Ulipristal Acetate and Extracellular Matrix Production in Human Leiomyomas In Vivo: A Laboratory Analysis of a Randomized Placebo Controlled Trial

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Abstract

In a prior randomized controlled study, patients treated with ulipristal acetate (UPA) or placebo for 3 months had a decrease in leiomyoma size. A total of 10 patients' tissue samples (5 placebo and 5 treated with 10 mg/d UPA) that underwent hysterectomy and tissue preservation were identified from this study. Quantitative real-time reverse transcriptase polymerase chain reaction and Western blotting were used to assess fold gene and protein expression of extracellular membrane (ECM) proteins: collagen IA (COLIA), fibronectin (FNI), and versican (VCAN) of the samples. Confirmatory immunohistochemical analysis was performed. Changes in total matrix collagen were examined using Masson trichrome staining. Multiplex measurement of the matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases was performed. Compared to placebo-treated surgical specimens, 80% of the treated specimens showed decrease in VCAN protein, 60% showed decrease in FNI, but no consistent alteration in COLIA. This effect was also supported by immunohistochemistry where leiomyoma surgical specimens demonstrated decreased amount of FNI and VCAN on UPA treatment. Increased MMP2 and decreased MMP9 in treated patient leiomyomas indicate both degradation of the matrix and inhibition of the pathway involved in matrix production. Treatment with UPA decreased fibroid volume in placebo-controlled, randomized trials. Treatment with UPA decreased gene expression and protein production in leiomyoma tissue, suggesting both an impact on water content and ECM protein concentration as a mechanism of ulipristal-mediated decrease in leiomyoma size.

Keywords

leiomyoma, extracellular matrix, ulipristal acetate, collagen-I, versican

Introduction

In reproductive-age women, uterine leiomyomas are the most common benign tumors^{1,2} and account for significant morbidity to include anemia, dysmenorrhea, bulk symptoms, and infertility. Due to lack of long-term effective medical therapies, leiomyomata account for over 200 000 hysterectomies annually in the United States.^{1,3} Despite the widespread prevalence and morbidity, the pathogenesis of leiomyoma has still not been fully identified, and surgical intervention with hysterectomy is the only cure.^{4,5} Medical options are available but have varying degrees of success to help reduce the bulk symptoms of fibroids.

Recent clinical studies have shown that gonadal steroid-blocking agents are efficacious in causing a decrease in leiomyoma volume over a period of treatment.⁶⁻¹³ Specifically, CDB-2914, also known as ulipristal acetate (UPA), is a selective progesterone receptor modulator (SPRM) that underwent phase I and II trials at the National Institutes of Health (NIH).⁸ These studies demonstrated 17% to 24% decrease in total volume of

fibroids over 3 months of treatment and improvement in quality-of-life measures.⁶⁻⁸ In multicenter European studies, Donnez and colleagues presented similar and more detailed clinical findings for UPA.⁹⁻¹¹ While UPA has been approved for clinical treatment of fibroids in the European Union and Canada, it is in phase III trials in the United States and is not yet approved for leiomyoma therapy by the Food and Drug Administration.

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The mechanism of UPA action on leiomyoma at cellular level has not been well elucidated. Depending on serum levels of progesterone, UPA can demonstrate both agonistic and antagonistic activity.¹⁴ This factor may also partially explain the differences in response to treatment as observed in clinical trials.⁶⁻⁸ Screening microarray experiments conducted in the early 2000s confirmed that the extracellular membrane (ECM) plays a critical role in the pathogenesis of leiomyomas.¹⁵ Increased production of proteoglycans and collagens participates in the fibrotic phenotype of leiomyomata,¹⁶⁻¹⁹ creating the significant bulk symptoms for patients. Limited studies with leiomyoma cell cultures suggest UPA affects the ECM components such as matrix metalloproteinases (MMPs) and tissue inhibitors of MMP (TIMPs) as well as collagen type I and III, and such studies potentially explain the decrease in size of leiomyoma in clinical trials.²⁰⁻²²

In the clinical trial done at the NIH, hysterectomy was performed at the conclusion of the study. Surgical specimens of uterine leiomyoma and patient-matched myometrium of women treated with UPA and placebo were available to characterize the impact of ulipristal treatment. Based on the limited UPA cellular studies and findings of the clinical trials, we hypothesized that we would find a difference in specific biomarkers including the ECM proteins known to be dysregulated in leiomyomas¹⁵⁻¹⁷ among surgical specimens exposed to UPA versus placebo. These findings could provide a mechanism for the decrease in observed size and volume after 3 months of treatment.

Materials and Methods

Patients and Clinical Study

This is a laboratory analysis of tissues obtained from a randomized, placebo-controlled double-blind clinical trials to evaluate therapeutic effect of UPA on uterine leiomyomas. Briefly, premenopausal patients with symptomatic leiomyomas were recruited for treatment with UPA (10 and 20 mg/d) or placebo for a total of 3 months. Baseline and presurgical magnetic resonance imaging results were obtained for each patient. Treatment effect on size was determined by measurement of the largest leiomyoma's growth change over the treatment time frame. As part of the study, patients underwent hysterectomy at 90 to 102 days of treatment, and leiomyoma and myometrium tissue samples were collected. These studies were performed under an institutional review board-approved protocol at the Uniformed Services University of the Health Sciences. The tissue was provided as a generous gift from Dr Lynnette Nie-man, as part of clinical trial NCT00290251.

Tissue Collection

For tissue analysis, a total of 10 patients (5 placebo and 5 treated with 10 mg/d UPA) were identified who had tissue samples collected in all forms including RNAlater, snap frozen, and paraffin embedded and were from the largest fibroid and adjacent myometrium of such patient.

Experimental Procedure: RNA and Protein Isolation

Total RNA was extracted from myometrium and leiomyoma tissue using TRIzol Reagent (Thermo Fisher Scientific, Waltham, Massachusetts). The method has been described previously^{23,24} with minor modifications. Briefly, tissue was diced into <2-mm pieces and submerged in TRIzol reagent for 1 hour on ice before homogenization. Samples were centrifuged at 10 000 rpm for 5 minutes at 4°C to pellet out the undigested tissue. Further steps were according to the manufacturers' protocol. Residual DNA was removed using Turbo DNase (Thermo Fisher), and the purified RNA was measured by NanoDrop (Thermo Fisher Scientific, Rockville, MD), aliquoted and stored at -80°C.

Leiomyoma and myometrial protein was extracted from the snap-frozen tissue or tissue stored in RNAlater. The snap-frozen tissue protocol has been detailed previously.²⁴ Briefly, the myometrium and leiomyoma tissue samples were cut into <1-mm pieces. The finely diced tissue was extracted overnight at 4°C in radioimmunoprecipitation assay buffer containing 1 × Halt proteinase and phosphatase inhibitors (Thermo Fisher Pierce, Rockville, MD). The homogenate was centrifuged at 13 000 rpm for 30 minutes in cold to pellet large cellular debris. The lysates were stored at -80°C. When snap-frozen tissue for patient match was not available, the Ambion PARIS kit (Thermo Fisher) was used to isolate both RNA and protein according to the manufacturers' protocol. The standard bicinchoninic acid assay was used to determine protein concentration.

Experimental Measurements

Quantitative real-time reverse transcriptase polymerase chain reaction. Expression of ECM genes, collagen 1A (COL1A), versican (VCAN), and fibronectin (FN1) using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) has been described previously.²³⁻²⁵ 18S ribosomal RNA gene was used as normalization control, and each sample was analyzed in triplicate.

Western blot analysis. Briefly, equal amounts of the proteins extracted from tissue were loaded onto Bio-Rad (Hercules, CA) 4% to 15% Tris-glycine gels and underwent electrophoreses under reducing conditions. For detection of proteins, the SNAP i.d. 2.0 (EMD Millipore, Massachusetts, Billerica, MA) was used according to instructions. Membranes were exposed to primary antibody against COL1A (sc-59772; 1:350), VCAN (V0/V2; ab19345; 1:500), and FN1 (ab6584; 1:10 000) at room temperature for 15 minutes followed by horseradish peroxidase (HRP)-conjugated secondary antibody 1:5000 to 1:10 000 (Thermo Fisher) for 10 minutes. Clarity Western ECL (Bio-Rad) was used for detection of the proteins. As an internal standard between samples, HRP-labeled antihuman β -actin (sc-1616; 1:50 000) was used.

Immunohistochemistry. Immunohistochemical staining was performed on tissue sections 6 to 8 μ m in thickness. The slides were deparaffinized in xylene, rehydrated in ethanol, and

placed in distilled water (DW). Proteins were evaluated using the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, California). Antigenic epitopes were chemically exposed using heated antigen unmasking solution (Vector Laboratories), followed by blocking of endogenous peroxidases. After equilibration (0.1% bovine serum albumin) and blocking (nonimmune serum), the sections were treated with primary antibodies: FN1 (1:500; ab299), VCAN (1:400; ab19345), COL1A (1:400; sc-59772), followed by secondary antibody and chromogenic development of proteins. Slides were dehydrated and mounted with HyperMount (Thermo Fisher).

Masson trichrome staining. Paraffin-embedded sections were deparaffinized and rehydrated in DW. The staining was performed according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Tissue sections were incubated with Bouin solution, stained with Weigert iron hematoxylin, and washed in DW followed by exposure to Biebrich scarlet-acid fuchsin. Washed slides were then incubated with 2.5% phosphomolybdic/phosphotungstic acid solution (vol/vol) followed by aniline blue staining. Slides were destained briefly in 1% acetic acid solution, dehydrated in 95% and 100% ethanol, and cleared in xylene before mounting.

Human MMP and TIMP assay. The Bio-Plex Pro Human MMP and TIMP Assay kits (Bio-Rad) were used to analyze the expression of MMPs (1-3, 7-10, 12, and 13) and TIMPs (1-4) in leiomyoma tissue from placebo ($n = 6$) and treated ($n = 8$) groups. The experiment was conducted twice with a minimum of 3 replicates per sample and according to manufacturers' protocol. Observed concentration values (pg/mL) were analyzed in the Bio-Plex Data Pro Package.

Statistical Analysis

Bio-Rad iCycler software 3.1 was used for gene expression analysis. Fold difference of leiomyoma to matched myometrium expression was compared between placebo and treated groups. For evaluation, Wilcoxon signed rank test for the non-parametric values was used, and $P < .05$ was considered statistically significant. The qRT-PCR data are reported as mean $\pm$ standard error of the mean (SEM). For Western blot analysis, Image Lab software 5.2.1 (Bio-Rad) was used, and fold difference between relative density units of treated and placebo samples was corrected for internal control, β -actin. Data are presented as fold difference $\pm$ SEM. For MMP and TIMP assay, the data were tested for significant differences using a Mann-Whitney U test, as normality could not be assured.

Results

Clinical Change in Size of Leiomyoma

The phase II trials for UPA showed interval decrease in uterine volume of patients treated with UPA versus placebo after 3

Table 1. Leiomyoma Tumor Characteristics.^a

Patient	Treatment	Race	Change	Initial Size, cm
1	Placebo	C	None	9 × 8 × 6.5
2	Placebo	AA	None	6 × 4.5 × 5
3	Placebo	AA	None	3 × 3 × 2.5
4	Placebo	AA	+18%	7 × 6 × 4.5
5	Placebo	AA	None	3 × 2 × 2
6	10 mg UPA	AA	-16%	1-5 cm
7	10 mg UPA	AA	-26%	4 × 5.5 × 6
8	10 mg UPA	AA	None	7.4 × 5.2 × 5.6
9	10 mg UPA	C	-20%	5.7 × 5.5 × 4.8
10	10 mg UPA	AA	+9%	5.3 × 4.8 × 3

Abbreviations: C, Caucasian; AA, African American; NIH, National Institutes of Health; UPA, ulipristal acetate.

^aLeiomyoma tumor characteristics and change in size of largest leiomyoma at the start of study of placebo or UPA-treated patients from NIH clinical trial over a 3-month treatment period.

months of treatment.^{6,7} As observed in Table 1, in the placebo group, only 1 of 5 patients demonstrated an increase in fibroid volume by 18%. In the treatment group, 1 of 5 patients demonstrated no change, 1 of 5 showed an increase of 9%, whereas 3 of 5 patients had a decrease in size ranging from 16% to 26% (Table 1). The maximum decrease of 26% was observed in patient 7 leiomyoma tissue.

Expression of ECM Gene in UPA-Treated Leiomyomas

It has been previously demonstrated that the expression of ECM genes, COL1A, FN1, and VCAN, were elevated in fibroids compared to patient-matched myometrium.^{23,26,27}

As observed in Table 2, compared to matched myometrium, the leiomyoma tissue of placebo patients (1-5) demonstrated a significant increased fold expression of COL1A transcripts ranging from 1.53 ± 0.11 (patient 2) to 7.12 ± 1.23 (patient 3), FN1 ranging from 1.48 ± 0.17 (patient 2) to 5.75 ± 1.78 (patient 3), and VCAN ranging from 3.14 ± 0.34 (patient 4) to 6.8 ± 0.96 (patient 2). Only 1 patient (patient 5) demonstrated 4-fold (0.247 ± 0.08) decreased expression of VCAN transcripts.

Compared to matched myometrium, fibroids from treated patients demonstrated a significant increased expression of all 3 ECM genes (Table 2). Fibroids from patient 7 demonstrated a 2.9 ± 0.1 -fold increased COL1A transcripts, 4.23 ± 1.79 -fold increased FN1 transcripts, and 13.55 ± 0.83 -fold increased VCAN transcripts. Leiomyoma from patient 10 in the treated group demonstrated significant 6-fold decrease in COL1A expression (0.168 ± 0.03) compared to matched myometrium, whereas no VCAN was detected at the transcript level. No significant association between the expressions of these genes was observed in treated and placebo patients (Table 2).

Expression of ECM Protein in UPA-Treated Leiomyomas

As observed in Table 2, compared to matched myometrium, significantly increased amount of COL1A protein was

Table 2. Transcript and Protein Changes of ECM Proteins in Placebo Versus UPA-Treated Tissue.^a

ECM Genes/Protein →	COL1A		FN1		VCAN	
	Transcript	Protein	Transcript	Protein	Transcript	Protein
1 (Placebo)	1.95 ± 0.07	1.56 ± 0.98 ^b	4.5 ± 1.15	2.64 ± 0.5 ^b	4.65 ± 0.75	2.44 ± 0.96 ^b
2 (Placebo)	1.53 ± 0.11	1.04 ± 0.31	1.48 ± 0.17	0.5 ± 0.2 ^b	6.80 ± 0.96	2.47 ± 0.42 ^b
3 (Placebo)	7.12 ± 1.23	6.50 ± 0.8 ^b	5.75 ± 1.78	7.25 ± 1.3 ^b	4.12 ± 0.42	3.5 ± 0.75 ^b
4 (Placebo)	2.56 ± 0.09	1.24 ± 0.18	1.93 ± 0.3	1.08 ± 0.42	3.14 ± 0.34	1.34 ± 0.27
5 (Placebo)	2.79 ± 0.16	1.02 ± 0.32	5.75 ± 1.5	0.91 ± 0.24	0.25 ± 0.08	1.87 ± 0.67 ^b
6 (UPA)	1.57 ± 0.11	1.44 ± 0.30 ^b	1.53 ± 0.06	1.21 ± 0.32	4.44 ± 0.33	0.6 ± 0.16 ^{b,c}
7 (UPA)	2.90 ± 0.1	1.38 ± 0.20 ^b	4.23 ± 1.79	1.6 ± 0.07	13.6 ± 0.83	0.83 ± 0.22 ^c
8 (UPA)	1.95 ± 0.18	1.23 ± 0.25 ^b	1.8 ± 0.006	0.8 ± 0.15	11.3 ± 1	2.1 ± 0.3 ^c
9 (UPA)	2.48 ± 0.3	1.18 ± 0.26	2.12 ± 0.23	0.86 ± 0.1	2.59 ± 0.33	0.73 ± 0.12 ^{b,c}
10 (UPA)	0.17 ± 0.03	2.12 ± 0.45 ^b	2.59 ± 0.95	0.68 ± 0.3 ^b	0 ± 0	1.1 ± 0.46 ^c

Abbreviations: COL1A, collagen 1A; ECM, extracellular membrane; FN1, fibronectin; UPA, ulipristal acetate; VCAN, versican.

^aFold changes in ECM genes and proteins (COL1A, FN1, VCAN) in 10 patient samples. Patient number and treatment type are noted in the left column, while the ECM-related genes and protein concentration are listed in columns to the right. Expression levels of each protein is relative to patient-matched myometrial samples. Data are presented as fold-expression ± standard error of the mean (SEM).

^bSignificant difference ($P < .05$), leiomyoma compared to patient-matched myometrium.

^cSignificant difference ($P < .05$), treated leiomyoma compared to placebo leiomyoma.

observed in both placebo and UPA-treated groups, with no significant difference between the 2 groups.

Fibronectin protein was significantly increased in 2 of 5 patients in placebo group; patient 1 (2.64 ± 0.5) and patient 3 (7.25 ± 1.35), whereas patient 2 demonstrated a 2-fold decrease (0.5 ± 0.2 ; Table 2). Compared to matched myometrium, in the treated group, 3 of 5 patients demonstrated >1-fold decrease in FN1 protein but was not significant. Similarly, no significant difference was found between fibroids of placebo or UPA-treated group.

The VCAN protein was significantly increased in 4 of 5 placebo patients, ranging from 1.87 ± 0.67 -fold (patient 5) to 3.5 ± 0.75 -fold (patient 3). In the treated group, VCAN protein was significantly decreased in 3 of 5 patients, whereas only 1 of 5 demonstrated an increase, patient 8 (2.1 ± 0.3), when compared to their respective myometrium (Table 2). A significant decrease ($P \leq .05$) in VCAN protein was observed in treated compared to placebo fibroids.

Immunohistochemistry of placebo patient myometrium and fibroid tissue demonstrated smooth muscle cells arranged in well-organized fascicles in which cells cut both longitudinally and in cross section are displayed (Figure 1A-D). The COL1A protein (Figure 1A) was detected in both placebo and treated fibroid samples, but no significant difference between the 2 groups was observed. Although FN1 protein demonstrated decreased expression in UPA-treated groups (Figure 1B; samples 6-10), the difference was variable between the 2 groups. Most patients in the treated group demonstrated substantially decreased VCAN protein (Figure 1C; samples 6-10) when compared to placebo patients (Figure 1C; samples 1-5). No staining was observed in tissue that was not exposed to either the primary or secondary antibodies (data not shown).

Hematoxylin-counterstained tissue sections of leiomyomas from placebo and treated patients demonstrated a degree of microscopic heterogeneity. Patient 3 (placebo; Figure 1A)

showed areas of pale, densely packed matrix that disrupted the fascicular arrangement of the cells most appreciated in 60% of the treated samples (Figure 1A and 1C; samples 6, 9, and 10). Representative sections of tissue from the majority of treated patients showed vast histologic differences to placebo patients. High-power view of treated samples revealed alternating areas of cellularity and comparatively less cellular areas (fibrosis) within the same tissue sections (data not shown). These areas range in appearance from a loosely organized fibrous stroma in which individual leiomyoma cells are seen as no longer a part of fascicles (Figure 1A; samples 6 and 7) but distinct from areas of fibrosis seen in typical leiomyomas.²⁸ Other areas show a very densely packed matrix with few cells whose cytoplasm is not easily distinguished from the surrounding matrix (Figure 1C; samples 6, 9 and 10). In particular, the nuclei of these cells are close together and appear as small nests of cells (data not shown). This type of fibrosis is dissimilar to the “hyalinized stroma” that has been described as the normal evolution of fibroids as phase 3 and 4 fibrosis.²⁸ These foci are accompanied by the presence of areas of karyorrhexis/karyolysis, indicating that the treated samples are experiencing apoptosis.

Representative sections of both placebo and treated leiomyoma tissue demonstrate a wide variation in ECM production as evidenced by trichrome stains. As observed in Figure 2, the production of collagen appears as a very dense, homogeneous ECM component in both placebo (1 and 3) and treated samples (6, 8 and 9), illustrating the remarkable heterogeneity of these tumors. Areas of hypercellularity and hypocellularity were present in both placebo and treated samples. Note the varying degree of hypercellularity of the untreated (2 and 5) and treated (7 and 10) samples. On close examination of the hypocellular areas within the leiomyoma tumors, the ECM ranges from densely compacted areas to those that appear as pale and loosely arranged matrix components.

Figure 1. Immunoreactivity of extracellular membrane (ECM) proteins in leiomyoma tissue. A, Collagen IA (COLIA): There is no change in the expression of COLIA in treated (6-10) leiomyoma tissue samples when compared to placebo samples (1-5). B, Fibronectin: Immunoreactivity for fibronectin (FNI) protein is decreased in treated patient samples (6-10). C, Versican: Treated samples (6-10) show a decrease in versican (VCAN) protein expression. D, Masson trichrome stain of leiomyoma tissue from placebo (patients 1-5) and ulipristal acetate (UPA)-treated (patients 6-10) specimen revealed less collagen production within the ECM of treated samples as indicated by the blue staining with aniline blue. These are the representative slides from a total of 4 staining protocols ($\times 20$).

Figure 2. The box whisker plot compares protein amount in ulipristal acetate (UPA)-treated leiomyomas from patients (T; n = 6) to fibroids from placebo patients (P; n = 8). The Bio-Plex Pro Human matrix metalloproteinase (MMP) assay kit was used to analyze expression of MMPs (1-3, 7-10, 12, and 13); MMP1, MMP2, MMP7, and MMP9 demonstrated significant differences in leiomyoma tissue from UPA-treated patients compared to placebo. The experiment was conducted twice with a minimum of 3 replicates per sample. *Significant difference ($P < .05$), UPA-treated leiomyoma compared to placebo.

Expression of MMPs and TIMPs in UPA-Treated Leiomyoma Tissue

As observed in Figure 2, in the placebo group, MMP2 (2876.54 ± 298.45 pg/mL) was highly expressed in leiomyoma tissue followed by MMP10 (358.67 ± 53.1 pg/mL) when compared to other MMPs. Comparing the protein concentration of the MMPs in treated versus the placebo leiomyoma tissue, significant differences were observed in MMP1 (z-score of -2.49 ; $P = .012$), MMP2 (z-score of $+2.92$; $P = .003$), MMP7 (z-score of $+2.46$; $P = .013$), and MMP9 (z-score of -3.09 ; $P = .002$). Patient 9 (treated) demonstrated >200 pg/mL of MMP3 compared to other treated or placebo leiomyoma tissue (10-100 pg/mL). This patient also demonstrated higher concentrations of MMP13 compared to other patients in both groups (data not shown). Most of other MMPs were comparable between each group.

We also examined the total protein amount of TIMPs in placebo and treated leiomyoma tissue. On an average, in placebo samples, TIMP2 was highly expressed in the leiomyoma tissue at 4862.3 ± 1380.2 pg/mL, followed by TIMP1 at 2612.3 ± 771.2 pg/mL, TIMP3 at 1046.8 ± 589.6 pg/mL, and TIMP4 at 37.6 ± 14.5 . Although a general decrease in total protein was observed on UPA treatment, the difference was not statistically significant (data not shown).

Discussion

The current study evaluated the effects of UPA on ECM production in clinically obtained leiomyoma and patient-matched myometrium tissue. In the placebo-treated group, 80% of the patients did not demonstrate any change in their largest leiomyoma volume. In the UPA-treated group, 60% of patients

demonstrated decrease in the volume of largest fibroid, whereas 1 (20%) of 5 patient demonstrated increase by 9%. Our subgroup analysis findings are comparable to the overall results observed by Nieman and coworkers.⁷ They reported a total of 7% increase in total fibroid volume of placebo group and a 17% decrease in UPA (10 mg/d) treatment group.^{6,7} In our study, the fibroids that showed the largest decrease in volume (patients 6, 7, and 9) were of the UPA group, and they also showed the largest decrease in VCAN protein production, respectively. Patient 9 in our UPA treatment group had an increase of 9% overall of largest fibroid, having a 2-fold increase in COL1A protein, decrease in FN protein, and no change in the VCAN protein production. Collagen 1A protein was not increased in any other of our UPA-treated patients. No correlation by treatment was observed based on size and location of individual fibroids. The subset of patients is inadequate to define a statistically valid relationship between race and treatment.

The UPA mechanism of action is not fully elucidated; however, it is categorized as an SPRM. Evidence suggests apparent importance of progesterone for growth and development of fibroids.²⁹⁻³¹ Clinical studies have established that UPA decreases fibroid volume.⁶⁻¹¹ Our study evaluated whether UPA was disrupting any major ECM component(s) that may contribute to its efficacy in decreasing fibroid volume.

Compared to patient-matched myometrium, most placebo-treated leiomyoma tissue consistently demonstrated an increase in RNA transcripts and/or protein expression of ECM components including collagens, FN1, and VCAN. This has been previously demonstrated in multiple studies and is a well-known likely mechanism of fibroid growth and dysfunction.^{15-19,27} Although patient 4 in the placebo group demonstrated an 18% increase in fibroid volume, no significant change was observed

in either the transcripts or the protein of analyzed genes when compared to other patients in the placebo group. No statistically significant conclusion could be reached based on size of the fibroid.

Collagen-1, a fibrillar collagen, makes up most of the disordered ECM protein found in uterine fibroids. Courtoy and coworkers³² reported a decrease in collagen abundance after long-term UPA treatment (10 mg/d for 2–4 cycles of 12 weeks) but no significant change in short-term (3 months) treatments of 5 and 10 mg/d. Our clinical samples did not demonstrate any significant effect of UPA on COL1A transcript or protein amount. This is further confirmed in our small study where patient 10 in our UPA-treated group had a 2-fold increase in COL1A protein production. No significant difference was observed in COL1A transcripts in myometrium as well from placebo and treated groups in our study (data not shown); thus, there appears to be no adverse effect of UPA on myometrium COL1A production. RNASeq analysis conducted in our patient samples indicated a decreased expression of collagens 2 and 3 (data not shown).

Xu and colleagues²⁰ demonstrated treatment with UPA changes the expression of MMPs and TIMPs, leading to decreased collagen formation of type I and type III *in vitro*. Increased expression of various MMPs and TIMPs has been reported in uterine fibroids,^{33–36} and the expression varies by size of the fibroid³⁷ and the phase of the menstrual cycle.³⁴ Based on microarray analysis, it has been suggested that MMPs may play a critical role in dysregulated ECM production in fibroids.¹⁹ In fact, increase in vitamin D3 treatment in fibroid cells can increase TIMPs and decrease MMPs expression and may be used as a potential therapeutic strategy for management of fibroids.³⁸ In the present study, we were able to measure MMP protein concentration in fibroids. Matrix metalloproteinase 2 was present in nanogram values per milliliter in these tissues compared to picogram per milliliter for other MMPs. Increased MMP2 (type IV collagenase) has been reported in uterine fibroids. It is considered an antifibrotic MMP in liver fibrosis³⁹ and is both an effector and a regulator of inflammatory response.⁴⁰ The UPA treatment in our subset of patients significantly increased the total MMP2 protein amount suggesting breakdown of ECM components. However, we also observed a significant decrease in MMP1 (collagenase for COL I, II, and III) protein in UPA-treated fibroids, which may explain no significant change in COL1A protein. A significant decrease was observed in MMP7 protein (matrilysin) known to be highly increased in fibrotic lungs.⁴¹ Matrix metalloproteinase 3 (stromelysin-1) is profibrotic and is known to process latent TGF β 1 to its active form. We did not see a significant change in protein concentration on UPA treatment. Notably, a submucosal fibroid high in matrix content and very acellular compared to other treated patient fibroids demonstrated an increased MMP3 protein (>250 pg/mL) as compared to other patients (<50 pg/mL). Matrix metalloproteinase 9 (gelatinase B) was also decreased by UPA treatment. Matrix metalloproteinase 9 is known to activate transforming growth factor β (TGF β) through proteolytic degradation,^{42,43} and decreased

activity of MMP9 may lead to decreased TGF β -mediated ECM production. Our subset of patients is insufficient to reach a definitive conclusion but provides direction for future study.

Fibronectin is an extracellular glycoprotein that acts as an adhesion molecule between matrix and cells. In the UPA-treated group, no significant relationship could be found between fibroids that demonstrated decrease or increase in total volume to FN1 expression. Although no direct effect of UPA on FN1 has been reported, Ciarmela and coworkers⁴⁴ observed that UPA blocked activin-A-induced FN1 and vascular endothelial growth factor A transcripts *in vitro*. In leiomyoma tissue, spatial arrangement (IHC) indicated areas of decreased FN1 amount. The discrepancy between total FN1 protein expression as observed by 2 different proteomic analyses and differences in areas of cellularity and matrix as observed by Trichrome staining emphasizes the importance of observing the fibroid tissue histologically and that protein should be collected from different parts of the leiomyoma to give a more realistic picture of protein changes.

Versican, a proteoglycan that forms high-molecular-weight aggregates with hyaluronic acid, has a large hydration potential in the ECM. Based on our subset of patient tissue, VCAN protein demonstrated maximum decrease with UPA treatment compared to other tested proteins as well as compared to the placebo-treated group. The UPA treatment significantly decreased VCAN protein in leiomyomas, and these leiomyomas demonstrated a decrease in total size, which was seen in patients 6, 7, and 9 who had the largest decrease in size of their total fibroid volume and largest decrease fold expression of VCAN protein. The VCAN proteins are highly negatively charged, with large number of hydrophilic glycan components, promoting hydrostatic swelling pressure in the interstitial space. The high density of negative charges and cations draw large amounts of water into the matrix. In leiomyomas, the concentration of VCAN is 4- to 6-fold higher than the surrounding myometrium and contributes to expansion of the ECM space. Loss or breakdown of these aggregates could lead to expulsion of water, decreasing the hydrostatic swelling potential and thus further lead to shrinkage of the fibroid tumor. Previous studies have demonstrated changes in osmoregulatory genes and increased proteoglycan composition of the leiomyoma ECM.⁴⁵ McCarthy-Keith and colleagues⁴⁵ showed that treatment with a gonadotropin-releasing hormone agonist (GnRHa) decreased osmoregulatory genes suggesting dehydration of leiomyomas when treated with a hormonal-blocking agent in the initial 3 months. While UPA has a different mechanism of action than GnRHa, perhaps both drugs initially decrease fibroid size clinically via an osmoregulation and dehydration effect rather than true dissolution of all ECM components. We also speculate that in the short-term treatment of the fibroids with UPA, activation of various components and pathways for ECM degradation, including changes in metalloproteinases, is initiated.

We worked with a subset of placebo and treated patient samples that demonstrated varied response at the end of 3 months. For our initial study, we limited our tissue analysis

to patients who had their largest leiomyoma and adjacent matched myometrium collected and stored in multiple forms. This may have limited our samples and not represented the overall patient population that was sampled in the phase II clinical trials by Nieman et al. This small tissue population showed varying results by each patient where in vivo environment is not controlled, emphasizing that many individualized factors such as hormone levels, race, and age may all have some implications to the response of each individual's tissue. Individualized changes in expression among the patients supports that leiomyomata are multifactorial, and despite changing the hormone environment, multiple pathways are involved.

We can conclude that in patients treated with 1 cycle of UPA (12 weeks), the fibroids initially likely respond by changes in the water content of the tumor, with VCAN as one notable protein change; however, it will be important to analyze other changes in osmoregulatory gene pathways involved in this process to further elucidate these processes. During this time frame, there is also activation of various pathways/genes that ultimately result in breakdown of the extracellular matrix.

Authors' Note

JC, MM, and JB have equally contributed to the manuscript. The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Army, Department of Defense, or the US Government.

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The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: WHC is consultant for Abbvie, Allergan, and Bayer.

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