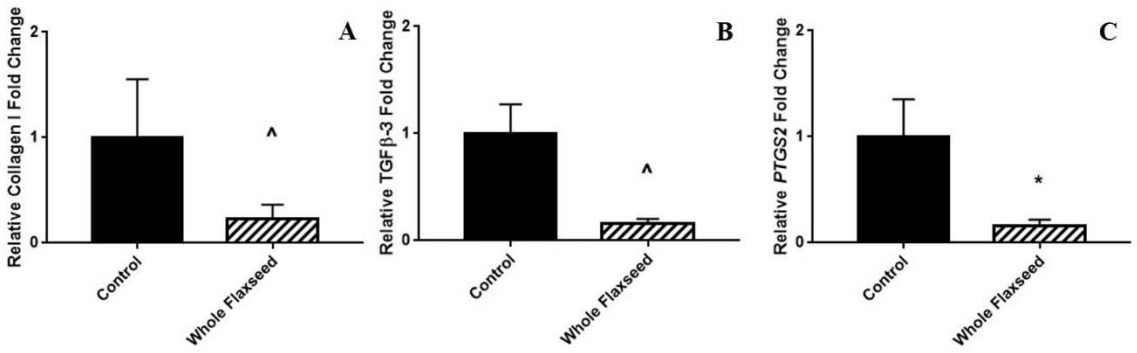

Figure 12: Relative Collagen I, TGFβ-3, and *PTGS2* Expression. A&B) Fibroids from the whole flaxseed group showed a trending decrease of collagen I and TGFβ-3 mRNA expression when compared to those from the control group. C) *PTGS2* was downregulated six-fold in fibroids from hens on the flaxseed diet when compared to those from hens fed the control diet. ^p=0.06 *p<0.05

Western Blot:

Western blot analysis for COX-2 was performed relative to β-actin to assess protein expression levels in leiomyomas from control-fed hens (n=9) and flaxseed-fed hens (n=9). COX-2 exists in two isoforms in the chicken, and we were able to detect both of them in our samples **(Figure 13A)**. While both the higher and lower molecular weight bands show a decrease in

COX-2 expression in fibroids in the whole flaxseed group, neither of these changes are significant (p=0.17 and p=0.23, respectively, **Figure 13B-C**).

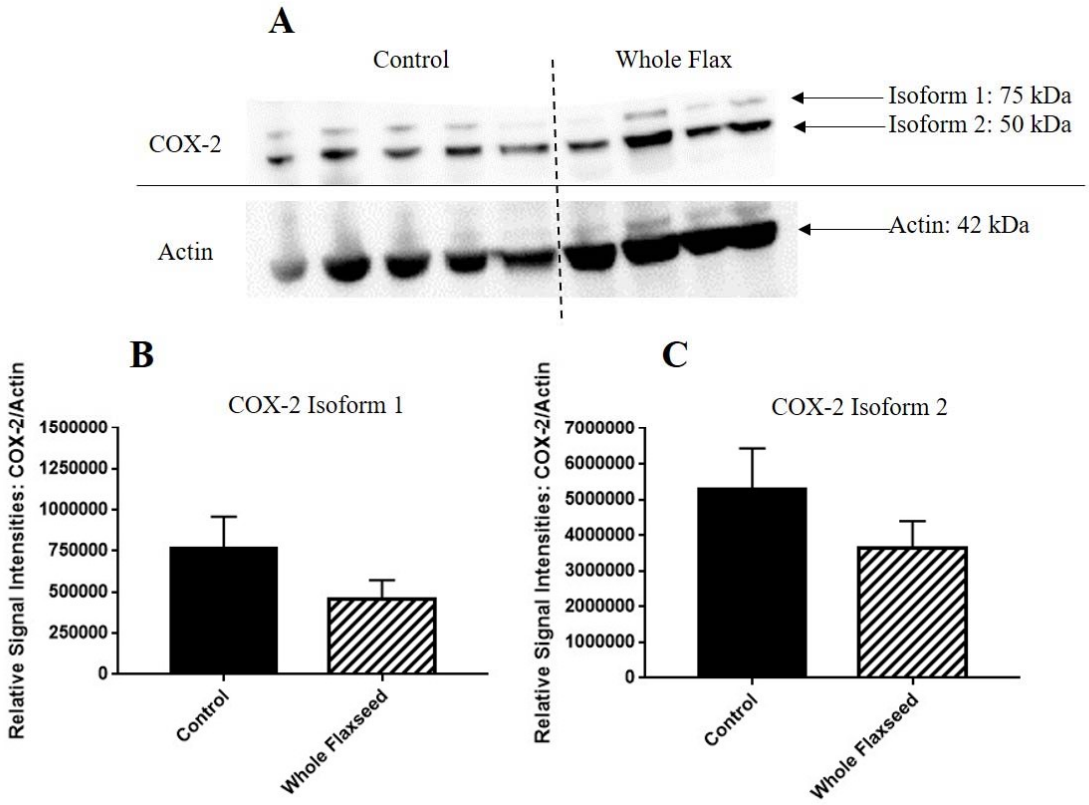

Figure 13: COX-2 protein expression relative to β-actin. A) Two isoforms of COX-2 were detected with molecular weights of ~75kDa and ~50kDa. B) Quantification of the isoform around 75kDa. Control n=9, Whole Flaxseed n=9, p=0.17. C) Quantification of the isoform around 50kDa. Control n=9, Whole Flaxseed n=9, p=0.23.

Discussion:

Currently, there are no effective long-term, non-surgical options for the treatment of uterine leiomyomas. The purpose of this study was to test the efficacy of dietary intervention with 15% whole flaxseed on leiomyomas by feeding White Leghorn hens either a control or 15% whole flaxseed-supplemented diet for 12 months. Overall, we found a modest decrease in proliferation in fibroids from hens fed the whole flaxseed diet compared to those fed the control diet. Our data also show fibroids from hens fed the flaxseed diet had significantly fewer blood

vessels per unit area than those from control hens. Finally, we demonstrated a modest decrease in collagen I and TGF-β3 gene expression and a significant decrease in *PTGS2* gene expression.

There were no differences in the number of fibroids per hen or the size of fibroids from hens fed either the control diet or the 15% whole flaxseed diet for 12 months. Based on the results of a previously conducted study by our group which demonstrated a significant decrease in the number of fibroids per hen and the size of leiomyomas from hens fed a similar diet consisting of 10% whole flaxseed for 18-24 months (data unpublished), there appears to be an effect of the length of time treatment is administered. Because hens in the previous study were fed this diet for twice the amount of time as our hens, we suspect that a longer period of time is required to see fibroid shrinkage with whole flaxseed supplementation. Furthermore, we witnessed a decreased incidence of fibroids in hens on the flaxseed diet after 12 months compared to the controls. The remainder of our findings suggest that molecular processes are occurring after 12 months that lead to the changes seen in leiomyomas after 18-24 months of dietary intervention with whole flaxseed.

Leiomyomas from hens fed the flaxseed diet had fewer proliferating cells than did those from hens on the control diet. Our results are consistent with various cancer studies that reported that flaxseed treatment decreases the number of proliferating cells in tumors[130–132]. In a study assessing the efficacy of flaxseed as a preventative for ovarian cancer, Dikshit et al. did not observe a decrease in cell proliferation within normal ovaries from hens fed a 5%, 10%, or 15% whole flaxseed diet compared to those fed a control diet[65]. However, unlike cancerous tumors, fibroids are not highly proliferative, and a decrease in cell proliferation within fibroids in the flaxseed group suggests that they are growing at a slower rate than those in the control group.

Nitrotyrosine is a general marker of nitrative stress, a form of oxidative stress[133]. Mast cells are granulocytes that release histamine and other factors during the inflammatory response. Oxidative stress and infiltration of mast cells were evaluated histologically in fibroids from both the control and whole flaxseed treatment groups. Fibroids from both groups exhibited generalized nitrotyrosine staining and infiltration of mast cells near tumor edges and blood vessels. Furthermore, nitrotyrosine staining was elevated in fibroids compared to normal oviduct tissue. These results are indicative of the occurrence of inflammatory responses in leiomyomas, supportive of the hypothesis that fibroids are caused, in part, by a chronic, pro-inflammatory state[8]. Consistent with our finding of elevated oxidative stress levels in leiomyomas versus normal oviduct tissue, Fletcher et al. investigated oxidative stress *in vitro* in cultured leiomyoma cells compared to cultured myometrial cells and found that myeloperoxidase, inducible nitric oxide synthase, and total nitrate and nitrite concentrations were increased in leiomyoma cells compared to myometrial cells[134]. Furthermore, this same group demonstrated upregulated nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4), a source of oxidative stress, in leiomyoma cells and tissues compared to normal myometrium[135]. Nitrotyrosine is only one indicator of oxidative stress, and further investigation into other markers (in addition to nitrotyrosine) could lead to new therapies for uterine fibroids that target oxidative stress pathways.

Cellular senescence is a state of growth arrest that can be indicative of the end of a cell's lifecycle. One study demonstrated induction of cellular senescence in three-dimensional leiomyoma cell cultures following Akt inhibition[128]. Because the fibroids we collected from our hens were formalin-fixed and embedded in paraffin, we decided to perform Sudan Black B staining to assess cellular senescence. No fibroids from hens fed the control or whole flaxseed

diet exhibited any Sudan Black B uptake, indicating that none of the fibroids we collected were undergoing senescence. Leiomyomas are common throughout the reproductive years of women, often regressing during menopause. This means that a woman could have a fibroid for a number of years, much longer than the hens on our study had them. It is possible that the tumors found in our hens were not 'aged' enough to be undergoing cellular senescence and therefore their cells are not near the end of their lifecycles. However, further studies are needed with our animal model to assess whether or not flaxseed could play a role in inducing senescence in leiomyomas.

Angiogenesis, the development of new blood vessels, is an important process for tumor survival and growth. Chronic inflammation causes increased endothelial cell activation, leading to angiogenesis[136]. Zanetta et al. demonstrated upregulation of von Willebrand Factor mRNA and protein levels by VEGF and FGF-2 in cultured human endothelial cells along with frozen human colon carcinoma tissues and normal colon tissue from patients undergoing a colon resection, suggesting VWF as a useful marker for endothelial cell activation and angiogenesis[137]. Immunohistochemical staining in our study for von Willebrand Factor showed significantly fewer blood vessels in fibroids from flax-fed hens compared to those from control hens. This result demonstrates the potential anti-inflammatory benefits of flaxseed on leiomyomas through the decrease of endothelial cell activation and angiogenesis. A study investigating the effects of diets high in polyunsaturated fatty acids and α-linolenic acid on cardiovascular disease risk found that a diet high in α-linolenic acid significantly reduced serum levels of C-reactive protein, intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin in men and women with high cholesterol[69]. They concluded that dietary α-linolenic acid exhibited vascular anti-inflammatory events to reduce the risk of cardiovascular disease[69]. This study,

alongside ours, support the anti-inflammatory properties of α-linolenic acid on endothelial cell activation.

In order to further investigate mechanisms through which flaxseed could exert its effects on leiomyomas in the laying hen, immunohistochemistry was performed to assess the translocation of p65 to the nucleus of fibroid smooth muscle cells, which would be indicative of NF-κB activation—a major pro-inflammatory pathway. We found no differences in the levels of p65 translocation between fibroids from hens fed either the control or 15% whole flaxseed diet, suggesting that suppressing NF-κB is not the main pathway by which flaxseed exerts its anti-inflammatory effects. In agreement with our finding, Heymach et al. showed NF-κB downregulation in men with prostate cancer consuming a low-fat diet versus those consuming a flaxseed-supplemented diet[138]. Another study found that whole flaxseed downregulated nuclear receptor coactivator 1 (NCOA1) mRNA expression, a NF-κB regulator, in the ovaries of White Leghorn hens, but had no effect on NF-κB mRNA expression[66]. They proposed that the flaxseed diet might regulate NF-κB activation by preventing nuclear translocation of NF-κB's transcriptional units[66]. However, our results show that this is not the case with regard to fibroids from hens fed a whole flaxseed-supplemented diet. Worthy of note, immunohistochemistry is generally recognized as a subjective and semi-quantitative analysis because it is up to the viewer to determine immunoreactive cells and the sections being analyzed are only microns thick compared to the actual size of the tissue. In an attempt to minimize error and ensure our results are representative of each sample, an average of ten high power fields per section and three sections per fibroids were counted. Samples were also analyzed blindly. However, it is possible that by not counting every nuclear-positive cell in the entire fibroid differences in NF-κB activation were missed. Furthermore, we only assessed one endpoint of the pathway using one

technique. Assessment of other effectors of the NF-κB pathway, such as NCOA1 mentioned above, through more quantitative methods could solidify our results.

We next investigated the effects of whole flaxseed supplementation on mRNA expression of three genes well-known to play a role in fibroid biology: collagen I, TGF-β3, and *PTGS2*. Collagen is a main component of ECM and TGF-β3 is a pro-fibrotic cytokine whose mRNA is expressed at higher levels in leiomyomas than normal myometrium and has been shown to induce fibronectin expression in leiomyoma cells[122]. TGF-β3 also increases the production of collagen I[124]. Previously, Machado et al. showed that leiomyomas from the laying hen expressed higher levels of collagen I and TGF-β3 compared to normal oviduct[93]. Collagen I and TGF-β3 mRNA expression were modestly decreased in fibroids from hens fed the whole flaxseed diet compared with those hens fed the control diet. These results suggest that flaxseed is having a beneficial effect on leiomyomas, however, consumption of the whole flaxseed diet over a longer period of time could lead to greater reductions. Future studies are needed to determine whether this is the case.

Fibroids from hens fed the 15% whole flaxseed diet showed a significant decrease in *PTGS2* mRNA expression compared to those from hens fed the control diet. *PTGS2* is the gene that codes for COX-2, the inducible enzyme in the prostaglandin pathway. The COX-2/prostaglandin pathway is implicated in a variety of inflammatory diseases, including cancer, and downregulating this pathway has been shown to be potentially beneficial to hinder disease progression[63–65,97,105]. Dr. D.B. Hales' group has extensively studied the effects of flaxseed supplementation on ovarian cancer in White Leghorn hens. According to their research, flaxseed reduces the severity of ovarian cancer by inhibiting COX-2 and subsequent PGE_2 production in the ovaries of the laying hen[63,64]. Our results suggest that the prostaglandin pathway is a target of

dietary flaxseed through decreased *PTGS2* expression, which could have a beneficial, therapeutic effect on fibroids.

It is important to note that our housekeeping gene didn't run as expected in that it there was a lot of variation between samples. There are a few possible reasons for this. First, it is possible that 18S isn't the best housekeeping gene for our samples and species. However, previous work with leiomyomas in the laying hen from our lab used 18S as a housekeeping gene successfully, so this is unlikely the case[93]. Second, our extracted RNA was analyzed on a Nanodrop Spectrophotometer prior to cDNA synthesis and were not treated with DNase. Because the Nanodrop cannot differentiate between RNA and DNA, it is possible that the amplification we witnessed was DNA amplification and not mRNA amplification. Our 18S primer-probe was designed within a single exon whereas our collagen I, TGFβ-3, and *PTGS2* primer-probes were designed across an exon junction, thus making DNA contamination and amplification in our housekeeping samples probable.

Western blot analysis showed a slight decrease in both isoforms of COX-2's expression relative to the β-actin loading control. As mentioned previously, COX-2 is the rate-limiting enzyme in the prostaglandin pathway and plays a role in various inflammatory diseases. Ke et al. investigated COX-2 expression in uterine leiomyomas in comparison with normal uterine tissue from women undergoing laparoscopy for their fibroids and found increased COX-2 expression in leiomyomas, suggesting an important role of COX-2 in uterine fibroids pathogenesis[139]. A different study simulated a menstruation-like environment by adding PGE_2 to cultured leiomyomas cells[140]. Their results demonstrate that PGE_2 increased COX-2 expression compared to non-treated cells[140]. Furthermore, they demonstrate that celecoxib (a COX-2 inhibitor) mediated this effect through decreasing inflammatory mediators associated with fibroid growth,

suggesting that celecoxib could inhibit leiomyoma growth[140]. In agreement with the aforementioned *in vitro* study, we saw a modest decrease in proliferation of our leiomyomas in the flaxseed group and a slight decrease in COX-2 expression *in vivo*, suggesting that flaxseed supplementation could be working to inhibit fibroid growth through the inhibition of COX-2. Furthermore, our western blot analysis agrees with our qRT-PCR analysis of *PTGS2*, the gene that codes for COX-2. While the changes in COX-2 expression were not significant, we suspect that a longer time on the 15% whole flaxseed diet will lead to a significant reduction in COX-2 protein expression and overall leiomyoma growth.

Conclusions and Future Directions:

Altogether, we conclude dietary intervention with whole flaxseed has therapeutic, anti-inflammatory effects on leiomyomas found in the oviduct of the laying hen, but the length of time in which the flaxseed diet is consumed plays a role in its ultimate efficacy. While our results depict an elementary peek into the mechanisms behind which flaxseed exerts its effects, a lot of future work needs to be completed to further elucidate these mechanisms and strengthen our findings. For example, assessing mRNA and protein expression of end products in the prostaglandin pathway, such as PGE_2 or $PGF_{2\alpha}$, between fibroids from control and flaxseed-fed hens would be valuable in determining if the prostaglandin pathway is a target of dietary flaxseed in the scope of leiomyoma treatment. Other valuable endpoints to assess in the elucidation of the mechanism of action behind dietary flaxseed would be mRNA and protein levels of inflammatory cytokines, such as IL-1β (pro), IL-6 (pro), and IL-10 (anti) in leiomyomas found in hens from each diet.

As mentioned in our discussion, our 18S housekeeping gene did not run properly during our qRT-PCR experiment. To validate the results we obtained, a few steps should be taken prior

to repeating this experiment. First, isolated RNA should be treated with DNase to digest any DNA in the sample. Secondly, more thorough quality control of our RNA should be conducted prior to running qRT-PCR, such as analysis using an Agilent Bioanalyzer machine. Third, a housekeeping gene designed across an exon junction would minimize the risk of DNA contamination in our samples interfering with the true results of the qRT-PCR experiment. Our 18S primer-probe was a Taqman assay from Applied Biosystems and was designed to target human 18S. Because of previous results from our lab with this specific primer in chicken samples, we chose to use this assay again. However, there is a GAPDH Taqman assay available that is designed for the chicken as a target species in addition to spanning an exon junction.

In addition to assessing the efficacy of dietary intervention with whole flaxseed as a therapy for leiomyomas, it would be interesting to test dietary intervention with whole flaxseed as a preventative for fibroids. To do this, White Leghorn pullets around 6 months of age should be fed either a 15% whole flaxseed or control diet and remain on their respective diets through their third year of lay. A subset of hens should be sacrificed and incidence of fibroids determined every six months. At the completion of the third year of lay, the remaining hens should be sacrificed, fibroid incidence determined, and fibroids collected for histological and molecular analysis.

There have not been many studies conducted analyzing the benefits of flaxseed supplementation as a treatment for uterine fibroids. After confirming our current results with further analyses on our samples in addition to conducting more animal trials, clinical trials should be carried out to test the efficacy of flaxseed supplementation in women with uterine fibroids.

CHAPTER 3

A Dietary Intervention Study Assessing the Effects of Supplementation with 10% Whole Flaxseed or Vitamin D_3 on Oviductal Leiomyomas in the Hen

Abstract:

Uterine leiomyomas, or fibroids, a leading cause of reproductive morbidity in reproductively-aged women, have a symptomatic incidence of around 40%. Symptoms include menorrhagia, pelvic pain, and infertility. These benign tumors are characterized by excessive production of extracellular matrix (ECM) components such as collagen. Leiomyomas are responsive to estrogen and progesterone, and occur more frequently as women age. Because the laying hen develops leiomyomas spontaneously as they age, they are a good animal model for studying the effects of diet on fibroids. White Leghorn hens, having completed their second year of lay, were randomly divided into three groups and fed either a control, 10% whole flaxseed-supplemented, or vitamin D_3-supplemented iso-caloric diet for 15 months. Blood was drawn from the same subset of hens every three months until the end of the trial. After 15 months on their respective diets, hens were euthanized. Oviducts were collected, and fibroids were counted, measured, and either flash frozen in liquid nitrogen or fixed in 10% neutral buffered formalin for further analysis. Leiomyoma incidence increased from 44% at the start of the trial to 78%, 74%, and 71% in the control, whole flaxseed, and vitamin D_3 groups, respectively after 15 months. The number of leiomyomas per hen in all three groups increased significantly after fifteen months when compared to the baseline; there were no differences between groups after fifteen months. Plasma carbonylated protein levels decreased significantly in the whole flaxseed and vitamin D_3-treated hens after 6 months on their respective diets and were significantly lower than those of the control hens after 9 months on the diets in both groups. qRT-PCR analysis showed a

downregulation of collagen I, TGF-β3, and *PTGS2* gene expression in leiomyomas from hens fed the vitamin D_3-supplemented diet after 15 months when compared to those from control hens. There were no differences between the whole flaxseed group and the control. Taken together, our data suggest an anti-inflammatory and anti-fibrotic effect of vitamin D_3 on oviductal leiomyomas of the laying hen. We conclude that dietary intervention with vitamin D_3 should be further pursued as an effective non-invasive, long-term, fertility-preserving treatment for uterine fibroids in women.

Introduction:

Uterine leiomyomas, or fibroids, have an incidence of approximately 77% and are the most common benign gynecological tumors in women of reproductive age[4]. Symptomatic fibroids cause pelvic pain, menorrhagia, and infertility[5]. Because of the significance of these symptoms and the lack of an effective, long-term treatment for fibroids, they are the leading cause of hysterectomies in the United States with an overall healthcare burden of approximately 34.37 billion dollars annually[2]. Leiomyomas form from smooth muscle cells of the myometrium, are characterized by excessive ECM deposition, and estrogen and progesterone play a key role in their development[3]. Furthermore, environmental and genetic factors such as injury, stress, obesity, and race increase the risk of developing fibroids[1,6]. While scientists have uncovered some of the mechanisms regulating fibroids, a large part of their etiology still remains unknown. However, chronic inflammation has been hypothesized to play a role in their development[7]. Unfortunately, no long-term, non-invasive treatments for fibroids exist. Hysterectomy and myomectomy have been the gold-standard for fibroid treatment, with the latter allowing for fertility preservation. Because of this, there has been a growing interest in the potential use of dietary supplements as a treatment for fibroids. Phytochemicals, such as genistein and curcumin,

in addition to vitamin D_3 have shown some potential as effective, long-term fibroid treatments due to their anti-inflammatory properites[52–56,77–84]. Previously, our laboratory validated the laying hen as an animal model for uterine fibroids[91]. The laying hen spontaneously develops leiomyomas on her oviduct as she ages. Furthermore, these tumors express higher levels of estrogen receptor (ER), progesterone receptor (PR), collagen I, and alpha smooth muscle actin (α-SMA) compared to normal oviduct tissue[91]. The purpose of our study was to determine the effects of dietary intervention with whole flaxseed and vitamin D_3 on leiomyomas using the laying hen model. We hypothesized that supplementation with whole flaxseed or vitamin D_3 would have beneficial anti-inflammatory and anti-fibrotic effects on leiomyomas found in the oviduct of the laying hen.

Materials and Methods:

Animals:

White Leghorn hens, aged 2 years and 9 months, were group-housed at the University of Illinois Poultry Research Facility. Hens were randomly assigned to treatment groups and were fed either a control (n=215), 10% whole flaxseed-supplemented (n=220), or vitamin D_3-supplemented (n=220) diet. The vitamin D_3 supplement, Rovimix® Hy-D®, was kindly donated by DSM Nutritional Products, Inc (Parsippany, NJ, USA) and was included at a dose of 69 µg/kg based on previous data from DSM showing this level of supplementation to have biological effects on bone[133]. **Table 3** shows the composition of each diet. Hens remained on their respective diets for a total of 15 months. At the beginning of the study, 25 hens were euthanized by CO_2 asphyxiation to obtain a baseline incidence of leiomyomas; oviducts and leiomyomas were measured and collected. Blood samples were collected from the same subset of hens (50 per treatment group) via the wing vein every three months and plasma was stored for future

analyses. After 15 months, hens were euthanized by CO_2 asphyxiation and oviducts and leiomyomas were measured and collected. All animal use was approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

Diet Composition			
Ingredient	**Control**	**10% Whole Flaxseed**	**Vitamin D_3**
Corn, %	67.64	59.04	67.59
Flaxseed (whole), %	0.00	10.00	0.00
Vitamin D_3, (%)*	0.00	0.00	0.05
SBM, %	18.30	18.30	18.30
Corn Gluten Meal, %	3.00	0.60	3.00
Solka Floc, %	0.00	1.00	0.00
Limestone, %	8.75	8.75	8.75
Dical, %	1.50	1.50	1.50
Salt, %	0.36	0.36	0.36
Vitamin Mix, %	0.20	0.20	0.20
Mineral Mix, %	0.15	0.15	0.15
DL-Met, %	0.10	0.10	0.10
*Rovimix® included at a dose of 69 µg/kg/day			

Table 4: Diet composition, % inclusion

Leiomyoma Incidence and Size:

Leiomyoma incidence was calculated by taking the number of hens per treatment group that had at least one fibroid, dividing that by the total number of hens per treatment group, and multiplying by 100. Data are presented as a percentage. Leiomyoma size was determined by measuring fibroids in three dimensions using millimeters as the unit. Volume was calculated using the formula $V=l \times w \times h \times 0.523$, which is the formula for the volume of an ellipsoid and has been used to calculate fibroid volume previously[124]. Data are presented as the mean fibroid volume per treatment group.

25-hydroxyvitamin D_3 via LC-MS/MS:

To confirm that the dose of vitamin D3 supplementation was significantly increasing vitamin D3 levels in in our hens throughout the trial, plasma samples were sent to Heartland Assays (Ames, IA, USA) for 25-hydroxyvitamin D_3 analysis via LC-MS/MS. 10 samples from each of the following time points and treatment groups were assayed: baseline (prior to start of trial), vitamin D3 3 months, vitamin D3 6 months, vitamin D3 9 months, vitamin D3 15 months, and control 15 months. We followed the same hens throughout as many time points as possible. DSM generously covered the costs of this analysis.

Carbonylated Protein Assay:

To obtain a measure systemic levels of oxidative stress in our hens, we measured levels of oxidized proteins in the plasma using the Protein Carbonyl Colorimetric Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA) per manufacturer's instructions. This kit uses the reaction between 2,4-dinitrophenylhydrazine and protein carbonyls to form a Schiff base thus producing a hydrazone[134]. The protein-hydrazone complex can be measured spectrophotometrically[134]. Samples were plated in duplicate with n=12 per treatment per time point. A pooled plasma sample was used to determine inter-assay variation across plates.

ELISA's:

Enzyme-linked immunosorbent assays were performed to measure systemic levels of the inflammatory cytokines TNF-α and interferon gamma (IFN-γ). The TNF-α kit was purchased from LifeSpan Biosciences, Inc. (LS-F23677, Seattle, WA, USA) and the IFN-γ kit was purchased from ThermoFisher (CAC1233, ThermoFisher Scientific, Waltham, MA, USA). Both

ELISAs were performed according to the manufacturer's instructions. Samples were plated in duplicate with n=7 per treatment per timepoint.

RNA Isolation and cDNA synthesis:

RNA was isolated from fibroids using the Trizol method. Briefly, 50-100 mg of tissue was homogenized in 1 mL of Trizol using 2mm Tungsten Carbide beads and the TissueLyserII, both from Qiagen. Fibroids were macerated for 25 second intervals with 5 seconds on ice between intervals. On average, it took 2-3 intervals for samples to be completely homogenized. Following homogenization, 200 µL of chloroform were added to sample tubes. Tubes were vortexed and incubated at room temperature for 10 minutes. Following incubation, tubes were centrifuged at 12,000xg for 15 minutes at 4ºC in a microcentrifuge. After centrifugation, the aqueous layer was extracted. 500 µL of isopropanol and 5 µL of glycogen (for pellet visualization) were added to the aqueous layer and samples were vortexed and incubated overnight at -20ºC. The following day, samples were centrifuged at 12,000xg for 15 minutes at 4ºC in a microcentrifuge and the supernatants were discarded. Pellets were washed with 75% ethanol and centrifuged at 12,000xg for 5 minutes at 4ºC. Supernatants were again discarded and pellets were allowed to air dry. Finally, pellets were re-suspended in 22 µL of nuclease free water. RNA concentration, A260/280, and A260/230 were measured using the Nanodrop. cDNA was synthesized from RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) per the manufacturer's instructions.

qRT-PCR:

Quantitative reverse transcription PCR was performed using a TaqMan ABI 7900 real time PCR machine at the Roy J. Carver Biotechnology Center at the University of Illinois at

Urbana-Champaign. Changes in mRNA levels of collagen I (Gg03325897_m1), *PTGS2* (Gg03320004_m1), and TGF-β3 (Gg03371523_m1) in leiomyomas of hens from each of the treatment groups (after 15 months on the diets) were determined. 18S (Hs99999901_s1) was used as the housekeeping gene. All primer probe sets were purchased from Applied Biosystems (TaqMan™, Foster City, CA, USA). Data are presented as relative fold change between the control and flaxseed and control and vitamin D3 treatments.

It is important to note that due to high variation in our housekeeping gene between samples, our relative fold change average of our control samples was not equal to 1. Therefore, we divided our control and flaxseed fold change averages by the average fold change value of the controls to accurately depict relative fold change in our graphs. Statistical analyses were not performed on these values as they induced artificial significance.

Statistical Analyses:

All statistical analyses were computed using GraphPad Prism 7.0. The D'Agonstino and Pearson normality test and the one-way ANOVA followed by multiple comparison's test were used. For data that did not follow Gaussian distribution, the Kruskal-Wallis non-parametric test was performed. A p value less than 0.05 was considered significant.

Results:

Measurement of 25-hydroxyvitamin D_3 via LC-MS/MS:

A subset of plasma samples from baseline, 3- 6- 9- 15- month vitamin D, and 15-month control hens were evaluated by LC-MS/MS for levels of 25-hydroxyvitamin D_3. The 25-hydroxyvitamin D_3 levels were significantly increased at all timepoints in the vitamin D3 group when compared to both the baseline and 15-month control hens, confirming that the 69 µg/kg/day dose of Rovimix was leading to increased blood levels in our laying hens throughout the entire trial **(Figure 14)**. There was no significant difference in 25-hydroxyvitamin D_3 levels between baseline control hens at the start of the trial and in control hens after 15 months **(Figure 14)**.

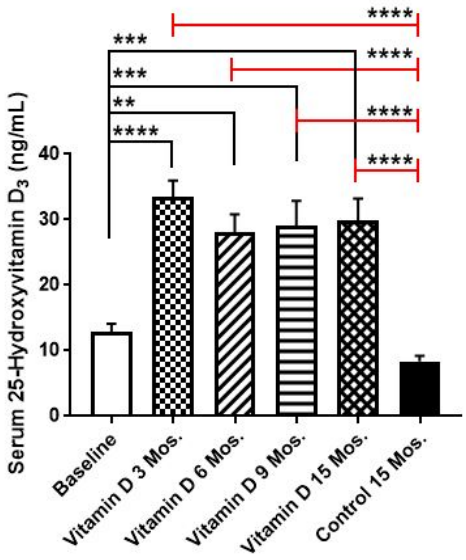

Figure 14: 25-Hydroxyvitamin D_3 via LC-MS/MS. Vitamin D supplemented at a dose of 69 µg/kg/day was biologically active in our laying hens throughout the course of the study. Black bars compare vitamin D time points versus the hens prior to the trial. Grey bars compare vitamin D time points versus the control hens at the conclusion of the study. **p<0.01 ***p<0.001 ****p<0.0001

Leiomyoma Incidence, Number of Leiomyomas per Hen, and Size:

Incidence of leiomyomas increased from 44% at the start of the trial to 78%, 74%, and 71% in the control, whole flaxseed, and vitamin D3 groups, respectively, after 15 months on each diet **(Figure 15A)**. While the average number of leiomyomas per hen increased significantly between the start of the trial and the end, the average number of fibroids per hen was slightly decreased in both the whole flaxseed (3.02 per hen) and vitamin D3 (2.72 per hen) groups when compared to the controls (3.84 per hen) after 15 months, although this change was not significant **(Figure 15B)**. There were no differences in the average size of leiomyomas between hens fed the control, whole flaxseed, or vitamin D3 diets after 15 months **(Figure 15C)**.

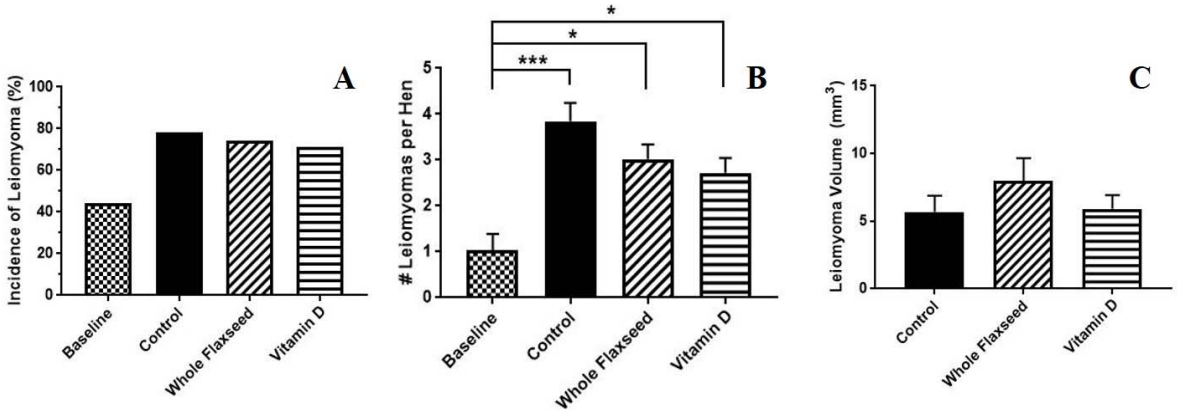

Figure 15: A) % Incidence of leiomyomas from hens before the trial compared with those from hens fed the control, whole flaxseed, and vitamin D diets. Incidence increased from ~40% to ~80% between the second and third year of lay in each of the treatment groups. B) Number of fibroids per hen was significantly increased between the baseline and all treatment groups after 15 months. There was no significant difference in number of fibroids per hen between the treatment groups. C) There were no significant differences in the average volume of leiomyomas between the three treatment groups. *$p<0.05$ ***$p<0.001$

Carbonylated Protein Levels in Plasma:

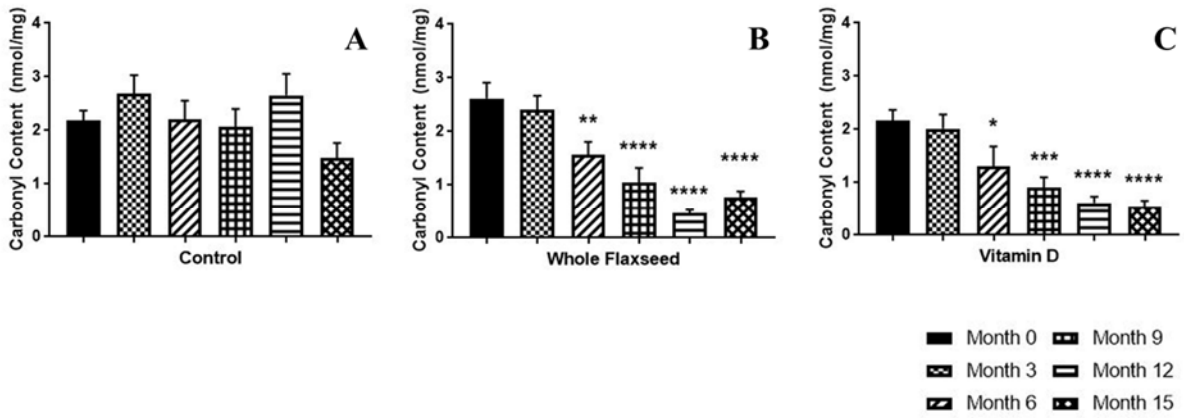

Figure 16: A Comparison of Protein Carbonyl Content by Treatment Group. A) Carbonylated protein content in control hens at 0 (n=11), 3 (n=10), 6 (n=9), 9 (n=12), 12 (n=11), and 15 (n=12) months. There were no differences in control hens at any time point when compared to month 0. B) Carbonylated protein content in hens fed the 10% whole flaxseed diet at 0 (n=12), 3 (n=12), 6 (n=11, p=0.0083), 9 (n=11, p=0.0001), 12 (n=11, p=0.0001), and 15 (n=12, p=0.0001) months. Hens had significantly reduced protein carbonyl content in the plasma after 6 months on the whole flaxseed diet when compared to month 0. These levels remained significantly lower at months 9, 12, and 15. C) Carbonylated protein content in hens fed the vitamin D diet at 0 (n=11), 3 (n=12), 6 (n=9, p=0.0439), 9 (n=11, p=0.0007), 12 (n=10, p=0.0001), and 15 (n=11, p=0.0001) months. Hens had significantly reduced protein carbonyl content in the plasma after 6 months on the vitamin D diet when compared to month 0. These levels remained significantly lower at months 9, 12, and 15. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

Carbonylated protein levels in plasma were measured to assess systemic levels of oxidative stress. We found that carbonylated protein levels in plasma were significantly reduced in hens fed the 10% whole flaxseed diet as well as in hens fed the vitamin D3 diet after 6 months on the diet, and these levels continued to remain significantly lower throughout the remaining time points of the trial **(Figure 16B-C)**. There were no changes in plasma levels of carbonylated proteins in the control hens throughout the course of the study **(Figure 16A)**. In addition, carbonylated protein levels in plasma were significantly reduced in both the whole flaxseed and vitamin D3 groups when compared to the control group after 9 months **(Figure 17D)**. These levels remained significantly lower than those of the control hens throughout the remainder of the trial **(Figure 17E-F)**.

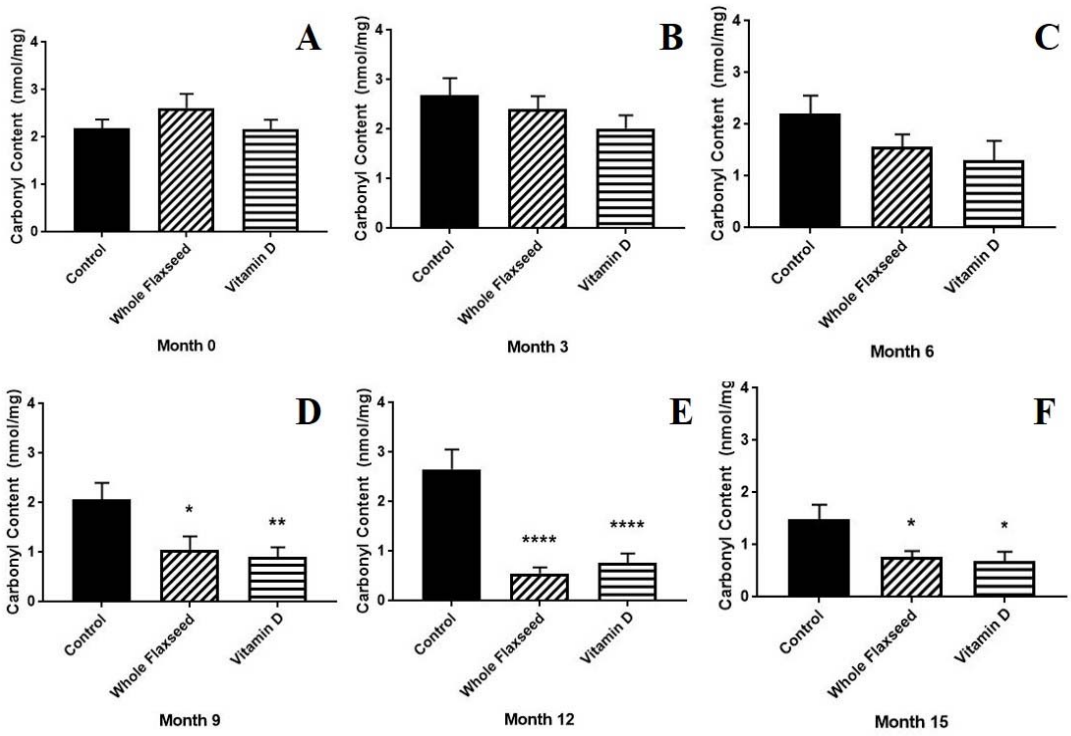

Figure 17: A Comparison of Protein Carbonyl Content between Treatment Groups. A–C) There were no significant differences in plasma levels of carbonylated proteins between treatment groups at months 0, 3, or 6. Control n=11,10,9 (month 0,3,6), Whole Flaxseed n=12,12,11 (month 0,3,6), and Vitamin D n=11,12,9 (month 0,3,6). D–F) Protein Carbonyl Content in the plasma of hens fed the 10% whole flaxseed (n=11,11,12; month 9,12,15) or vitamin D (n=11,11,12; month 9,12,15) diets were significantly reduced at month 9, 12, and 15 when compared to that of hens fed the control diet (n=12,11,12; month 9,12,15). *p<0.05 **p<0.01 ****p<0.0001

ELISAs:

In addition to assessing oxidative stress, we also wanted to determine whether there were changes in systemic levels of the inflammatory cytokines TNF-α and IFN-γ, and thus, performed ELISA assays for each. Unfortunately, due to the high variation between samples (all CV's were ≥30%) of the same treatment group, at the same time points, and within duplicates, we were not able to draw any conclusions about levels of TNF-α in the plasma. Furthermore, several samples from all three treatment groups were too low to detect. This occurred at all time points measured (0, 3, 6, 9, 12, and 15 months). We were not able to detect TNF-α in any of our 15-month samples. The upper detection limit of this assay is 2000 pg/mL and the lower detection limit is

31.25 pg/mL. It does not appear that there were any differences in plasma levels of TNF-α between treatment groups at any time during the study **(Figure 18)**. **Figure 18** does depict a significant increase in plasma TNF-α levels after three months in the vitamin D group, however, because the coefficient of variation was ~50%, we do not believe these values are representative of actual levels of TNF-α in these hens. This experiment should be repeated to determine the true levels of TNF-α in the plasma of hens from each treatment group at all time points. IFN-γ was not detectable in the plasma of our hens in any treatment group or time point (data not shown).

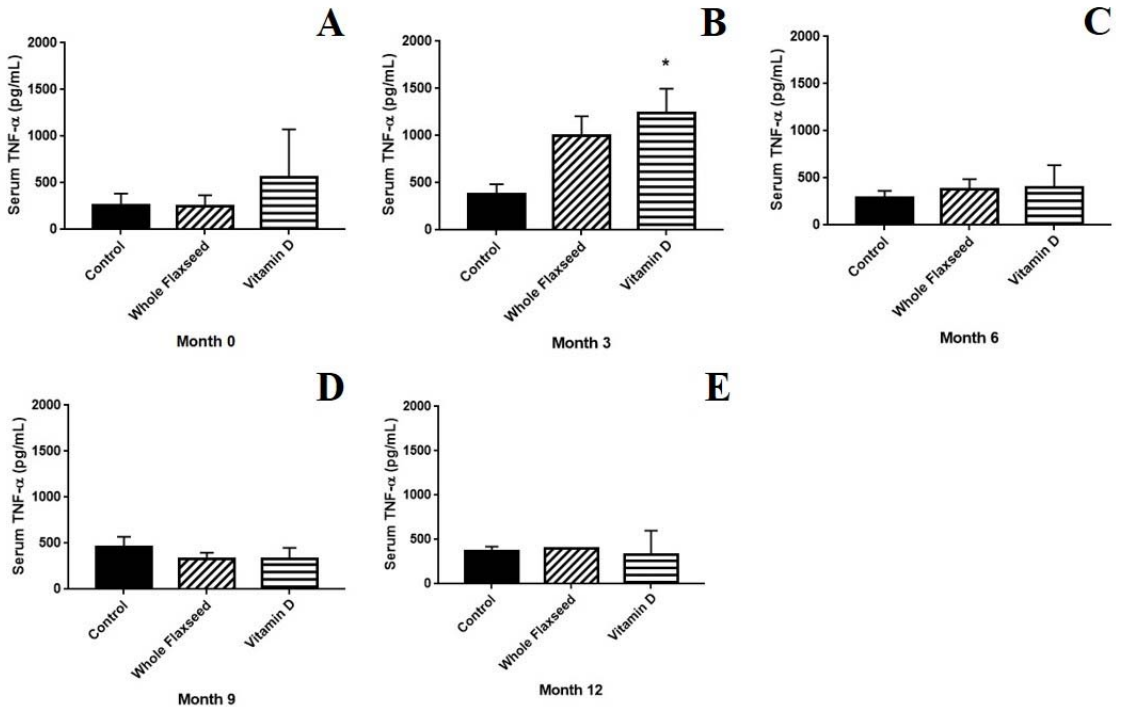

Figure 18: Plasma levels of TNF-α from hens fed either the control, 10% whole flaxseed, or vitamin D diet after A) 0 months, B) 3 months, C) 6 months, D) 9 months, and E) 12 months. Number of plasma samples ran for analysis: N=7/trt/timepoint. Number of plasma samples with levels of TNF-α on the linear portion of the standard curve: Month 0: control n=7, whole flax n=5, vit D n=2. Month 3: control n=7, whole flax n=6, vit D n=6. Month 6: control n=7, whole flax n=6, vit D n=5. Month 9: control n=4, whole flax n=6, vit D n=4. Month 12: control n=6, whole flax n=1, vit D n=3. Upper limit of detection: 2000 pg/mL. Lower limit of detection: 31.25 pg/mL.

qRT-PCR Analysis of Gene Expression:

mRNA expression levels of collagen I, TGF-β3, and *PTGS2* were compared in leiomyomas from hens fed either the control, 10% whole flaxseed, or vitamin D3 diets. Fibroids from hens fed the vitamin D3 diet showed significant downregulation of all three genes when compared to those from control hens, whereas there were no changes in collagen I (p=0.88) expression in addition to non-significant decreases in expression of TGFβ-3 (p=0.48) and *PTGS2* (p=0.49) in fibroids from hens fed the whole flaxseed diet when compared to the controls **(Figure 19)**.

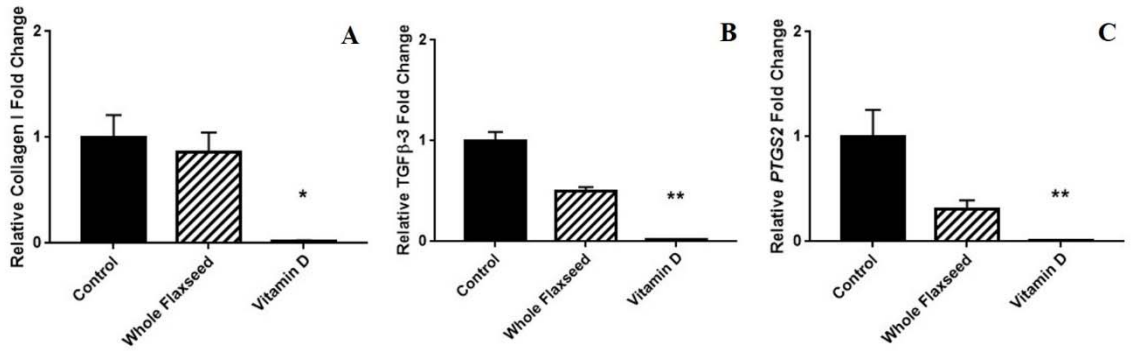

Figure 19: Collagen I, TGF-β3, and *PTGS2* mRNA Expression via qRT-PCR. A) Collagen I was significantly downregulated in fibroids from hens fed the vitamin D diet (n=12) when compared to those from hens on the control diet (n=8) (p=0.02). B) Leiomyomas from the vitamin D group (n=12) expressed lower levels of TGF-β3 than those from the control group (n=8) (p=0.003). C) *PTGS2* was significantly downregulated in vitamin D fibroids (n=12) than in fibroids from hens fed the control diet (n=8) (p=0.001). There were no significant differences in collagen I, TGF-β3, or *PTGS2* expression between fibroids from hens on the flaxseed diet (n=8/gene) and fibroids from control hens. *p<0.05 **p<0.01

Discussion:

Uterine leiomyomas are the most common benign gynecological tumors found in women of reproductive age and cause a significant burden on women's healthcare due to the high cost of treatment. Hysterectomy, myomectomy, and uterine artery embolization have become routine treatments for fibroids, and currently no effective long-term, non-surgical options for

leiomyomas exist. Thus, the purpose of our study was to assess the efficacy of dietary intervention with whole flaxseed and vitamin D as a therapy for fibroids found in the oviduct of the laying hen. Overall, our data show that dietary supplementation with whole flaxseed is beneficial to the general health of the laying hen while vitamin D supplementation has a therapeutic effect on oviductal leiomyomas. Hens fed a diet supplemented with whole flaxseed or vitamin D_3 had significantly lower plasma levels of carbonylated proteins compared to hens fed the control diet. Hens fed the vitamin D_3 diet had significantly decreased levels of collagen I, TGF-β3, and *PTGS2* mRNA expression.

Consistent with our laboratory's previous finding of an increase in leiomyoma incidence (from 45% to 73.19%) in White Leghorn hens between their second and third years of lay, we also found that leiomyoma incidence increased in White Leghorn hens from 44% at the start of our trial (end of the second year of lay) to 78%, 74%, and 71% in the control, 10% whole flaxseed, and vitamin D_3 treatments after 15 months, respectively[91]. Another study reported a 100% incidence of leiomyomas in 5-year-old White Leghorn hens[90]. While this incidence is approximately 25% higher than our reported findings, the hens in this study were two years older than ours. We suspect that we would have reported similar increased incidences had our study continued past 15 months.

We did not find any significant differences in number of fibroids per hen or leiomyoma volume between the three treatment groups. This is consistent with our reported findings in Chapter 2 of this thesis for hens fed a similar, 15% flaxseed diet for 12 months. However, a previous trial assessing the effects of 10% whole flaxseed on oviductal leiomyomas in the laying hen found a significant decrease in the size of leiomyomas from hens fed the flaxseed diet versus the control diet. This suggests that the beneficial effect of 10% flaxseed diet on leiomyomas

require a longer period of treatment (18-24 months). In contrast to our results, Halder et al. found that 1,25-dihydroxyvitamin D_3 reduced the size of leiomyomas in the Eker rat model after 3 weeks[83]. This difference could be due to a variety of factors. First of all, species differences could account for the discrepancy in results since the rat is a mammal and the chicken an avian species. Secondly, and the more likely reason, is that the tumors found in the Eker rat are more similar to leiomyosarcomas, which are malignant tumors, rather than the benign leiomyomas found in our laying hen model and may be more responsive to changes in Vitamin D[87].

To measure oxidative stress, we assessed changes in levels of carbonylated proteins in the plasma of a subgroup of our hens. Carbonylated protein levels in the blood is a recognized marker of oxidative stress[134–136]. Plasma was collected every three months from the start of the trial from the same birds. We found that the hens fed the whole flaxseed and vitamin D_3 diets had a significantly lower carbonylated protein level in their plasma nine months after beginning their respective diets compared to hens fed the control diet. These levels remained significantly lower throughout the rest of the trial. Oxidative stress is associated with inflammation[137–139]. The lower levels of carbonylated proteins found in our flaxseed and vitamin D_3 treated hens suggests that these hens were experiencing less oxidative stress and inflammation compared to hens fed the control diet. Unfortunately, we were not able to obtain any accurate measures of inflammatory cytokines to confirm these results. In the future, we would like to measure the pro-inflammatory cytokines IL-1β and IL-6 along with the anti-inflammatory cytokine IL-10.

We also investigated potential mechanisms by which flaxseed and vitamin D_3 exert their effects on leiomyomas in the laying hen, by performing qRT-PCR to assess effects on mRNA expression levels of collagen I, TGF-β3, and *PTGS2*. It is important to note that our housekeeping gene didn't run as expected in that there was a lot of variation between samples.

There are a few possible reasons for this. First, it is possible that 18S isn't the best housekeeping gene for our samples and species. However, previous work with leiomyomas in the laying hen from our lab used 18S as a housekeeping gene successfully, so this is unlikely the case[93]. Second, our extracted RNA was analyzed on a Nanodrop Spectrophotometer prior to cDNA synthesis and were not treated with DNase. Because the Nanodrop cannot differentiate between RNA and DNA, it is possible that the amplification we witnessed was DNA amplification and not mRNA amplification. Our 18S primer-probe was designed within a single exon whereas our collagen I, TGFβ-3, and *PTGS2* primer-probes were designed across an exon junction, thus making DNA contamination and amplification in our housekeeping samples probable.

Leiomyomas from hens fed a diet supplemented with vitamin D_3 for 15 months exhibited significantly decreased expression levels of all three genes when compared to leiomyomas from hens fed the control diet. *PTGS2* is the gene that encodes COX-2, the inducible enzyme in the prostaglandin pathway. The COX-2/prostaglandin pathway has been strongly implicated in a variety of inflammatory diseases, including cancer, and downregulating COX-2 expression or action has been shown to be potentially beneficial to inhibiting disease progression[61–63,95,103]. Leiomyomas from hens fed the whole flaxseed treatment showed a modest, non-significant decrease in *PTGS2* mRNA expression compared to the control group; which is inconsistent with our data presented in the previous chapter. Due to limited resources, we were not able to run qRT-PCR on an identical number of fibroids from the control and whole flaxseed groups (n=8/group) compared to the vitamin D group (n=12). It is possible that increasing our n would lower the variation in our control treatment group, improving our statistical rigor.

Previously, Machado et al. reported that leiomyomas from the laying hen expressed higher levels of collagen I and TGF-β3 than normal oviduct smooth muscle[91]. Collagen is a main

component of ECM and TGF-β is a pro-fibrotic cytokine with higher levels of expression in leiomyomas than in normal myometrium and has been shown to induce fibronectin expression in leiomyoma smooth muscle cells[120]. TGF-β3 has also been shown to stimulate the production of collagen I[122]. The decreased expression of collagen I and TGF-β3 in hens fed the diet supplemented with vitamin D_3 suggests that vitamin D_3 may exert its effects directly on TGF-β3 which then leads to a decrease in collagen I production quite possibly through the Smad signaling pathway. The Smad signaling pathway consists of eight intracellular proteins, known as Smads (Smad1 through Smad8) that transport signals from the cell membrane to the nucleus[121]. TGF-β is an activator of the Smad pathway and regulates the transcription of genes responsible for cellular proliferation and ECM deposition[121]. Several papers have shown vitamin D_3's negative regulation of TGF-β and Smad signaling in fibrosis-related diseases, many of which are reviewed by Shany et al[140]. Induction of vitamin D receptor (VDR) has been shown to be important in vitamin D_3's regulation of TGF-β/Smad in cutaneous injury, systemic sclerosis, and intestinal fibrosis[69–71]. Halder et al. clearly demonstrated the ability of vitamin D_3 to downregulate pro-fibrotic genes induced by TGF-β through Smad signaling in cultured human leiomyoma cells[84]. We have not yet investigated specific signaling pathways in regard to vitamin D_3's effects on fibroids. Other pathways, such as MAPK/ERK and wingless-type (Wnt)/β-catenin (or several pathways acting simultaneously through an additive effect), could explain the mechanism behind vitamin D_3's potential therapeutic effects on leiomyomas. Further research is needed to elucidate the mechanisms involved. However, our *in vivo* data suggest that investigating TGF-β/Smad signaling is a good future avenue to pursue.

Conclusions and Future Directions:

Our data show that dietary supplementation with whole flaxseed and vitamin D_3 decreases systemic oxidative stress, an indicator of inflammation and general health status, in the laying hen model of uterine fibroids. We also provide evidence that vitamin D_3 has beneficial, therapeutic effects on leiomyomas found in the oviduct of the laying hen and that TGF-β3 is a possible target of vitamin D_3. Based on these findings, we recommend the pursuit of dietary intervention with vitamin D_3 as a treatment for women with uterine fibroids because it is inexpensive and easily accessible.

While our evidence is supportive for an anti-inflammatory effect of vitamin D_3 and of flaxseed, there is more work that should be done to confirm our findings. As mentioned in our discussion, our 18S housekeeping gene did not run properly during our qRT-PCR experiment. To validate the results we obtained, a few steps should be taken prior to repeating the experiment. First, isolated RNA should be treated with DNase to digest any DNA in the sample. Secondly, more thorough quality control of our RNA should be conducted prior to running qRT-PCR, such as analysis using an Agilent Bioanalyzer machine. Third, a housekeeping gene designed across an exon junction would minimize the risk of DNA contamination in our samples interfering with the true results of the qRT-PCR experiment. Our 18S primer-probe was a Taqman assay from Applied Biosystems and was designed to target human 18S. Because of previous results from our lab with this specific primer in chicken samples, we chose to use this assay again. However, there is a GAPDH Taqman assay available that is designed for the chicken as a target species in addition to spanning an exon junction.

We also looked at oxidative stress as a marker of inflammation in our plasma samples, but unfortunately, were unable to obtain results for TNF-α and IFN-γ through ELISA assay of

these same blood samples. In further reading of the literature, it seems that measuring plasma levels of IL-1β, IL-6, and IL-10 would provide a more comprehensive picture of the inflammatory state of these hens. Therefore, we would like to perform additional ELISAs to measure these cytokines. In addition, we quantitated collagen I, TGF-β3, and *PTGS2* gene expression using qRT-PCR, but we did not look at protein expression of these markers. Performing a western blot for the proteins translated from these genes (or a hydroxyproline assay to assess collagen) would be a good way to determine if the changes we saw at the mRNA level are translated to functional protein, thus strengthening our evidence. In our discussion, we mentioned vitamin D_3 exerting its effects through the TGF-β/Smad signaling pathway. To test this hypothesis, performing western blots to assess changes in functional protein levels of pSmad2, pSmad3, total Smad2, and total Smad3 would give us more detailed information on the regulation of this pathway by vitamin D_3. It would also be beneficial to use this method to look at potential changes in VDR levels.

In our study we had a limited number of hens available to us. Therefore, we had to limit our treatment groups to control, whole flaxseed, and vitamin D_3 and were unable to include a combined treatment group of 10% whole flaxseed plus vitamin D_3. We are very interested in determining whether the combined dietary supplements would have an additive protective effect on leiomyomas in the laying hen. Furthermore, we had to limit our current study to only 15 months of treatment. Our findings in Chapter 2 suggest that the length of time on these diets is the key to their success. Thus, we would like to examine the effects of these diets after 24 months or more. Additionally, our diets were initiated at the completion of the second year of lay. It is possible that these agents might work better as preventatives for the development of fibroids. It would be interesting to see the effects of whole flaxseed and vitamin D_3 on the

development of leiomyomas by starting hens on these diets when they reach sexual maturity (~6 months of age) and keeping them on these diets until after their third year of lay.

Clinical trials are an important part of medical research to confirm that therapies found to be safe and effective in animal models are safe and effective in humans and work better than a placebo. Therefore, we are interested to see the implications of dietary supplementation with whole flaxseed or vitamin D_3 on women with uterine fibroids. We hypothesize that long-term treatment with these supplements will shrink fibroids and reduce symptoms such as pelvic pain or discomfort and menorrhagia. Furthermore, vitamin supplementation is something that can begin at any stage in life which poses the question: would supplementation with vitamin D_3 during the peripubertal or teenage years (and continuation throughout adulthood) prevent uterine leiomyoma development in women? Moving forward with clinical studies to answer these questions will help to provide long-term, non-surgical treatment strategies to women suffering from symptomatic uterine leiomyomas and their clinicians.

In summary, we saw an improvement in overall health indicators of our laying hens after 15 months of either whole flaxseed or vitamin D_3 supplementation, and vitamin D_3 significantly reduced the expression of genes important to the development of leiomyomas. However, we did not see any changes in the size and incidence of oviductal leiomyomas in our laying hens after 15 months on their diets. Additional experiments should be completed to confirm our findings and add to the growing body of evidence supporting dietary intervention as a relatively cheap, effective, non-surgical, long-term treatment for uterine fibroids that allows women to preserve their fertility.

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APPENDIX

Experimental Protocols

Gomori's Trichrome Stain:

Reagent Preparation:

- Meyer's Hematoxylin: filter before use
- Bouin's Solution: **VERY VOLATILE, USE IN FUME HOOD!!!!!**
- 0.2% Acetic Acid (250 mL):
  - Add 0.5 mL Glacial Acetic Acid to 250 mL MilliQ water
  - ALWAYS **DO WHAT YOU OUGHTA, ADD ACID TO WATER**
- Gomori's Trichrome Stain (**store in refrigerator, good for approx. 2 months)**:
  - Chrometrope 2R – 0.6 g
  - Fast Green FCF – 0.3 g
  - 10% Phosphotungstic Acid – 10.0 mL
  - Deionized Water – 89.0 mL
  - Glacial Acetic Acid – 1.0 mL
  - **Adjust pH to 3.4 using 1 M NaOH**

Procedure:

- Put water bath into fume hood and turn on to 60°C
- Fill staining tub with Bouin's solution, place into water bath, and heat to 60°C
  - DO NOT OPEN BOUIN'S OUTSIDE OF FUME HOOD!!!!
- Deparaffinization & Rehydration:
  - Xylenes I – **5 min**
  - Xylenes II – **5 min**
  - Xylenes III – **5 min**
  - 100% EtOH I – **1 min**
  - 100% EtOH II – **1 min**
  - 70% EtOH – **1 min**
  - Tap water – **1 min**
- Incubate slides for 1 hour in Bouin's solution at 60°C
  - **Check periodically- Bouin's solution is very volatile due to its picric acid content!!!**
  - After incubation is complete, allow Bouin's to cool down in fume hood before cleanup during later step
- Wash slides in running deionized water until liquid is clear
- Place slides into MilliQ water – **2 min**
- Place slides into Meyer's Hematoxylin – **5 min**
  - During this step, clean up Bouin's materials

    - Bouin's solution is good for ~2 days, so you can reuse this solution if you will be doing multiple runs, otherwise, dispose of into liquid waste container labeled "Bouin's"
    - Use a large beaker of deionized water to rinse Bouin's container in the fume hood. Dump from Bouin's container into liquid waste container labeled "Bouin's" until water comes out clear.
    - Place any contaminated paper towels, gloves, etc. into a solid waste container labeled "Bouin's"
- Wash slides in running tap water until liquid is clear
- Incubate in Gomori's Trichrome Stain – **10 min**
- Dip 3 times in 0.2% Acetic Acid
- Dehydration and Clearing:
    - 70% EtOH – **1 min**
    - 80% EtOH – **1 min**
    - 90% EtOH – **1 min**
    - 100% EtOH II – **1 min**
    - 100% EtOH I – **1 min**
    - Xylenes I – **1 min**
    - Xylenes II – **1 min**
    - Xylenes III – **1 min**
- Mount slides with Clearmount

Immunohistochemsitry—Proliferating Cell Nuclear Antigen:

PCNA Antibody (PC10), Novus Biologicals, NB500-106

ABC Method, Mouse IgG kit

Perform all steps at room temperature unless otherwise noted

Incubation must be performed in moist chamber

Do not allow sections to dry out

Day 1:

- Deparaffinization & rehydration:
  - Xylenes I – **5 min**
  - Xylenes II – **5 min**
  - Xylenes III – **5 min**
  - 100% EtOH I – **1 min**
  - 100% EtOH II – **1 min**
  - 70% EtOH – **1 min**
  - Tap water – **1 min**
- Antigen retrieval:
  - Boil in 1:10 DAKO – **20 min**
- Cool down to room temperature
- Rinse in PBST – **5 min**
- Inactivation of endogenous peroxidase:
  - Place in 0.3% H_2O_2/MeOH in dark – **15 min**
- Rinse in PBST – **3 x 5 min**
  - Encircle each section with a PAP pen while rinsing in PBST
- Blocking of non-specific binding:
  - Incubate in 5% normal horse serum (diluted in PBST) from mouse kit – **60 min**
- Incubate in **1:1000** PCNA antibody (diluted in 1% BSA/PBST) – **Overnight @ 4°C**

Day 2:

- Rinse in PBST – **3 x 5 min**
- Incubate in secondary antibody:
  - **1:100** Biotinylated Anti-Mouse (diluted in 1% BSA/PBST) – **60 min**
- Prepare ABC solution 30 min before use:
  - 50:1:1 – 2.5 mL 1% BSA/PBST: 1 drop A: 1 drop B
- Rinse in PBST – **3 x 5 min**
- Incubate in ABC solution – **30 min**
- Rinse in PBST – **3 x 5 min**

- DAB reaction (see kit for preparation) – **30 seconds per slide**
    - Stop DAB reaction in tap water
- Counterstain in Meyer's Hematoxylin – **1 min**
- Wash in tap water until water runs clear
- Dehydration & clearing:
    - 70% EtOH – **1 min**
    - 80% EtOH – **1 min**
    - 90% EtOH – **1 min**
    - 100% EtOH II – **1 min**
    - 100% EtOH I – **1 min**
    - Xylenes I – **1 min**
    - Xylenes II – **1 min**
    - Xylenes III – **1 min**
- Mount slides with Clearmount

Immunohistochemistry—Von Willebrand Factor:

VWF Antibody, Agilent Pathology Solutions, A008229-5

ABC Method, Rabbit IgG kit

Perform all steps at room temperature unless otherwise noted

Incubation must be performed in moist chamber

Do not allow sections to dry out

Day 1:

- Deparaffinization & rehydration:
  - Xylenes I – **5 min**
  - Xylenes II – **5 min**
  - Xylenes III – **5 min**
  - 100% EtOH I – **1 min**
  - 100% EtOH II – **1 min**
  - 70% EtOH – **1 min**
  - Tap water – **1 min**
- Antigen retrieval:
  - Boil in 1:10 DAKO – **20 min**
- Cool down to room temperature
- Rinse in PBST – **5 min**
- Inactivation of endogenous peroxidase:
  - Place in 0.3% H_2O_2/MeOH in dark – **15 min**
- Rinse in PBST – **3 x 5 min**
  - Encircle each section with a PAP pen while rinsing in PBST
- Blocking of non-specific binding:
  - Incubate in 5% normal goat serum (diluted in PBST) from rabbit kit – **60 min**
- Incubate in **1:400** VWF antibody (diluted in 1% BSA/PBST) – **Overnight @ 4°C**

Day 2:

- Rinse in PBST – **3 x 5 min**
- Incubate in secondary antibody:
  - **1:100** Biotinylated Anti-Rabbit (diluted in 1% BSA/PBST) – **60 min**
- Prepare ABC solution 30 min before use:
  - 50:1:1 – 2.5 mL 1% BSA/PBST: 1 drop A: 1 drop B
- Rinse in PBST – **3 x 5 min**
- Incubate in ABC solution – **30 min**
- Rinse in PBST – **3 x 5 min**

- DAB reaction (see kit for preparation) – **30 seconds per slide**
    - Stop DAB reaction in tap water
- Counterstain in Meyer's Hematoxylin – **1 min**
- Wash in tap water until water runs clear
- Dehydration & clearing:
    - 70% EtOH – **1 min**
    - 80% EtOH – **1 min**
    - 90% EtOH – **1 min**
    - 100% EtOH II – **1 min**
    - 100% EtOH I – **1 min**
    - Xylenes I – **1 min**
    - Xylenes II – **1 min**
    - Xylenes III – **1 min**

Mount slides with Clearmount

Immunohistochemistry—NF-κB p65:

NF-κB p65 Antibody, Abcam, AB16502

ABC Method, Rabbit IgG kit

Perform all steps at room temperature unless otherwise noted

Incubation must be performed in moist chamber

Do not allow sections to dry out

Day 1:

- Deparaffinization & rehydration:
  - Xylenes I – **5 min**
  - Xylenes II – **5 min**
  - Xylenes III – **5 min**
  - 100% EtOH I – **1 min**
  - 100% EtOH II – **1 min**
  - 70% EtOH – **1 min**
  - Tap water – **1 min**
- Antigen retrieval:
  - Boil in 1:10 DAKO – **20 min**
- Cool down to room temperature
- Rinse in PBST – **5 min**
- Inactivation of endogenous peroxidase:
  - Place in 0.3% H_2O_2/MeOH in dark – **15 min**
- Rinse in PBST – **3 x 5 min**
  - Encircle each section with a PAP pen while rinsing in PBST
- Blocking of non-specific binding:
  - Incubate in 5% normal goat serum (diluted in PBST) from rabbit kit – **60 min**
- Incubate in **1:1000** NF-κB p65 antibody (diluted in 1% BSA/PBST) – **Overnight @ 4°C**

Day 2:

- Rinse in PBST – **3 x 5 min**
- Incubate in secondary antibody:
  - **1:100** Biotinylated Anti-Rabbit (diluted in 1% BSA/PBST) – **60 min**
- Prepare ABC solution 30 min before use:
  - 50:1:1 – 2.5 mL 1% BSA/PBST: 1 drop A: 1 drop B
- Rinse in PBST – **3 x 5 min**
- Incubate in ABC solution – **30 min**
- Rinse in PBST – **3 x 5 min**

- DAB reaction (see kit for preparation) – **30 seconds per slide**
    - Stop DAB reaction in tap water
- Counterstain in Meyer's Hematoxylin – **1 min**
- Wash in tap water until water runs clear
- Dehydration & clearing:
    - 70% EtOH – **1 min**
    - 80% EtOH – **1 min**
    - 90% EtOH – **1 min**
    - 100% EtOH II – **1 min**
    - 100% EtOH I – **1 min**
    - Xylenes I – **1 min**
    - Xylenes II – **1 min**
    - Xylenes III – **1 min**

Mount slides with Clearmount

Immunohistochemistry—Nitrotyrosine:

Nitrotyrosine Antibody, Millipore Sigma, 06-284

ABC Method, Rabbit IgG kit

Perform all steps at room temperature unless otherwise noted

Incubation must be performed in moist chamber

Do not allow sections to dry out

Day 1:

- Deparaffinization & rehydration:
  - Xylenes I – **5 min**
  - Xylenes II – **5 min**
  - Xylenes III – **5 min**
  - 100% EtOH I – **1 min**
  - 100% EtOH II – **1 min**
  - 70% EtOH – **1 min**
  - Tap water – **1 min**
- Antigen retrieval:
  - Boil in 1:10 DAKO – **20 min**
- Cool down to room temperature
- Rinse in PBST – **5 min**
- Inactivation of endogenous peroxidase:
  - Place in 0.3% H_2O_2/MeOH in dark – **30 min**
- Rinse in PBST – **3 x 5 min**
  - Encircle each section with a PAP pen while rinsing in PBST
- Blocking of non-specific binding:
  - Incubate in 5% normal goat serum (diluted in PBST) from rabbit kit – **60 min**
- Incubate in **1:400** Nitrotyrosine antibody (diluted in 1% BSA/PBST) – **Overnight @ 4°C**

Day 2:

- Rinse in PBST – **3 x 5 min**
- Incubate in secondary antibody:
  - **1:100** Biotinylated Anti-Rabbit (diluted in 1% BSA/PBST) – **60 min**
- Prepare ABC solution 30 min before use:
  - 50:1:1 – 2.5 mL 1% BSA/PBST: 1 drop A: 1 drop B
- Rinse in PBST – **3 x 5 min**
- Incubate in ABC solution – **30 min**
- Rinse in PBST – **3 x 5 min**

- Incubate in AEC Solution – **5 min in dark**
    - Rinse in 3 changes of DI water
- Counterstain in Meyer's Hematoxylin – **2 min**
- Wash in DI water until water runs clear

Mount slides with VectaMount AQ Aqueous Mounting Medium

Sudan Black B Stain:

Reagent Preparation:

- Sudan Black B Stain:
    - Sudan Black B – 2.1 g
    - 70% EtOH – 300 mL
    - Mix reagents in low-light conditions.
    - Filter twice with Whatman Filter Paper.
    - Store at room temperature in dark.

Procedure:

- Deparaffinization & Rehydration:
    - Xylenes I – **5 min**
    - Xylenes II – **5 min**
    - Xylenes III – **5 min**
    - 100% EtOH I – **1 min**
    - 100% EtOH II – **1 min**
    - 70% EtOH – **1 min**
- Place slides in Sudan Black B solution at room temperature overnight
- Dehydration and Clearing:
    - 70% EtOH – **until sections are pale grey**
    - 100% EtOH II – **1 min**
    - 100% EtOH I – **1 min**
    - Xylenes I – **1 min**
    - Xylenes II – **1 min**
    - Xylenes III – **1 min**

Mount slides with Clearmount

Toluidine Blue Stain (courtesy of Karen Doty):

Procedure:

- Deparaffinize sections and hydrate through graded alcohols to water.
- Stain slides in 0.1% aqueous toluidine blue solution for 10 min.
- Rinse in distilled water.
- Dehydrate quickly through 95% and 100% alcohol. This step differentiates the stain and must be checked carefully.
- Clear in xylene and mount.

RNA Isolation and cDNA Synthesis:

Homogenization

- Add 50-100 mg fibroid tissue to a 2.0-ml Safe-Lock Eppendorf tube containing 1 ml of Trizol Reagent and enough 2mm Tungsten Carbide beads (Qiagen) to fill the conical portion of the tube.

- Macerate fibroid tissue using TissueLyserII from Qiagen for 25 second intervals. Place on wet ice for 5 seconds between intervals.

    - Typically takes 2-3 intervals of homogenization.

- Remove supernatant and add to fresh tubes containing **0.2ml** chloroform per ml Tri Reagent; mix vigorously by shaking/vortexing for 15 sec.

- Incubate tubes at RT for at least 10 min.

- Centrifuge mixture at 12,000xg for 15 min at 4ºC.

RNA isolation

- Transfer upper aqueous (clear) phase to fresh tubes containing **0.5 ml** of isopropanol (2-propanol) per ml of Tri Reagent. **NOTE**: be sure to leave "buffy coat" undisturbed to minimize incidence of a contaminant "bubble" during RNA isolation. **Vortex well. Save interphase and phenol/chloroform layer for DNA extraction and store in either -20 ºC (processing within the month) or -80 ºC (processing past a month).**

    - Note: To see pellet when using a small amount of cells/tissue, dilute glycogen 1:10 and add 5ul to isopropanol before transferring upper aqueous phase.

- Allow sample to stand for at least 10 min at –80ºC, then at least 10 min at –20ºC. Alternatively, hold sample at –20ºC for at least 20 min or overnight at 4ºC or –20ºC. The longer the samples are at a lower temperature the better the RNA yield (**this is the first good stopping point**).

- Centrifuge mixture at 12,000xg for 15 min at 4ºC. RNA precipitate will form a pellet on side and bottom of the tube.

- Remove contaminant "bubble" from beneath pellet (if present) and re-centrifuge at 12,000xg for 15 min at 4ºC (repeat if necessary). Once contaminant "bubble" no longer remains, remove and discard supernatant in appropriate ISO waste container.

- Wash RNA pellet by adding at least **1 ml** of 75% ethanol per ml of Tri Reagent (use 100% ethanol and DEPC water to make 75% ethanol).

- Vortex sample well but do not dissolve the pellet (short vortex to float pellet).

- Centrifuge at 12,000xg for 5 min at 4ºC. Samples can be stored in ethanol at 4ºC for 1 week and up to a year at –20ºC.

- Remove and discard supernatant in appropriate EtOH waste container without disturbing RNA pellet.

- Dry RNA pellet for 5-10 min (will probably take 15-30 if not more depending on how much supernatant is removed) by air-drying or by using a P10 pipetteman to remove any remaining ethanol. Do not let RNA pellet dry completely and do not dry under Speed-vacuum; complete drying greatly decreases RNA solubility.

- Add 20 µL of RNAase-free (DEPC) H_2O to RNA pellet, mix well, and quick-spin; store at -80 ºC.

Nanodrop Spec procedure

- If proceeding directly from RNA isolation, let samples sit on ice for at least 15-20 min. Otherwise get out samples from the freezer and thaw on ice (~30 min).

- Vortex and quick-spin samples to ensure sufficient mixing.

- Log onto computer and start Nanodrop software. Select Nucleic Acid.

- Clean both parts of Nanodrop detector with ddiH_2O and dry with Chemwipe.

- Add 2 µl drop of ddiH_2O and click OK to calibrate.

- Change sample type to RNA (software defaults to DNA).

- Add 2 µl drop of ddiH_2O and click Blank (will automatically give it sample number of 0).

- Change sample ID and add 2 µl drop of sample; click Measure. Once complete, open detector, clean with ddiH_2O and dry. Repeat for remainder of samples.

- Upon analyzing all samples, transfer data file(s) [located in C:/nanodrop data/default/(choose folder corresponding to type of data)/] to your computer and import using Excel. Normal 260/280 and 260/230 ratios: 1.8 – 2.1

cDNA synthesis

- cDNA was synthesized from RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) per the manufacturer's instructions. 2 µg of RNA from each sample was used to synthesize the cDNA.

Quantitative Real-Time PCR—Collagen I, TGF-β3, PTGS2:

Gene	TaqMan Assay
18S	Hs99999901_s1
Collagen I	Gg03325897_m1
TGF-β3	Gg03371523_m1
PTGS2	Gg03320004_m1

Table 5: TaqMan primer-probes used for qRT-PCR.

Preparation Steps

- Make plate maps
- Sign up to use rtPCR machine in Keck Center
- Make all calculations and label all tubes

- RNA Isolation: follow Tri Reagent protocol
- *cDNA samples should be stored at -20ºC unless performing rtPCR on same day.*
- ***PRODUCT = 40 µL cDNA***

Dilutions and Master Mixes

Dilute cDNA 1:5 (add 160µL DEPC-H_2O per sample, for a total of 200µL) – gives you ~3 plates (probably less)

OR

Dilute cDNA 1:10 (add 360µL DEPC-H_2O per sample, for a total of 400µL) – gives you 5 plates worth

- Thaw cDNA on ice (if needed)
- Dilute cDNA with appropriate amount of DEPC-H_2O
    - Quick spin when needed

- Label tubes for each primer/probe MM
    - A 384-well plate has 24 columns and 16 rows. Each column is a different sample, so duplicates run vertically; thus, you can fit 8 genes on one plate and 24 different samples. We round up to 28 in order to account for volume lost in transfer/when using repeater pipettes.

Master Mix (per gene): ** Note, this breakdown is for a full plate (24 columns) **

Taqman = 280µL
primer/probes = 28µL
TOTAL = 308µL per gene

- Vortex then centrifuge Master Mixes
- Dispense 5.5µL MM for each well using repeater pipette
- Turn plate and dispense 4.5µL sample (diluted cDNA) into appropriate wells
- Tap the plate gently to get all volumes to fall to the bottom of the wells
- Cover plate with pressure-adhesive plate cover and cleanly tear off edges

PRODUCT = 10µL (cDNA with primer/probe MM)

- Protect from light and store at 4ºC until ready to run

Running qRT-PCR

- Take foil-wrapped 384-well optical plate to Keck Center
- Remove foil, balance plate in centrifuge (use provided balance plates)
- Run centrifuge using Program 1 (20,000rpm at 1min) – use instructions on centrifuge
- In other room, open QuantStudio rtPCR program
- Click "New Experiment" at the top left
    - Enter all appropriate fields for each of the tabs on the left
    - Create Experiment Name
    - Scan your barcode
    - Create username
        - Default settings for "Experiment Properties" should be fine EXCEPT #5
            - QuantStudio 7
            - 384-well
            - Comparative CT or Standard Curve
            - TaqMan Reagents
            - Standard (NOT "Fast"!)
        - On "Assign" tab, create your plate map (highlight all occupied wells)
        - On "Run Method" tab, re-adjust reaction volume to 10µL
        - Save the experiment under Nowak folder, under your folder
        - Click "File -> Close" – this will close the experiment but not the program
- NOTE: You HAVE to close the experiment in order to run the next steps
    - Open Automation Controller program
        - Click YES to the serial number prompt and the prompts on the following pop-up
        - Once the window opens, click "add plates" at the bottom right
        - Find the experiment that you just saved from QuantStudio (make sure the barcodes match)
        - Load your plate into Rack 1
        - Remove any completed plates from Rack 3
        - Click "start batch" to start the PCR machine
            - The robotic arm will go through its own trouble shooting motions, then check to make sure Rack 3 and the plate holder is empty; then

it will lower into Rack 1, grab your plate, scan the barcode, and load it in the plate rack. MAKE SURE THE ARM DOESN'T DISLODGE YOUR PLATE FROM THE PLATE HOLDER!

- Everything should be running, you can double check the read-out on the machine itself and on the computer screen.

Getting Your Data

- When your run is complete, you can access the file and send it to yourself through Box:
  - Open chrome, Box will automatically come up
  - Log in with your UIUC credentials
  - Upload your file

Protein Isolation:

- Put 50-100 mg of fibroid tissue into 2 mL of NP-40 lysis buffer.
- Homogenize with 2mm Tungsten Carbide beads and TissueLysterII (both from Qiagen) for 25 seconds followed by 5 seconds on wet ice.
    - Repeat until fibroids are completely dissociated (approx. 2-3 intervals)
- Centrifuge samples at 12,000xg for 10 min at 4°C.
- Aliquot the supernatant and store at -80°C until needed for future applications.

Western Blot—COX-2:

- Perform BCA Protein Assay (Pierce™, ThermoFisher, Waltham, MA, USA) to determine protein concentration of samples
- Calculate 3:1 ratios of protein (15 µg) to 4xLSB & mix protein and 4xLSB
- Denature protein for 5 min at 95°C
- Load gel into running box & fill with running buffer
- Load protein diluted in 4xLSB to wells
- Load 5µL BioRad protein ladder to one well
- Run gel at 120V for 1 hour
- Transfer to PVDF membrane:
    - Build sandwich from black to white IN THIS ORDER (be sure sponges and filter paper are soaked in transfer buffer):
        - 2 sponges
        - Filter paper
        - Gel
        - PVDF membrane
            - Activate in methanol for 30 sec
        - Filter paper
        - 2 sponges
- Insert sandwich into transfer box, add ice pack and stir bar, fill with transfer buffer
- Run on stir plate at 100V for 1 hour
- Block membrane in 3% BSA/TBST overnight at 4°C on plate shaker
- Incubate in primary antibody (COX-2, 1:1000) diluted in 2% BSA/TBST overnight at 4°C on plate shaker
- Wash in TBST 3x10 min on plate shaker
- Incubate in secondary antibody (HRP-Anti-Rabbit, 1:5000) diluted in 2% BSA/TBST for 1 hour at room temp. on plate shaker
- Wash in TBST 3x10 min on plate shaker
- Incubate in chemiluminescence for 5 min in the dark
- Visualize on ImageQuant machine
- Place membrane in stripping buffer for 15 min at room temp on plate shaker
- Block in 3% BSA/TBST overnight at 4°C on plate shaker
- Incubate in primary antibody (β-actin, 1:1000) diluted in 2% BSA/TBST overnight at 4°C on plate shaker
- Wash in TBST 3x10 min on plate shaker
- Incubate in secondary antibody (HRP-Anti-Rabbit, 1:5000) diluted in 2% BSA/TBST for 1 hour at room temp. on plate shaker
- Wash in TBST 3x10 min on plate shaker
- Incubate in chemiluminescence for 5 min in the dark
- Visualize on ImageQuant machine