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XXXI Cycle

Doctoral Thesis

"The effect of phytochemical compounds on uterine myometrial and leiomyoma cells"

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Summary (English)

Uterine leiomyomas (fibroids or myomas) are the most common benign tumors of the female reproductive tract. Fibroids cause pelvic pain, abnormal vaginal bleeding, pressure on the bladder, miscarriage, and infertility. They are the leading indication for hysterectomy. Unfortunately, no long-term medical treatments are available. In modern pharmaceutical industries, dietary phytochemicals are used as a source of new potential drugs for many kinds of tumors. Dietary phytochemicals may exert therapeutic effects by interfering with key cellular events of the tumorigenesis.

Since uterine leiomyoma is characterized by excessive accumulation of extracellular matrix (ECM) including collagens and fibronectin, in the present study, we aimed to investigate if dietary phytochemicals can regulate the ECM in human myometrium and leiomyoma cells.

In the present study, initially, we aimed to test the antifibrotic effect of 2 different strawberry cultivars (*Alba* and *Romina*) and the *anthocyanin enriched fraction(ACY)* of Romina in uterine leiomyoma and myometrial cells.

Leiomyoma and myometrial cells were treated with 2 different concentrations (100 µg/ml and 250 µg/ml) and with 250 µg/ml anthocyanin enriched fraction (ACY), for 48 h to measure gene expression and/or protein associated with fibrosis. We show, significantly decreased of collagen1A1, fibronectin, versican, and activin-A mRNA expression, in leiomyoma cells as well as in myometrial cells. The reduced protein expression of fibronectin was also observed after treatments with those cultivars in leiomyoma cells compared with untreated control.

Afterward, I has been involved in testing other phytochemical compounds on leiomyoma cells such as Quercetin and Indole-3-Carbinol.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a plant bioflavonoid, found in most edible fruits and vegetables. Indole-3-Carbinol (I3C; 1H-indole-3-ylmethanol) is produced from naturally occurring glucosinolates contained in a wide variety of plants, including members of the family Cruciferae and particularly members of the genus *Brassica.*

Leiomyoma and myometrial cells were treated with Quercetin and I3C (10 µg/ml; 50 µg/ml; 100 µg/ml; 250 µg/ml) for 48 h to measure mRNA and protein expressions of ECM, as well as to evaluate the migration (by wound healing assay) and the proliferation rate. Quercetin and I3C significantly decreased collagen1A1 and fibronectin mRNAs expression and protein expression of fibronectin in leiomyoma cells. We also, obtained a significant reduction of the migration and proliferation in leiomyoma and myometrium cells, after treatment with quercetin and I3C.

In conclusion, this study shows the in *vitro* effects of some phytochemical compounds and lays the scientific bases for the development of new therapeutic and/or preventive agents for the uterine leiomyomas.

Summary (Italian)

Il fibroma uterino o leiomioma è il tumore benigno più comune che colpisce l'utero nelle donne in età fertile. I fibromi causano, dolore o pressione pelvica, menorragia ossia una perdita ematica mestruale abbondante nonché dolorosa, ed è una delle principali cause d'infertilità e aborti ricorrenti. I fibromi uterini, sono l'indicazione principale d'isterectomia. Nonostante, l'alta prevalenza di questa patologia nelle donne, la patogenesi dei leiomiomi uterini non è stata ancora del tutto chiarita. Recenti studi, hanno dimostrato che una dieta basata su composti fitochimici ha proprietà preventive su diverse malattie.

Il fibroma uterino è caratterizzato da un'alta componente extracellulare (ECM), in particolare collagene, fibronectina e proteoglicani, e da fattori di crescita, da citochine e chemochine, da fattori angiogenici e da mediatori di risposta infiammatoria. Nel presente studio, abbiamo cercato di indagare sull'effetto dei composti fitochimici su cellule di fibroma e di miometrio *in vitro*, in particolare se fossero in grado di regolare l'espressione genica e proteica dei componenti della matrice extracellulare. Per il nostro studio abbiamo utilizzato 2 diverse cultivar di fragole (*Alba* e *Romina*) e la *frazione di Romina arricchita di antociani o ACY*, per valutare l'effetto antifibrotico sia sulle cellule di fibroma che su quelle di miometrio. Le cellule di fibroma e di miometrio sono state trattate con estratti delle diverse cultivar, utilizzando per entrambi 2 diverse concentrazioni (100 μg/ml e 250 μg/ml) e 250 μg/ml per ACY, per 48 ore, con lo scopo di valutare la variazione dell'espressione genica e proteica dei componenti associati alla fibrosi. Abbiamo ottenuto, che sia nelle cellule di leiomioma che in quelle di miometrio, l'espressione genica di collagene1A1, fibronectina, versican e activin-A è diminuita significativamente; inoltre, si ha una riduzione significativa dell'espressione proteica della fibronectina rispetto al controllo.

Successivamente, abbiamo voluto testare, altri costituenti fitochimici tra cui la Quercetina e l'Indolo-3-Carbinolo.

La quercetina (3,3 ', 4', 5,7-pentahydroxyflavone) è un bioflavonoide vegetale, comune nella maggior parte dei frutti e vegetali, mentre l'indolo-3-carbinolo (I3C; 1H-indolo-3-ilmetanolo) è un derivato dei glucosinolati presente in numerose piante, compresi i membri della famiglia Cruciferae e in particolare i membri del genere *Brassica*.

Le cellule di leiomioma e di miometrio sono state trattate, *in vitro*, con la quercetina e l'indolo-3-carbinolo a diverse concentrazioni (10 μg/ml, 50 μg/ml, 100 μg/ml, 250 μg/ml) per 48 ore, per misurare l'espressione genica e proteica delle componenti della matrice extracellulare. Si è visto, che sia la quercetina che l'indolo-3-carbinolo riducono significativamente l'espressione genica di collagene1A1 e della fibronectina nelle cellule di

leiomioma, l'espressione proteica della fibronectina nelle cellule di leiomioma. Inoltre, grazie ai saggi di wound healing e di proliferazione, si è vista un'inibizione della migrazione e della proliferazione cellulare, delle cellule di leiomioma, in coltura successivamente al trattamento con la quercetina e l'indolo-3-carbinolo.
In conclusione, questo studio mostra gli effetti in *vitro* di alcuni costituenti fitochimici e getta le basi scientifiche per lo sviluppo di nuovi agenti terapeutici e/o preventivi per i leiomiomi uterini.

List of publications

1. Islam MS, **Greco S**, Janjusevic M, Ciavattini A, Giannubilo SR, D'Adderio A, Biagini A, Fiorini R, Castellucci M, Ciarmela P. Growth factors and pathogenesis. Best Pract Res Clin Obstet Gynaecol. 2016 Jul;34:25-36. doi: 10.1016/j.bpobgyn.2015.08.018. PubMed PMID: 26527305.

2. Janjusevic M, **Greco S**, Islam MS, Castellucci C, Ciavattini A, Toti P, Petraglia F, Ciarmela P. Locostatin, a disrupter of Raf kinase inhibitor protein, inhibits extracellular matrix production, proliferation, and migration in human uterine leiomyoma and myometrial cells. Fertil Steril. 2016 Nov;106(6):1530-1538.e1. doi: 10.1016/j.fertnstert.2016.08.010. PubMed PMID: 27565262.

3. Islam MS, Giampieri F, Janjusevic M, Gasparrini M, Forbes-Hernandez TY, Mazzoni L, **Greco S**, Giannubilo SR, Ciavattini A, Mezzetti B, Capocasa F, Castellucci M, Battino M, Ciarmela P. An anthocyanin rich strawberry extract induces apoptosis and ROS while decreases glycolysis and fibrosis in human uterine leiomyoma cells. Oncotarget. 2017 Apr 4;8(14):23575-23587. doi: 10.18632/oncotarget.15333. PubMed PMID: 28212568; PubMed Central PMCID: PMC5410328

4. Protic O, Islam MS, **Greco S**, Giannubilo SR, Lamanna P, Petraglia F, Ciavattini A, Castellucci M, Hinz B, Ciarmela P. Activin A in Inflammation, Tissue Repair, and Fibrosis: Possible Role as Inflammatory and Fibrotic Mediator of Uterine Fibroid Development and Growth. Semin Reprod Med. 2017 Nov;35(6):499-509. doi:10.1055/s-0037-1607265. PubMed PMID: 29100238.

5. Islam MS, Castellucci C, Fiorini R, **Greco S**, Gagliardi R, Zannotti A, Giannubilo SR, Ciavattini A, Frega NG, Pacetti D, Ciarmela P. Omega-3 fatty acids modulate the lipid profile, membrane architecture, and gene expression of leiomyoma cells. J Cell Physiol. 2018 Sep;233(9):7143-7156. doi:10.1002/jcp.26537. PubMed PMID: 29574773.

6. Giampieri F*, Islam MS*, **Greco S***, Gasparrini M, Forbes Hernandez TY, Delli Carpini G, Giannubilo SR, Ciavattini A, Mezzetti B, Mazzoni L, Capocasa F, Castellucci M, Battino M, Ciarmela P. Romina: A powerful strawberry with in vitro efficacy against uterine leiomyoma cells. J Cell Physiol. 2018 Oct 14. doi: 10.1002/jcp.27524. [PubMed PMID: 30317591 *=Equally contributed

1 Introduction

1.1 Uterine fibroids

The uterine leiomyomas or fibroids are the most common benign, monoclonal, gynecological neoplasms originating from the pelvis in women, in particular from smooth muscle cells layer of the uterus or myometrium [1, 2]. The tumors incidence was estimated around 77% of women in the reproductive age [1, 3], and 25% of them bear clinically apparent tumors causing significant morbidity, including pelvic pain or pressure, infertility, and reproductive dysfunction in rare case [4]. Symptomatic uterine fibroids remain the most cited indication for hysterectomy. The leiomyoma causes a considerable impact on the quality of life and an increased economic burden on the healthcare system [5].

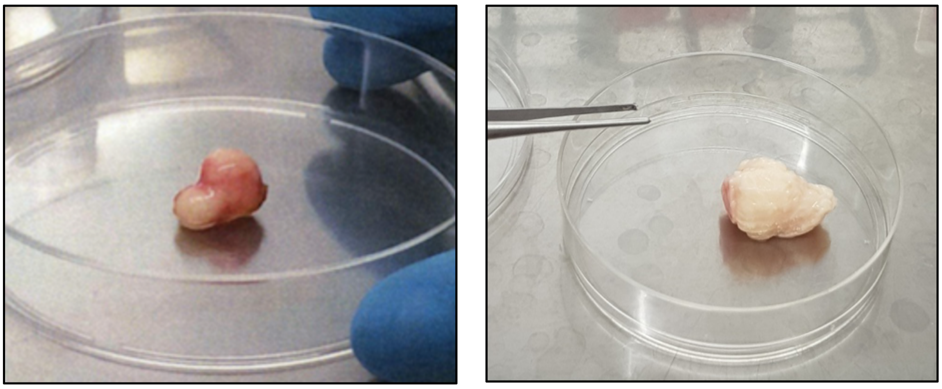


Figure 1. Samples of leiomyoma from laparoscopic extraction

1.1.1 Classification of uterine fibroids

The leiomyomas are usually spherical fibrotic tissue masses composed by a large amount of extracellular matrix (ECM) with size varying between a few millimeters up to multiple centimeters. The International Federation of Gynecology and Obstetrics (FIGO) developed a classification according to location [6, 7].

Intramural fibroid develops in uterine wall thickness, behaving according to its volume a modification of the uterus in terms of shape and volume. Subserosal fibroid tends to accrue in the outer portion of the uterine wall, developing mostly outdoors in the peritoneal earnest that lines the uterus. If the subserosal fibroid develops by keeping contact with the wall by a stalk, it would be facing a pedunculated fibroid. Finally, there is the submucosal fibroid, which grows into the uterine wall thickness, growing into the endometrium. In addition, pedunculated fibroids can wring on its stalk, causing blockage of blood flow [8-10].

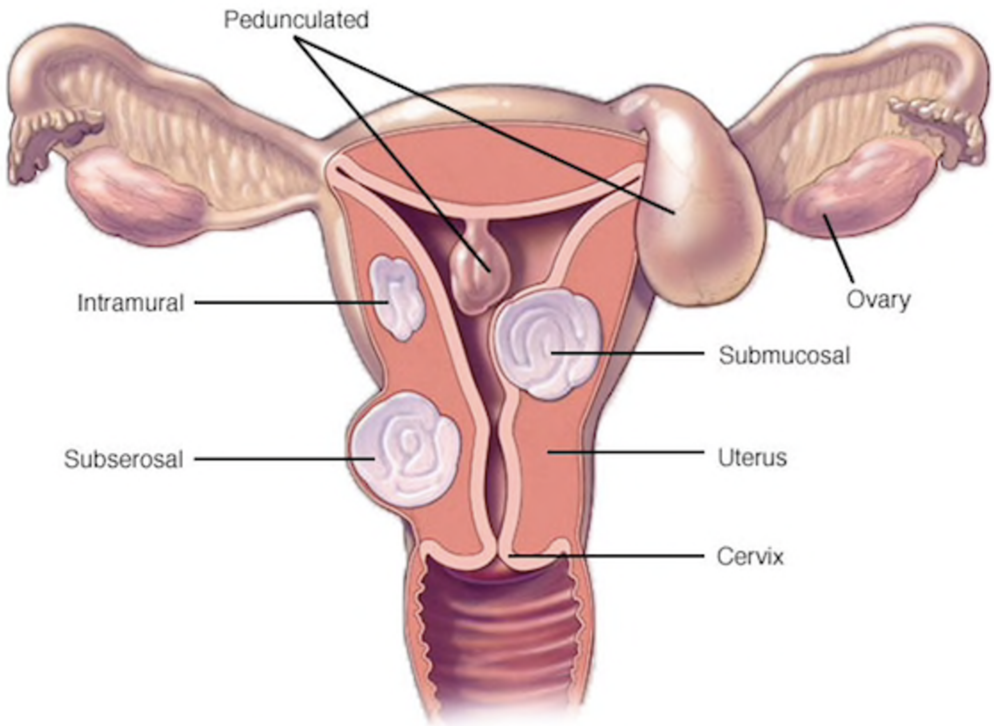


Figure 2. Location of uterine myomas

A second classification can be made according to histological characteristics of leiomyomas. Although, usual leiomyoma is the most common, other histological types exist. We can distinguish into atypical leiomyoma that is characterized by a marked polymorphic nucleus, which is always benign with bizarre nuclei and it is also called leiomyoma bizarre; usual leiomyoma; leiomyoma with high mitotic activity and cellular leiomyoma, which are two other histological types of benign neoplasm. Other types are haemorrhagic cellular (apoplectic) leiomyoma, epithelioid leiomyoma, myxoid leiomyoma, leiomyristioma with haematopoietic elements, benign metastasizing leiomyoma, perinodular hydropic leiomyoma, multinodular hydropic leiomyoma and cotyledonoid dissecting leiomyoma and lipoleiomyoma [11, 12].

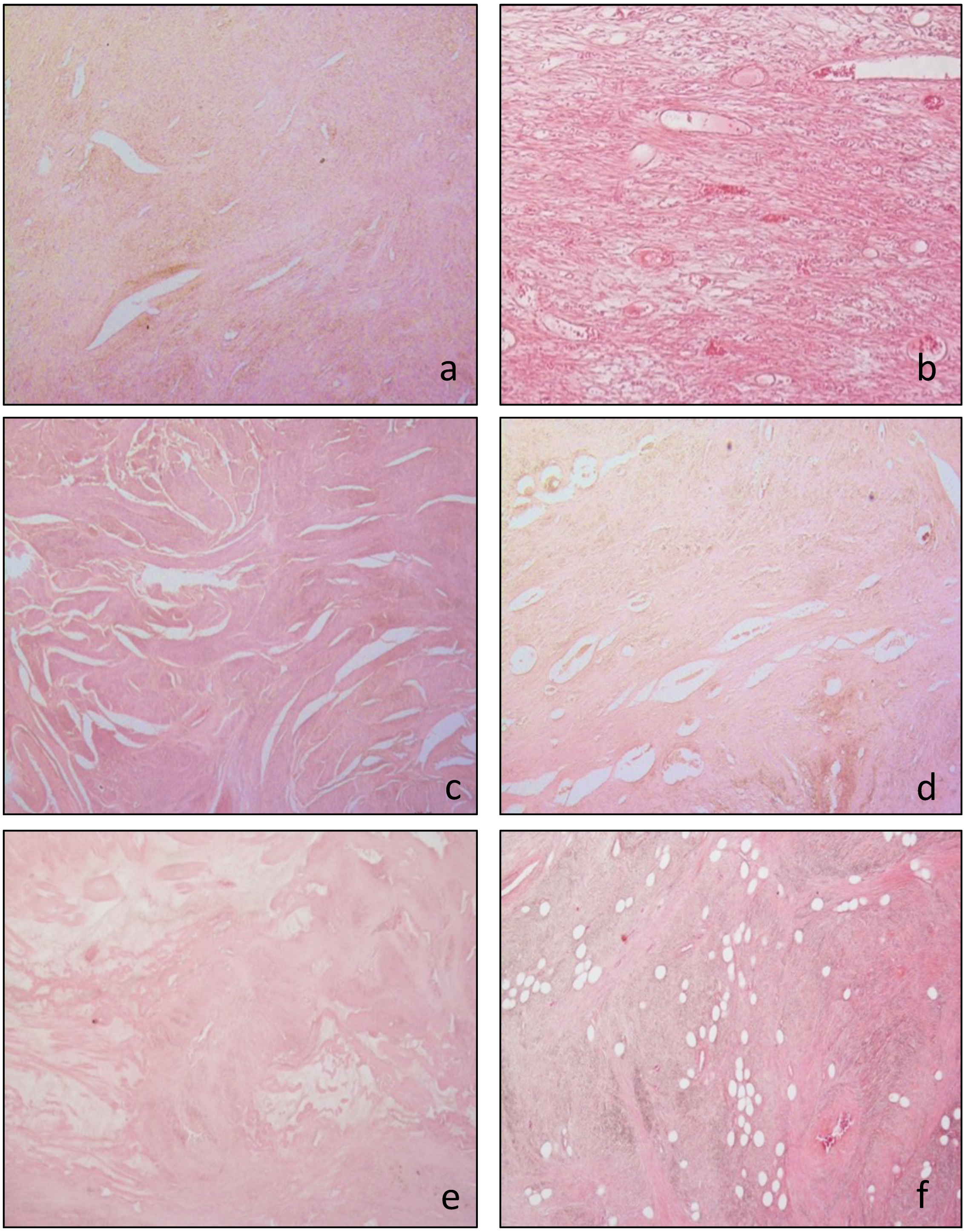


Figure 3. Different histological types of leiomyomas with hematoxylin and eosin staining (H&E): a) usual leiomyoma, b) cellular leiomyoma, c) leiomyoma bizarre, d) leiomyoma with high mitotic activity, e) leiomyoma with edema, f) lipoleiomyoma.

1.1.2 Symptoms and therapeutic options

The presence of uterine fibroids can be asymptomatic, or as it happens in about 30-40% of women, can be symptomatic with a presence of menorrhagia (heavy and prolonged menstrual bleeding) [13]. Usually, this type of symptomatology is caused by the presence of intramural fibroid or submucosal fibroid. Other symptoms that indicate the presence of fibroids is the feeling of a pelvic pressure [14, 15]. In addition, the occurrence of uterine fibroids can result in 20% of cases in abortion, or infertility. Although for asymptomatic fibroids it is preferable to withhold care and undergo regular gynecological examinations to control their growth, for symptomatic fibroids the patients have to resort to medical treatment and/or surgery [15]. There are different approaches and different therapies for a woman with symptomatic fibroids depending on the localization and the size of tumors, and it depends on the age and race of the patient.

Levonorgestrel releasing intrauterine system (Mirena), is an oral contraceptive treatment which is indicated for a woman with an abnormal uterine bleeding linked with an abnormal endometrium [16]. Tranexamic acid or Cyklokapron is a non-hormonal treatment, which significantly reduces menstrual blood, indicated for woman manifesting intralesional trombing, necrosis [17, 18]. Hormonal therapies are the most commonly used for management of leiomyoma. These are some preoperative treatments to decrease the size of a tumor and bleeding before surgery such as gonadotropin-releasing hormone agonists (GnRHa) which cause a downregulation of gonadotropic and gonadal hormones leading [19].

Other treatments include progesterone receptor modulators (SPRMs) such as ulipristal acetate. This treatment reduces the size of the uterine fibroid. The used dose for this indication can inhibit ovulation and lead to amenorrhea which will be of benefit to the woman who has menstrual bleeding related to their fibroids [20, 21]. Compared with placebo, ulipristal significantly reduces mean blood loss or induce amenorrhea and reduce fibroid volume in these patients.

The treatment is limited for a short time, three months, and used for preoperative adjuvant.

Other options include aromatase inhibitors (ex. Letrozole, anastrozole, fadrozole) reducing the size of tumors, but with side effects including hot flashes, vaginal dryness, and musculoskeletal pain [21-24].

Surgery such as hysterectomy, which consists of the complete removal of the uterus, is the only therapy that guarantees the definitive disappearance of disorders. The indication of

hysterectomy depends on the size, localization, and number of leiomyomas, but it determines infertility and it can led to psychological and sexual problems [25].
The laparoscopic extraction of the uterus may be performed with morcellation, however, the U.S. Food and Drug Administration, recently recommends limiting the use of laparoscopic morcellation to reproductive-aged women [26].
Other surgical procedures for women include myomectomy and uterine artery embolization. The first technique guarantees and preserve the fertility; it is indicated for submucosal fibroids whose dimensions are less than 3 cm when more than 50% of the tumor is intracavitary. The second one is an option for a woman who wants to preserve the uterus after surgery; it is an interventional radiologic procedure in which occluding agents are injected into one or both of the uterine arteries, limiting blood supply to the uterus and fibroids. It is a minimally invasive procedure targeting the destruction of fibroids via a focused energy delivery system such as heat, laser, or more recently, magnetic resonance–guided focused ultrasound surgery (MRgFUS).
Overall, this less-invasive procedure is well tolerated, although it includes some risks such as localized pain and heavy bleeding [26].

1.1.3 Epidemiology of uterine fibroids

The presence of leiomyoma is related to several factors and its incidence depends on ethnicity, it is estimated to be 3-4 times higher in African American women compared to Caucasian women [27]. In addition, there are other factors which are considered to be associated with uterine leiomyomas such as early age, obesity, polycystic ovary syndrome, diabetes, hypertension, and alcohol intake [28-31].

1.1.4 Etiology and pathogenesis of uterine fibroids

The cause of uterine leiomyomas remains unknown, but some recent studies have shown that the onset of cancer is linked to hormonal factors (sex steroid), epigenetic and genetic factors, growth factors and the extracellular matrix component.
Genetic studies have shown that approximately 20-50% of uterine myomas have chromosomal rearrangements [32, 33], and molecular studies have shown that in leiomyomas about 226 genes have an altered expression, 152 genes induce apoptosis while 74 are involved in cell proliferation. Recent studies documented also the involvement of

epigenetic mechanism (DNA methylation, histone modification, and microRNAs) in uterine fibroids [34-36].

Moreover, it shows that stem-cell population is responsible for the proliferation of normal myometrium smooth muscle cells. This process accounts in part for the physiologic enlargement of the uterus during pregnancy. Mature myometrial cells express much higher levels of estrogen receptor a (ERa) and progesterone receptors (PR) than do stem cells. Thus, it is likely that estrogen – and progesterone-dependent cell proliferation is primarily mediated by the ERa and PRs that reside in mature cells. Paracrine factors, such as WNT ligands, that are released by mature cells may act on stem cells to induce their self-renewal and proliferation [37].

A genetic hit, such as a MED mutation or a chromosomal rearrangement affecting HMGA2, may transform a myometrial stem cell into a fibroid stem cell. This fibroid cell may self-renew and start dividing in an uncontrolled fashion until it differentiates into a mature fibroid smooth-muscle cell. During this process, fibroid smooth-muscle cells acquire many epigenetic and phenotypic abnormalities. ERa and PRs are concentrated primarily in mature fibroid cells and they pass on estrogenic or progestogenic signals to stem cells through paracrine mechanisms. The single, transformed fibroid stem cell eventually gives rise to a benign fibroid tumor with well-demarcated margins, which expands within the myometrial tissue.

Extensive studies have established that ovarian steroids (estrogen and progesterone) have a significant effect on the growth of leiomyomas and their actions are partly mediated by local production of growth factors. In particular, steroids may modulate the producing of different growth factors (EGF, HB-EGF, PDGF, IGF, TGF-α, TGF-β, VEGF, aFGF, bFGF, activin-A and myostatin) [38, 39], cytokines (IL-1, IL-6, IL-11, IL-13, IL-15, TNF-α, GM-CSF, and erythropoietin) as well as chemokines and their receptors (MIP-1α, MIP-1β, RANTES, Eotaxin, Eotaxin-2, IL-8, CCR1, CCR3, CCR5, CXCR1, CXCR2, MCP-1) [40, 41] which are involved in myometrial and leiomyoma biology.

These elements are essential in the control of cell proliferation rate and deposition of extracellular matrix. Infact, it has been shown that uterine leiomyomas usual type are characterized by a high component of ECM, especially collagen, fibronectin, and proteoglycans and growth factors, cytokines and chemokines, angiogenic factors and as mediators of response inflammatory. As a matter of fact, it is regarded as a fibrotic disease type [42]. Fibrosis is a mechanism that includes the recruitment inflammatory cells to the site of injury and the activation of collagen producing cell-myofibroblast.

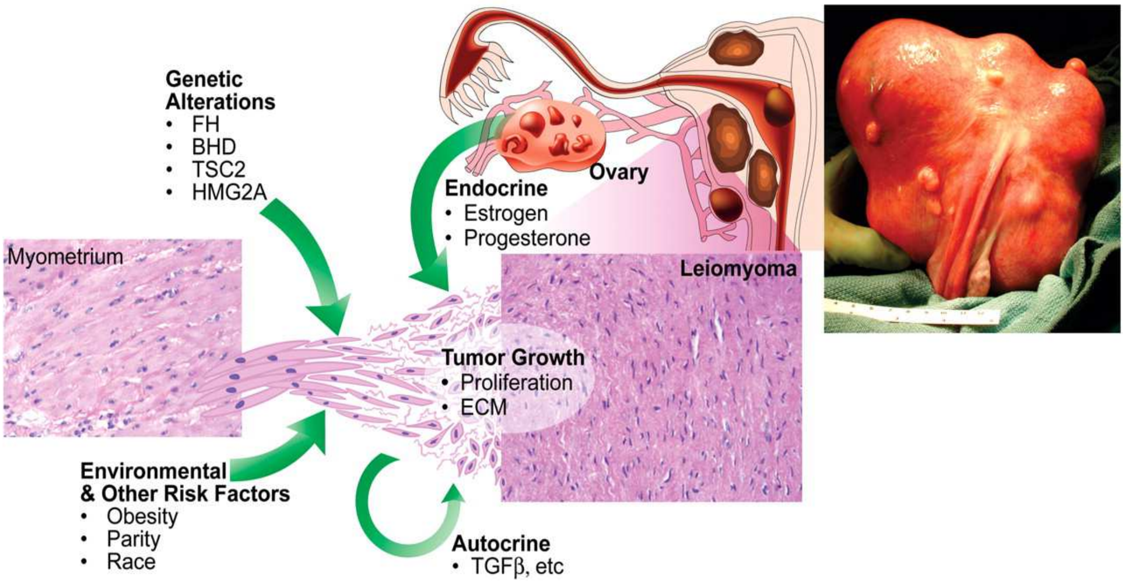


Figure 4. Etiology of uterine fibroids.

The knowledge on the pathogenesis of uterine fibroids is still limited, so it is important to gain a better understanding of the disease in order to define new therapeutic targets.

In the last decade our research group, investigated the role of growth factors such as activin-A and myostatin in uterine biology. Activin is a dimeric protein that consists of two activin β_A subunits [43].

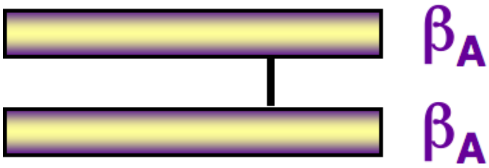


Figure 5. Subunit structure of activin A

Myostatin, or growth differentiation factor 8 (GDF-8), is a secreted protein member of the TGF-ß superfamily. Myostatin is a negative regulator of skeletal muscle growth, in addition, it is found in the circulation and it may have functions in another process, such as adipogenesis, skeletal muscle fibrosis, and myometrial cell proliferation.

First, we showed that activin-A and myostatin have a cytostatic effect on myometrial cell proliferation using a cell line model [pregnant human myometrial 1 (PHM1)] [44, 45]. In addition, we investigated the expression levels of activin-A and myostatin in human

leiomyoma and adjacent healthy myometrial tissue and we found increased expression levels of both activin-A and myostatin in leiomyoma [46].
Leiomyoma cells are resistant to the cytostatic effect of activin-A and myostatin. Later, we demonstrated that the ability of activin-A to increase fibronectin, collagen1A1, and versican expression in leiomyoma cells demonstrate also its potential profibrotic role in leiomyoma growth [42, 47].

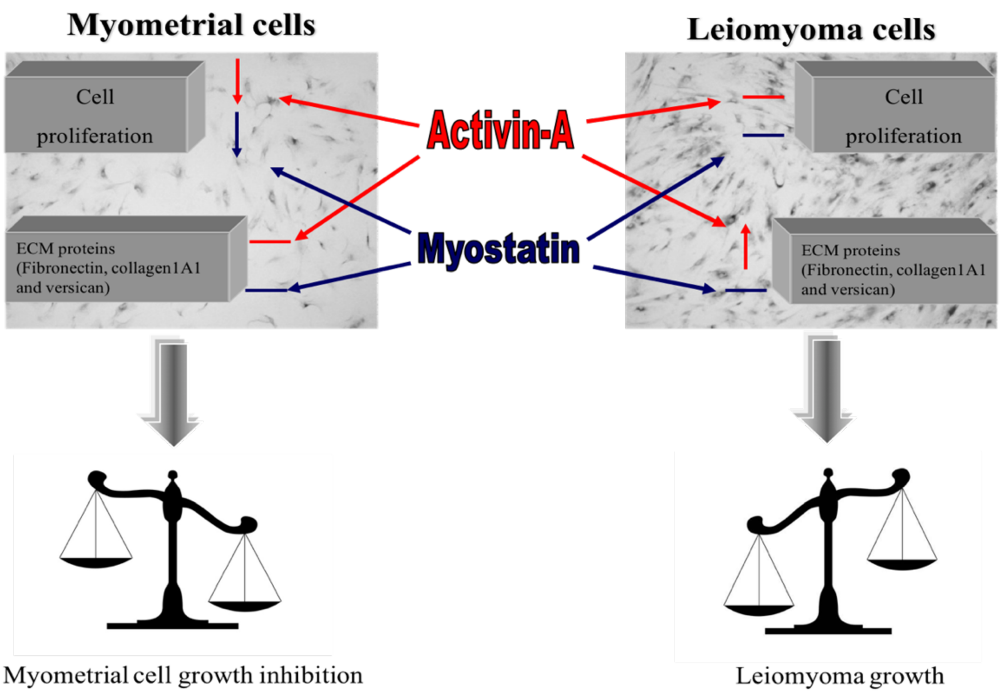


Figure 6. Effect of activin-A and myostatin on leiomyoma and myometrial cells

Our research group, then focused on inflammatory processes involving the activin-A. Our results showed the presence of a large number of inflammatory cells inside the leiomyomas and in the surrounding tissue, when compared to autologous myometrium far from the leiomyoma. In particular, there was a large increase in macrophages and in myofibroblast recruitment and activation. Furthermore, it was seen that activin-A significantly increase the mRNA expression of the ECM components in leiomyoma cells and significantly increase fibronectin mRNA expression in primary myometrial cells. Based on these results, it is stated that activin-A has a pro-fibrotic role in uterine leiomyoma. In fact, treating the cells with TNF-α, both primary and immortalized cells, there is an increased mRNA expression of activin-A. These data support the presence of inflammation within the uterine fibroid [48].

Another important protein associated with an increasing number of disease through its involvement with signal transduction pathways is Raf Kinase Protein Inhibitor (RKIP). We demonstrated that RKIP is expressed in human myometrial and leiomyoma tissue. We performed in vitro experiments with the chemical compound locostatin, known to bind and block RKIP. We showed that locostatin treatment results in the activation of the MAPK signal pathway and the RKIP inhibition by locostatin reduce ECM components, moreover, the inhibition of RKIP impairs cell proliferation and migration in leiomyoma and myometrial cells [49].

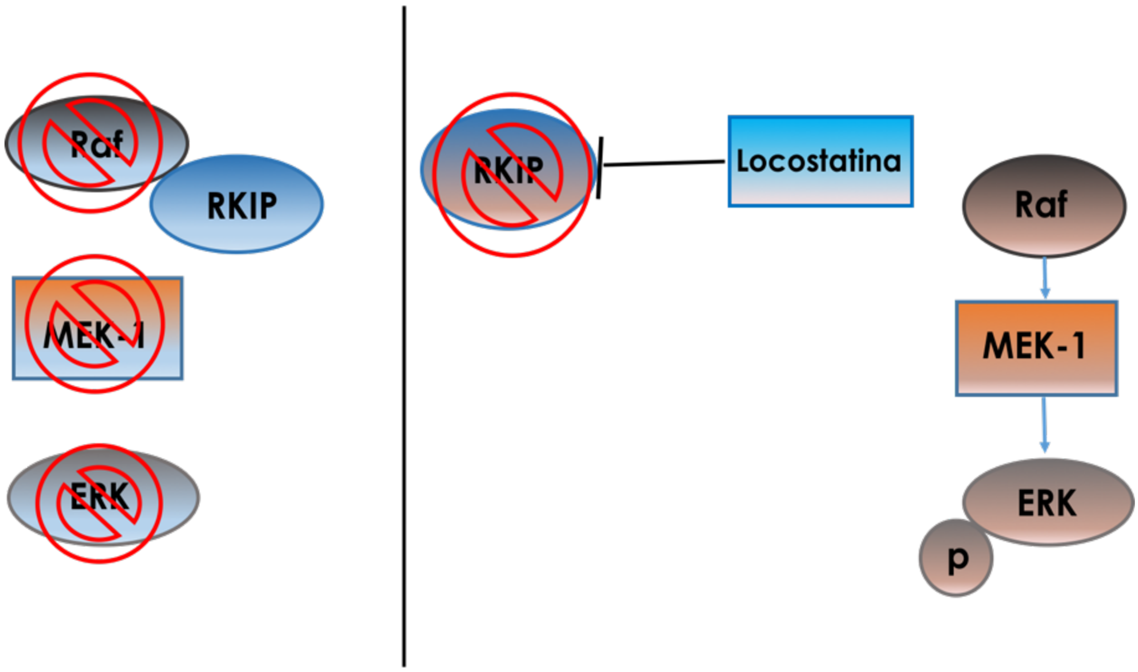


Figure 7. Effect of RKIP inhibition by locostatin treatment

Other important study, which was performed by our group, is the effects of omega-3 fatty acids on the lipid profile, membrane architecture and specific gene expression in myometrial and leiomyoma cells.

Primary myometrial and leiomyoma cells were treated with omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (50 µM) for 48 hours. We obtained that EPA and DHA reduced the monounsaturated fatty acid content and they lead to an increase of polyunsaturated fatty acids in both, myometrial and leiomyoma cells *in vitro*. Afterward, we used Gas-chromatography with flame ionization detector (GC-FID) to measure fatty acids content and, by applying the method of Laurdan fluorescence, we assessed membrane fluidity or rigidity. Untreated myometrial and leiomyoma cell membranes were in the liquid-crystalline phase. When treated with EPA and DHA, both cell types had higher Laurdan excitation generalized polarization values indicating an increased rigidity and by Real-time PCR we showed the decrease of the gene expression of extracellular matrix components (Collagen1A1, fibronectin, versican), pro-fibrotic factor (activin-A), mechanical signaling (integrin β1, FAK, and AKAP13) and sterol regulatory expression of EMC components, EPA and DHA lead to reduced levels of ABCG1, ABCA1 and AKAP13 in both cell types and also decreased FAK and CYP11A1 production in myometrial cells. Our study shows that omega-3 fatty acids are able to modify the fatty acids profile, restructuring the cell membrane architecture and downregulating the expression of genes required for mechanical signaling and cellular lipid accumulation in myometrial and leiomyoma cells [50].

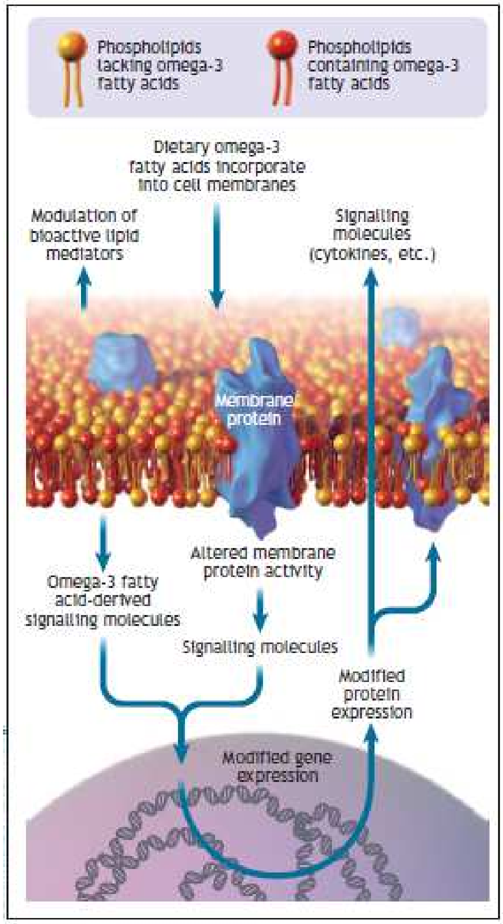


Figure 8. The image show that Omega-3 fatty acids can modify gene and protein expression, modulate membrane protein activity and act as a reservoir for bioactive molecules.

1.2 Dietary Phytochemicals

Dietary phytochemicals can be found in plants, fruits, vegetables, grain, and tea. In modern pharmaceutical industries, dietary phytochemicals are used as a source of new potential drugs for many kinds of tumors, and they are known for their antiproliferative effects [51, 52].

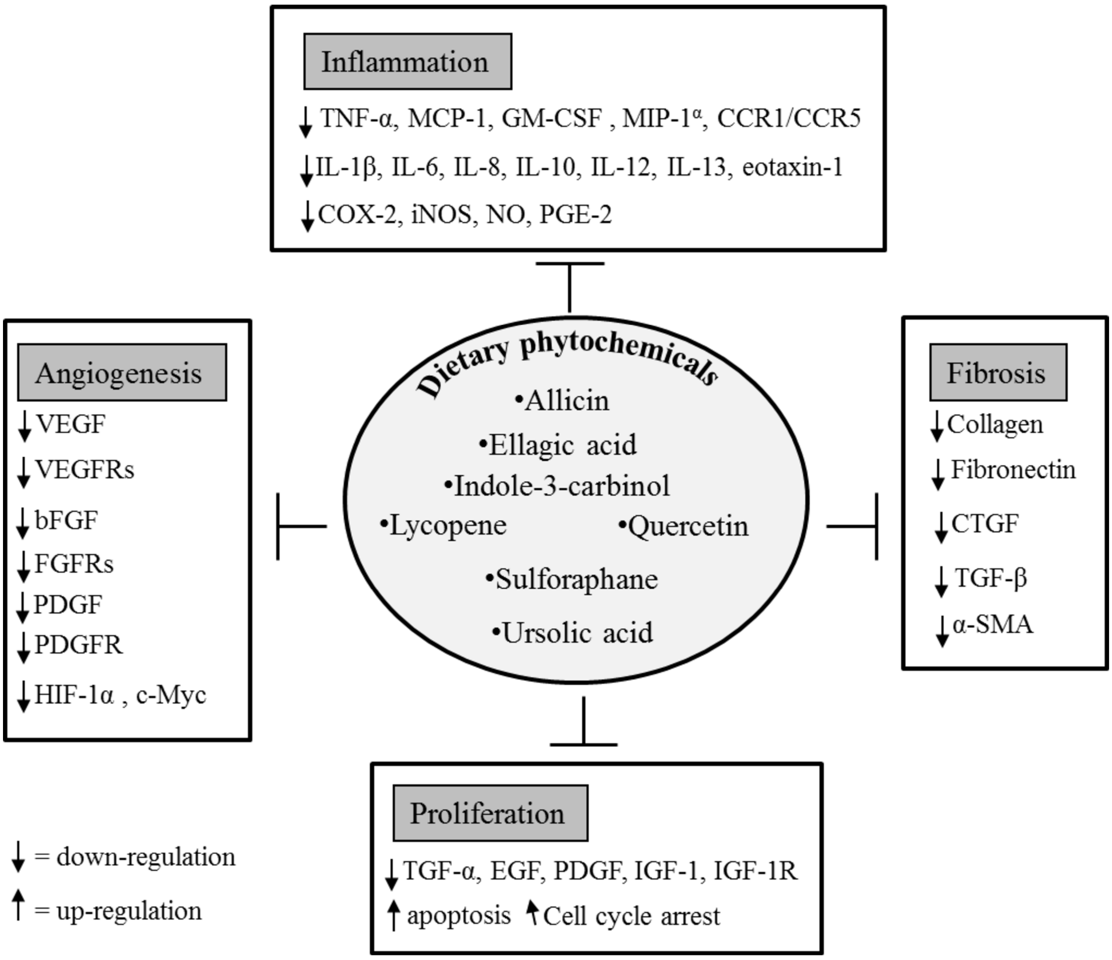


Figure 9. Effects of dietary phytochemicals

1.2.1 Strawberries

Strawberries are fruits common in the Mediterranean diet, they are rich of essential bioactive molecules like anthocyanins, flavonols, flavanols, tannins such as proanthocyanidins, ellagitannins, and gallotannins, tannins [53].

Recent studies show the capability of these fruits, their anti-inflammatory effects, the suppression of mutagenesis, the induction of apoptosis, and the inhibition of proliferation of several types of cancer cells [54].

Figure 10. Example of phytochemical compounds

In this thesis, we aimed to study the *in vitro* effects of the different variety of strawberries on leiomyoma and myometrial cells. The selection of variety depends for higher sensorial and nutritional quality [55]. We used 2 different variety of strawberry, *Alba* and *Romina* and the *anthocyanin enriched fraction* of Romina.

The strawberry of cultivar Alba has been selected since it is a very interesting variety from the nutritional point of view, with a well-balanced micronutrient and phytochemical composition. In addition, this cultivar has a considerable healthy effect, thanks to its phenolic contents and its ability to maintain the cell membrane integrity, to reduce the free radical-dependent lipid peroxidation and to preserve and/or activate endogenous antioxidant enzymes [36, 37].

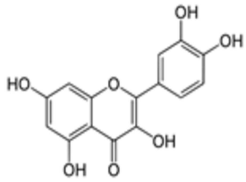
The strawberry cultivar Romina was released in 2011 by Università Politecnica delle Marche and it is particularly characterized by an high adaptability to climatic conditions from the mid-Adriatic to the Center-North of Europe, resistance to the major strawberry diseases, very early ripening, good taste with high sweetness and high firmness and shelf life [56]. The high sweetness of Romina fruit is well appreciated by the consumers and it is recognized by its high content of soluble solids (SS) (7.7° Brix) combined with low total acidity (10.1 mEq NaOH/100 g) [56]. The high nutritional quality and healthy benefits of Romina fruit

are also recognized by its good levels of vitamin C, folic acid and flavonols, as well as high content of anthocyanin and antioxidant capacity [56].
The strawberry cultivar Romina is a new promising cultivar in terms of nutritional composition, phytochemical content and biological properties.
These cultivars (Alba and Romina) were cultivated by the "Pasquale Rosati" Research and Service Center for Didactic-Experimental Agriculture of the Università Politecnica delle Marche. Furthermore, the extraction and preparation of the anthocyanin enriched fraction of Romina has been provided by the research group of agrarians (Prof. Mezzetti) and biochemistry (Prof. Battino) of the Università Politecnica delle Marche.

1.2.2 Quercetin and Indole-3-Carbinol (I3C)

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a plant bioflavonoid, found in most edible fruits and vegetables. It is present in tea, lemon, tomato, onion leaves, and strawberries [57]. It has anti-fibrotic properties in hepatic fibrosis, pulmonary fibrosis, kidney fibroblasts and it was shown to improve liver histology and reduce collagen content in rats with carbon tetrachloride-induced cirrhosis *in vivo* [58].
Quercetin was reported to reduce both the risk and the progression of cancer through its free radical scavenging activity [59]. It protects cells from oxidative stress, inflammation, and DNA damage due to its antioxidant properties and it modulates the growth of many cancer cell lines through the blocking cell cycle progression and tumor cell proliferation and the induction of apoptosis. Epidemiological studies report that the intake of quercetin rich food reduces the risk of gastric cancer by 43% and colon cancer by 32%. The consumption of quercetin was also reported to reduce lung cancer risk by 51% and even in heavy smokers by 65% [59, 60].

Quercetin



Red onions

Red onions, tea, tomato and onion leaves, lemon and strawberries

Figure 11. Quercetin: molecular structure

Indole-3-Carbinol is found in cruciferous vegetables such as broccoli, cabbage, cauliflower, brussels sprouts, bok choy, collard greens, mustard greens, kale, Chinese cabbage, radishes, turnips, kohlrabi, arugula, watercress, and daikon [61]. I3C inhibits the proliferation of hepatic stellate cells (with or without PDGF-BB stimulation) [62], it suppresses the angiogenesis by inhibiting tube formation and VEGF secretion in Human Endothelial cells ECs (HUVECs Human umbilical vein endothelial cells) [63] and, at least in part, via inactivation of ERK1/2 in human umbilical vein ECs [64]. The antiangiogenic activity of I3C in ECs stimulated with activated macrophages has also been reported [52, 65].

The Indole-3-Carbinol (I3C) is a biologically active dimer diindolylmethane (DIM) [66], are promising agents for the prevention of estrogen-enhanced cancers. A combination of epidemiological and experimental data provides suggestive evidence that a high intake of cruciferous vegetables protects against some cancers at different stages. In a nationwide study of postmenopausal women in Sweden, consumption of cruciferous vegetables was inversely related to breast cancer risk. Although cruciferous vegetables have a number of cancer-preventing compounds, I3C alone shows efficacy for the prevention of breast, endometrial and cervical cancers in animal models. Importantly, I3C shows efficacy for treatment of precancerous lesions of the cervix in translational human studies [67].

Figure 12. Indole-3-Carbinol: molecular structure

2 Materials and method

2.1 Sample collection and primary cell culture

The study includes samples of myometrial and usual type leiomyoma tissue excised from women undergoing hysterectomy for fibroids used to established primary cell culture. Considering the high variability, for example, the different age, race, hormonal milieu, tumor size, and location of tumors, we including in this study the most homogeneous sample.
Infact, all patients were Caucasian and in proliferative phase of the menstrual cycle.
The location of leiomyomas was intramural, and their size was 7-10 cm in diameter. We considered all patients who has not received exogenous hormones for the previous 3 months. All patients gave their informed consent and the permission of the Human Investigation Committee was granted.
After surgery, the myometrial and leiomyoma sample were collected in Hank's Balanced Salt Solution (HBSS, Euroclone, Milan, Italy), and immediately transported to the laboratory.
The samples were washed several times with Dulbecco's PBS (Invitrogen, ThermoFischer, Carlsbad, CA, USA) to remove excess blood. After cutting tissue into small pieces, sample were mixed in 0.1% collagenase type 8 (Serva Electrophoresis GmbH, Heidelburg, Germany) in serum free Dulbecco Modified Eagle Medium (DMEM) (Lonza, Walkersville, MD, USA), and incubated at 37°C for 3-5 h in water bath with manual shaking.
After having digested the cells suspension it was centrifuged at 1200 rpm for 10 minutes, and the collagenase was inactivated with fetal bovine serum (FBS) (Gibco, ThermoFischer). Finally, the cell pellet was dispersed in DMEM containing 10% fetal bovine serum (FBS; Gibco, ThermoFischer), 1% antibiotic (penicillin-streptomycin; Euroclone), 1% fungizone (Amphotericin B; Lonza), and 1% glutamine (Gibco, ThermoFischer) in plastic dishes at 37°C in 95% air, 5% CO2. The growth medium was changed after 24 or 48 hours to remove unattached cells and subsequently twice a week. The purity of cells was assessed by staining with a specific smooth muscle cells marker (anti-a-smooth muscle actin) (Sigma-Aldrich, Milan, Italy). All cells were strongly positive for alfa-sma.
The lower passage number (P0-P4) of cells was used for experiments to avoid changes in phenotype and gene expression. Leiomyoma and myometrial cells were treated with 2 different cultivars (Alba and Romina), with 2 different concentrations for both (100 µg/ml e 250 µg/ml) and with 250 µg/ml anthocyanin enriched fraction of Romina (ACY), Quercetin and Indole-3-Carbinol I3C (10 µg/ml; 50 µg/ml; 100 µg/ml; 250 µg/ml) (Sigma-Aldrich) for

48 h. After treatment, the cells were detached from the petri dish by the TRIzol® reagent (Invitrogen, ThermoFisher). This reagent is known for the production of 3 phases. The upper white phase is used for the extraction of the RNA, a white disk containing DNA, and finally there is a lower pink phase that will be used for the extraction of proteins.

2.2 Cell lines culture

The myometrial and leiomyoma cell lines were provided by William H. Catherino, M.D., Ph.D. (Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Bethesda, Maryland). The primary myometrial and leiomyoma cells were immortalized following the modified protocol of Rhim [68] using human papillomavirus type 16 as previously described by Malik et al. [69].

Cells were cultured in fresh DMEM-F12 supplemented with 10% fetal bovine serum, 1% antibiotic (penicillin–streptomycin; EuroClone), 1% fungizone (amphotericin B;Euroclone), and 1% glutamine (Gibco, Life Technologies) at 37°C in 95% air 5% CO2.

2.3 RNA Extraction and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted using the white phase from TRIzol® reagent (Invitrogen, ThermoFisher), according to the manufacturer's instructions. Samples were digested with a ribonuclease-free deoxyribonuclease (Promega Corp., Madison, WI, USA), and the RNA was cleaned up and concentrated using ReliaPrep™ RNA Cell Miniprep System (Promega Corp.). We performed the reverse transcriptase (RT) using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, ThermoFischer, Foster, CA, USA) with 1μg RNA, and we performed the TaqMan real-time PCR for all the genes analyzed.

We used the TaqMan gene expression assays (Applied Biosystems, ThermoFischer): Collagen 1A1 (Hs00164004_m1), Fibronectin (Hs00365052_m1), Versican (Hs00171642_m1), Activin A (Hs00170103_m1) and the housekeeping genes, hypoxanthine phosphoribosyltransferase 1 (HPRT1), (Hs99999909_m1) and β-actin (ACTB) (Hs99999903_m1), performing the following thermal cycle protocol (initial denaturation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds) using 100 ng cDNA in a final reaction volume of 10 μL.

The blank for each reaction, consisting of amplifications performed in the absences of reverse transcriptase enzyme, was performed.

2.4 Western Blotting

Proteins were extracted using the pink phase from TRIzol® reagent (Invitrogen, ThermoFisher), following the manufacturer's instructions. Soluble protein was quantified using a Bradford protein assay (Bio-Rad, Milan, Italy), and equal amounts of proteins were loaded to 4-12% NuPAGE gels (Invitrogen, ThermoFischer) and resolved by SDS-PAGE under reducing conditions. Proteins were transferred to 0.2 µm nitrocellulose membranes in an X-cell II apparatus (Invitrogen, ThermoFischer) according to the manufacturer's instructions. Ponceau S solution (Euroclone) was used for the detection of proteins on nitrocellulose membranes. After blocking the membrane with 5% (wt/vol) nonfat milk powder in Tris-buffered saline with Tween 20 (TBST; 50 mm Tris-HCl, pH 7.4, 150 mm NaCl, 0.05% Tween 20), the membrane was incubated overnight with a primary antibody 1:30000 dilution for monoclonal mouse Anti-human fibronectin (Sigma-Aldrich) and 1:3000 dilution for monoclonal mouse tubulin (Sigma-Aldrich). On the next day, a membrane was washed 4 times in 5% (wt/vol) nonfat milk powder in TBST and incubated with 1:10000 horseradish peroxidase linked antimouse (Amershan) against Anti-human fibronectin and tubulin for 2 hours. The membrane was washed 4 times in 5% (wt/vol) nonfat milk powder in TBST, and immunoreactive proteins were visualized using Clarity ™ western ECL substrate (Bio-Rad). Protein levels were measured using Image J 1.49n software (National Institutes of Health, http://imagej.nih.gov/ij).

2.5 Immunocytochemistry

Myometrial and leiomyoma cells were seeded in chamber tissue culture slides and allowed to divide. Cells were washed 3 times with PBS, treated with 0.2% Triton X-100 in PBS for 5 minutes, and washed 3 times with PBS. To inhibit endogenous peroxidase activity, cells were incubated for 10 minutes with 3% hydrogen peroxide in deionized water. Cells were washed 3 times with PBS, and to block nonspecific background, cells were incubated for 20 minutes at RT with NHS (normal horse serum) diluted 1:75 in 1% bovine serum albumin in PBS. Cells were then incubated with fibronectin (Sigma-Aldrich) monoclonal mouse antibody at 1:600 and monoclonal mouse anti-alfa-smooth muscle actin (alfa-sma) at 1:000 (Dako, Agilent Tecnologies, Santa Clara, California, Stati Uniti) for 1 hour at RT. After washing with PBS, cells were incubated with biotinylated anti-mouse IgG made in horse diluted 1:200 against fibronectin and alfa-sma. The peroxidase ABC method was performed for 1 hour at RT using DAB as chromogen. Sections were counterstained in Mayer's hematoxylin, dehydrated, and mounted with Eukitt solution.

2.6 Wound closure assay

Leiomyomas and myometrial cells were grown to confluence and then scratch wounded with a sterile plastic micropipette tip. Cells were rinsed three times with warm media to wash away scraped off cells in the wound and then kept in supplemented media in the absence or presence of quercetin and indole-3-carbinol in different concentrations: 10 µg/ml; 50 µg/ml; 100 µg/ml; 250 µg/ml for 48h. Digital images were taken of the initial wound and at 12 h, 24h and 48h [70-72]. The area (mm^2) of the wound not occupied by cells was measured using a morphological imaging system Image J 1.49n software (National Institutes of Health, http://imagej.nih.gov/ij). Closure percentage was calculated as:

$$\left[1 - \frac{area\ of\ remmaing\ wound}{area\ of\ initial\ wound}\right] \times 100$$

2.7 Cell proliferation assay

Cellular growth curves were measured using the CyQuant cell proliferation assay kit according to the manufacturer’s instructions (Invitrogen, Life Technologies). Myometrial and leiomyoma cells were seeded in 96-well plates either at a various initial density of 150 cells/well; 100 cells/well; 50 cells/well in a total volume of 300 µl DMEM supplemented with 10% FBS. Cells were treated with 250 µg of quercetin (Q-250) and 250 µg indole-3-carbinol (I-250), on left untreated (NTD, no treatment with DMSO Dimethyl sulfoxide) (Invitrogen, Life Technologies) and allowed to divide for the number of days indicated (days 0, 1, 2 and 4). At the indicated times, media were discarded, and plates were frozen at 80°C. To assay, cells were lysed, and total cellular nucleic acid was measured using fluorescence at 520 nm emission after excitation at 480 nm [44, 45, 47].

2.8 Data analysis

Statistical analyses were performed using GraphPad Prism version 6.01 for Windows (GraphPad, San Diego, CA). The data were analyzed using non-parametric ‘Kruskal-Wallis’ ANOVA, followed by post hoc ‘Dunn’ test for multiple comparisons. Results are expressed as mean ±SD. Differences were considered significant when $p < 0.05$.

3 Results and discussion

3.1 The effects of strawberries on leiomyoma cells

In the first study, we analyzed the *in vitro* effect of Alba cultivar, focusing on the antifibrotic effect. Treating the primary leiomyoma and myometrial cells with different concentrations (100 μg/ml -250 μg/ml) of Alba cultivar, we studied the effect on extracellular matrix genes expressions and proteins expressions. We found that this cultivar significantly reduces the expression of collagen1A1, Fibronectin, and Versican (Fig.13) and it decreases the protein expression of Fibronectin in leiomyoma primary cells (Fig.14).

Then, we investigated whether the antifibrotic effect of a strawberry extract is mediated by regulation of activin-A expression and if it can modulate the expression of PAI-1, an important activin-A target. We found that strawberries extracts produce a significant downregulation of the expression of activin-A mRNA in leiomyoma cells (Fig.15). In addition, activin-A induces mRNA expression of PAI-1 in human uterine primary leiomyoma cells, which was inhibited by strawberry extracts (Fig.16). PAI-1 is induced by activin-A [20] and it is been implied in the pathology of fibrosis in different organs including, heart, lung, kidney, liver, and skin [19]. The role of PAI-1 in leiomyoma was demonstrated by the observation that TGF-β3 induce protein expression of PAI-1 in human uterine leiomyoma cells which was inhibited by an antifibrotic compound, 1,25-dihydroxyvitamin D3 [35]. The downregulation of activin-A and PAI-1 expression by strawberry extracts suggests that the antifibrotic effect is mediated, by regulation of activin-A and PAI-1 expression. The uterine leiomyoma is characterized by the accumulation of the excessive amount of extracellular matrix (ECM) components which includes collagens, fibronectin, and versican [27–29]. Collagen is an important component known to maintain cellular morphology, moreover, it regulates cellular proliferation, migration, differentiation, and survival as well as wound healing and fibrotic processes [73]. Leiomyoma cells demonstrate an overexpression of collagen types I mRNA compared to the adjacent myometrium cells. Fibronectin is a glycoprotein of the ECM, which regulates the cell adhesion, migration, growth, and differentiation as well as fibrosis. Leiomyoma cells express elevated levels of fibronectin compared to myometrial cells [74]. Versican, a large chondroitin sulfate proteoglycan, plays important roles in cell migration, cell adhesion, cell proliferation, tissue stabilization, tissue homeostasis and inflammation [75].

Activin-A, is a pleiotropic growth factor, belonging to the TGF-β superfamily. It was reported to increase ECM components, including collagen1A1, fibronectin, and versican mRNA expression in leiomyoma cells [76]. The role of ECM proteins, in producing leiomyoma bulk structure and growth, provides an important way to control this tumor. Therefore, the development of anti-fibrotic agents could be a good solution for uterine fibroids.

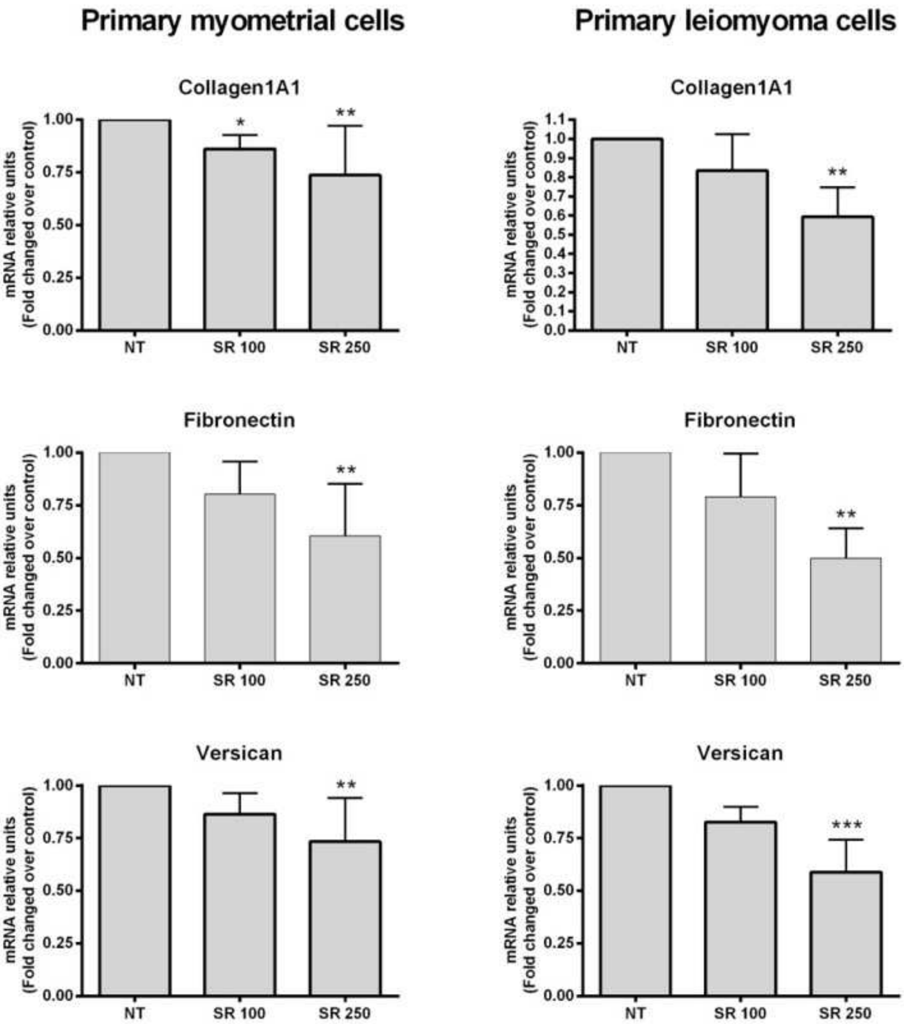


Figure 13. Effect of strawberry extract on extracellular matrix components mRNA in myometrial and leiomyoma cells. Data are expressed as mean ± SD (n=6). NT, no treatment; SR 100, strawberry extract at 100 µg/ml; SR 250, strawberry extract at 250 µg/ml. *P<0.05; **P<0.01; ***P<0.001.

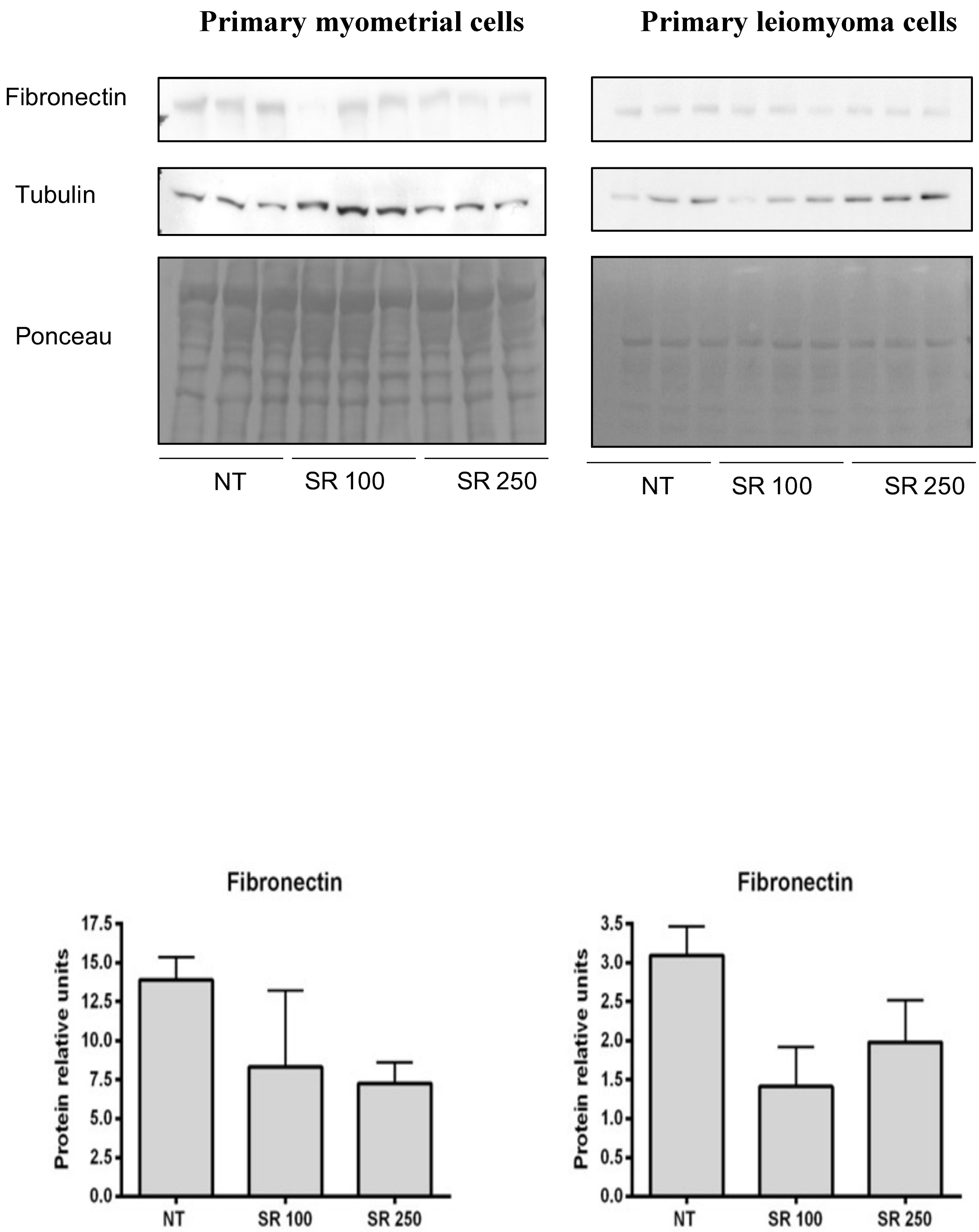


Figure 14. Effect of strawberry extract on extracellular matrix protein expression in myometrial and leiomyoma cells. Data are expressed as mean ± SD (n=6). NT, no treatment; SR 100, strawberry extract at 100 µg/ml; SR 250, strawberry at 250 µg/ml.

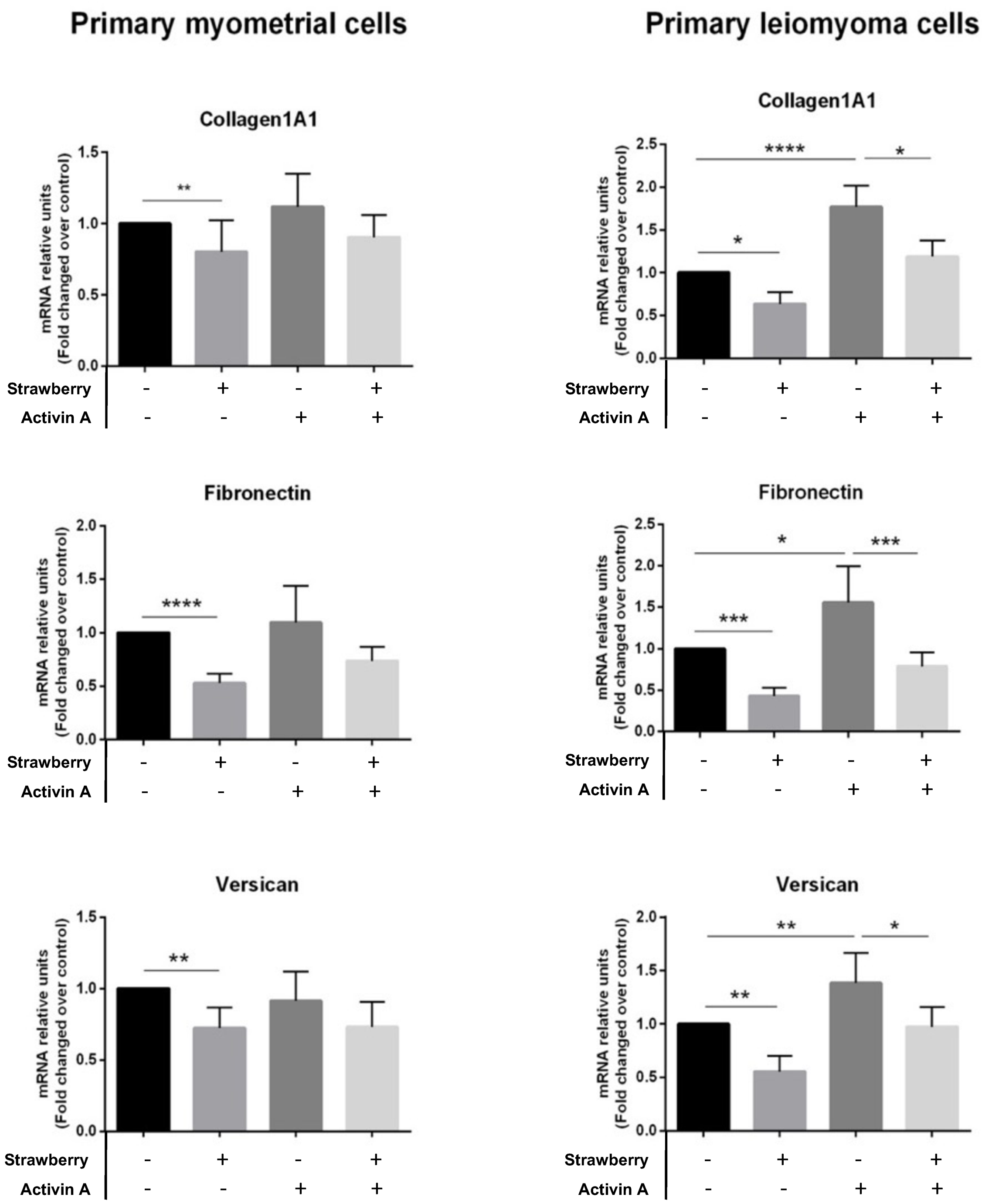


Figure 15. Effect of strawberry extract (250 µg/ml) on activin-A induced ECM components mRNA expression in myometrial and leiomyoma cells. Data are expressed as mean ± SD (n=9). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

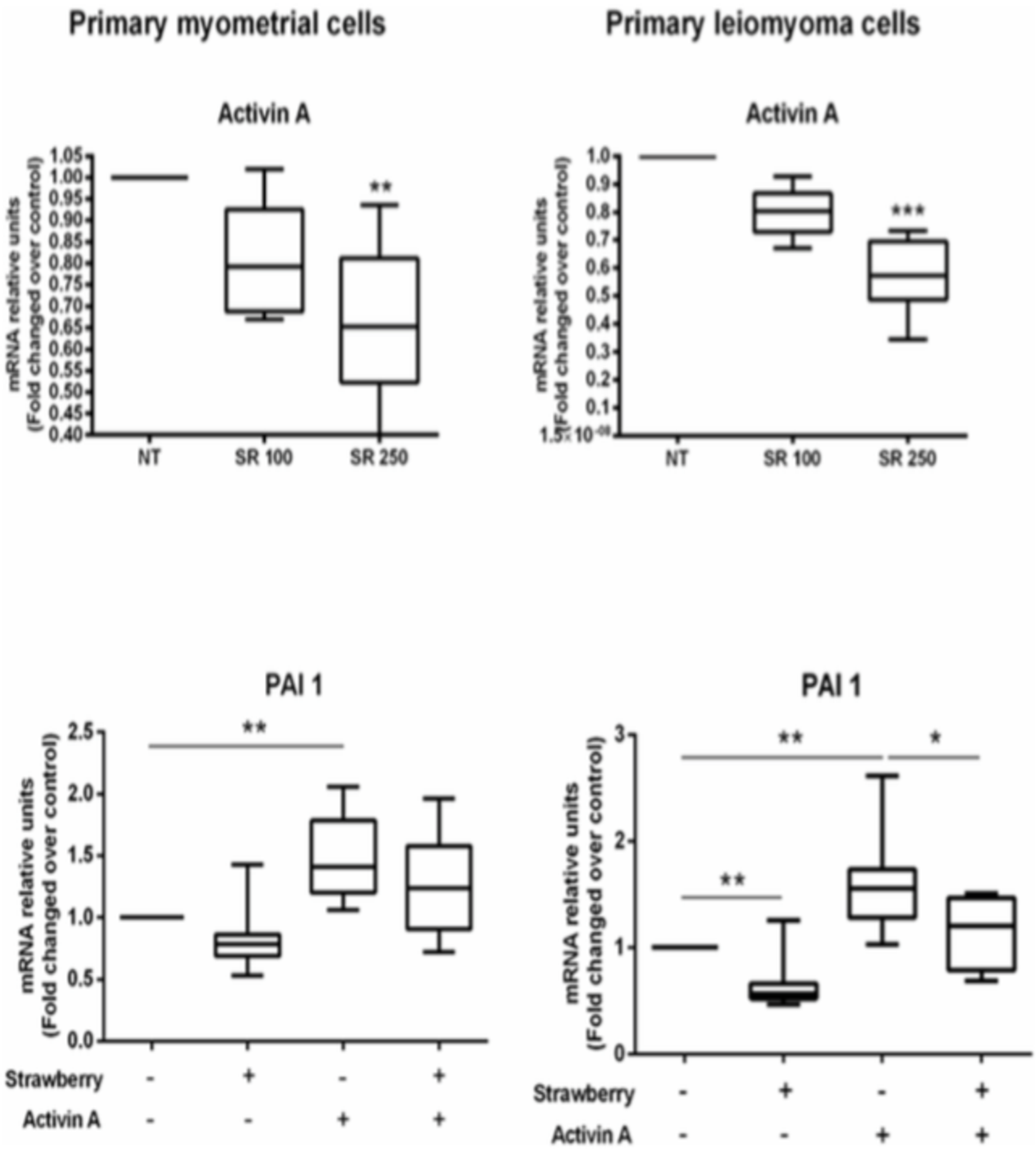


Figure 16. Effect of strawberry extract on activin-A (n=6) and activin-A induced PAI-1 (n=9) mRNA expression in myometrial and leiomyoma cells. Data are expressed as mean ± SD. NT, no treatment; SR 100, strawberry extract at 100 µg/ml; SR 250, strawberry extract at 250 µg/ml; activin-A. *P<0.05; **P<0.01; ***P<0.001.

Gaining positive data through Alba cultivar, we planned to test *in vitro* another cultivar of strawberry extracts on leiomyoma. Since the cultivar Romina has recently been patented by the Università Politecnica delle Marche, we wanted to test if its effectiveness on leiomyoma cells, and if its more efficient than cultivar Alba. Therefore, our study focused on comparing the Alba cultivar with the Romina cultivar and in addition we used also an anthocyanin enriched fraction of Romina (indicated with ACY).

We treated the primary leiomyoma and myometrial cells with different extracts and concentrations of strawberries: Alba cultivar (100 µg/ml -250 µg/ml); Romina cultivar (100 µg/ml -250 µg/ml); anthocyanin enriched fraction of Romina or ACY (250 µg/ml).

We found that the Romina strawberry extract and its anthocyanin enriched fraction are able to inhibit ECM components gene expression, including collagen 1A1, fibronectin, and versican expression in leiomyoma cells (Fig.17); we also found that it reduces the protein expression of fibronectin in leiomyoma cells (Figs.18; 19). This result suggests that Romina strawberry extract or its anthocyanin fraction has a greater effect on the leiomyoma cells *in vitro* than the Alba cultivar, and that it could be developed as potential antifibrotic agents for treatment and/or prevention of uterine leiomyomas. As regard Romina fruit, its therapeutic effect against leiomyoma cells may be attributed to its high levels of anthocyanin and flavonol content. The health benefits of strawberry consumption are justified by a plethora of studies [55-57]. Strawberry has therapeutic effects against multiple diseases, including inflammatory disorders, obesity, cardiovascular diseases, neurodegenerative diseases, and tumors through the combined effects of their bioactive compounds, such as anthocyanins, flavonols, flavanols, phenolic acids and ellagitannins [33, 55, 57].

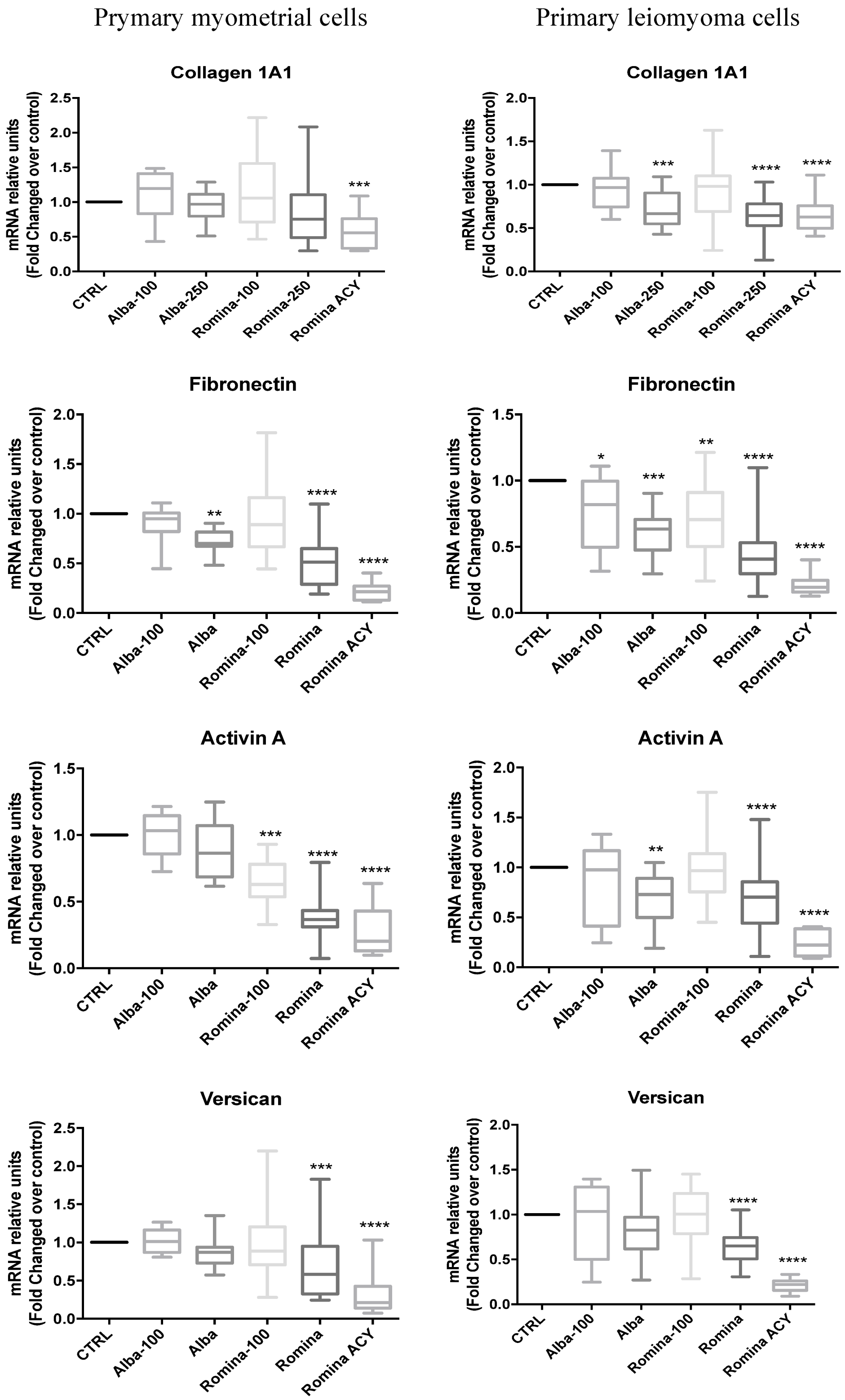


Figure 17. Results of real time PCR for relative amounts of collagen1A1, fibronectin, activin-A and Versican in myometrial and leiomyoma cells in response to strawberry Alba cultivar extract at 100 µg/ml (Alba 100); strawberry Alba cultivar extract at 250 µg/ml (Alba); strawberry Romina extract at 100 µg/ml (Romina 100); strawberry Romina extract at 250 µg/ml (Romina); Romina anthocyanin enriched fraction at 250 µg/ml (Romina ACY). Data are expressed as mean ± SD (n=6 for RNA) *P<0.05; **P<0.01; ***P<0.001.

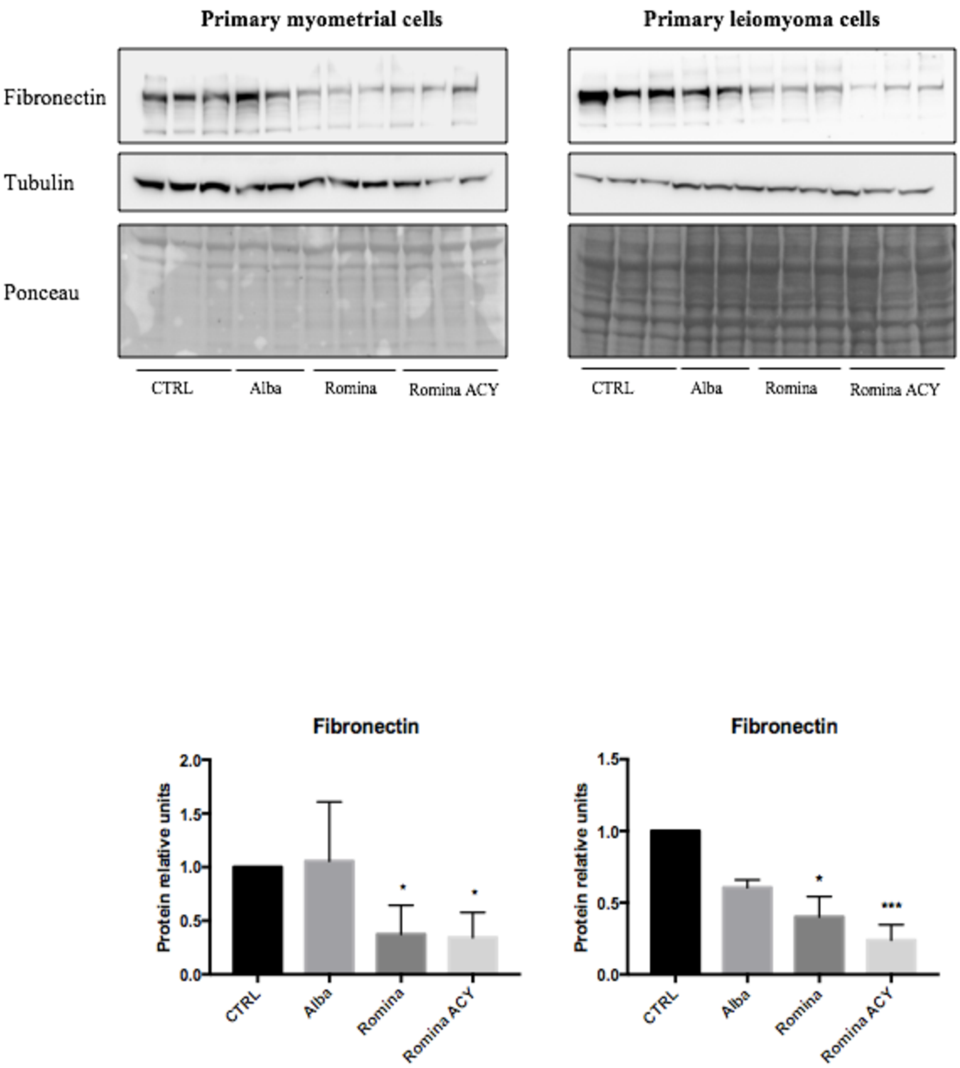


Figure 18. Effect of strawberry extract on fibronectin expression protein in myometrial and leiomyoma cells, in response to strawberry Alba cultivar extract at 250 µg/ml (Alba); strawberry Romina extract at 250 µg/ml (Romina); Romina anthocyanin enriched fraction at 250 µg/ml (Romina ACY) on left untreated. Data are expressed as mean ± SD (n=3 for protein) *P<0.05; **P<0.01; ***P<0.001.

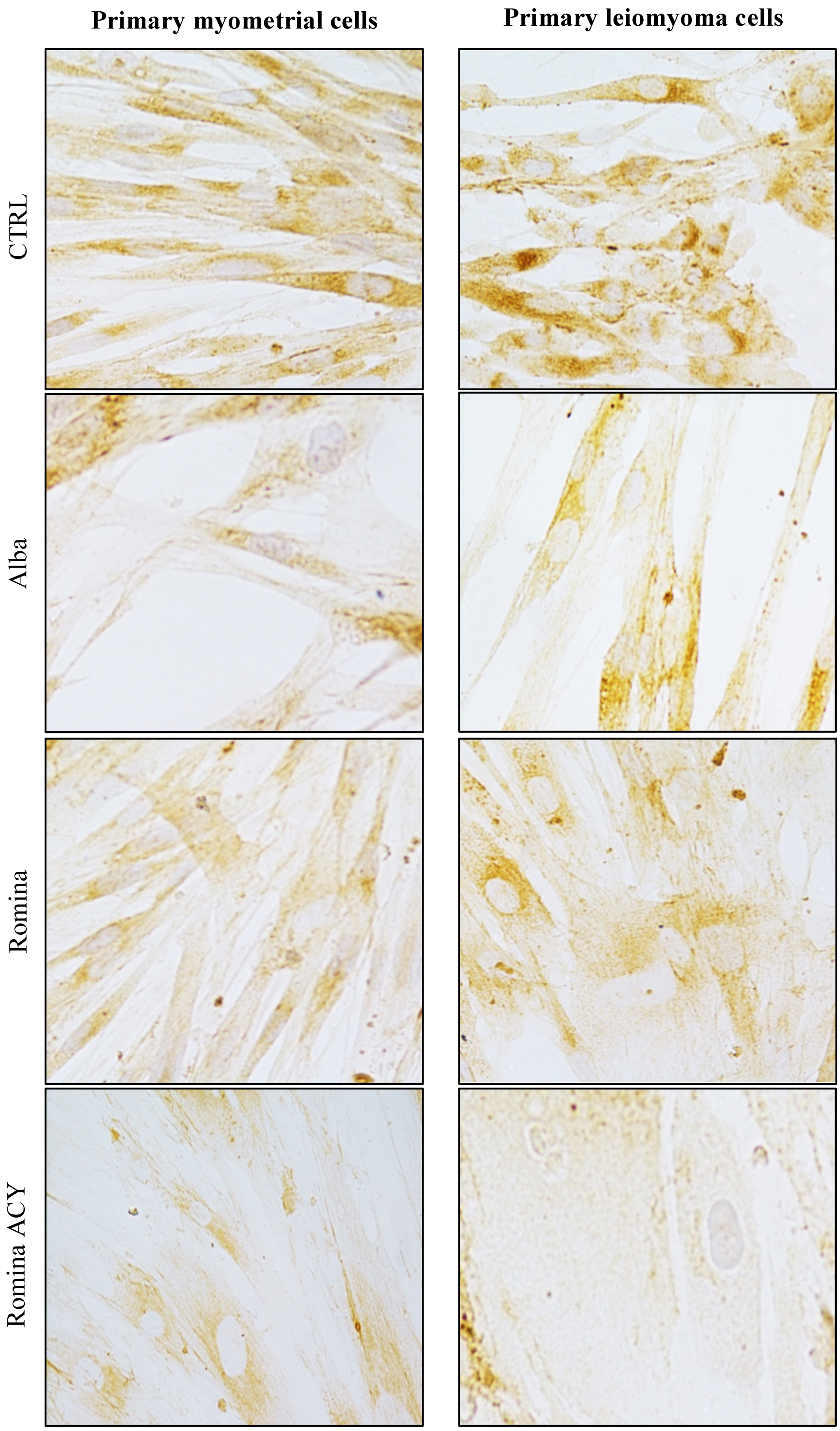


Figure 19. Immunocytochemistry shows the effect of strawberry extract on Fibronectin expression in myometrial and leiomyoma cells in response to strawberry Alba cultivar extract at 250 µg/ml (Alba); strawberry Romina extract at 250 µg/ml (Romina); Romina anthocyanin enriched fraction at 250 µg/ml (Romina ACY) on left untreated.

3.2 The effects of Quercetin and Indole-3-Carbinol on leiomyoma cells

In our second work we tested other phytochemical compounds on leiomyoma cells *in vitro*. We treated leiomyoma and myometrial cells with different concentrations of both extracts, 250 μg/ml of quercetin and indole-3-carbinol (I3C) compared with untreated control with DMSO. We found that quercetin and I3C are able to reduce ECM collagen1A1 and fibronectin, although there is no significant effect on versican and activin-A mRNAs expression in primary leiomyoma cells (Fig.20), as well as on the protein expression of fibronectin (Figs.21; 22). This is a crucial aspect due to the ECM key role in the growth of this tumor. We also obtained from wound healing and proliferation assay on leiomyoma and myometrium cells line and primary cells, after treatment with quercetin and indole-3-carbinol (250 μg/ml), a significant reduction of the migration (Figs.23, 24; Figs.25, 26) and proliferation (Figs.27; 28) in leiomyoma and myometrial cell lines and primary cells compared with untreated control. There are various natural compounds that have been analyzed in regards to their therapeutic potential in modulating and preventing myometrial tissue turning into leiomyomas. Among them, we can find epigallocatechin gallate, the ester of epigallocatechin and gallic acid, which can be found in green tea, which has been shown to induce apoptosis and inhibit the proliferation of leiomyoma cells and a phase II clinical trial of this compound has been completed [77]. Isoliquiritigenin, which is a chalcone flavonoid found in licorice, onions and bean sprouts, can also prevent growth while it upregulates cell death in leiomyoma cells [78]. Another natural compound, curcumin (diferuloylmethane, a polyphenol originating from the plant *Curcuma longa*), also known as turmeric, has shown to have significant anti-inflammatory, anti-oxidant and anti-carcinogenic characteristics. Turmeric inhibits mitosis by targeting the apoptotic pathway and leads to a decreased production of fibronectin [79]. In this article, we studied *in vitro* effect of quercetin and indole-3-carbinol on myometrial and leiomyoma cells. The results that we found in the present study are in line with the already known properties of quercetin and indole-3-carbinol.

Quercetin is a flavonol present in tea, lemon, tomato, onion leaves, and strawberries [57] and has antifibrotic properties in hepatic fibrosis, pulmonary fibrosis, kidney fibroblasts. It was shown to improve liver histology and reduce collagen content in rats with carbon tetrachloride-induced cirrhosis *in vivo* [58].

Indole-3-Carbinol is found in cruciferous vegetables such as broccoli, cabbage, cauliflower, brussels sprouts, bok choy, collard greens, mustard greens, kale, Chinese cabbage, radishes, turnips, kohlrabi, arugula, watercress, and daikon [61]. I3C inhibited the proliferation of hepatic stellate cells (with or without PDGF-BB stimulation) [62]. I3C suppress angiogenesis by inhibiting tube formation and VEGF secretion in ECs (HUVECs) [63] and via inactivation of ERK1/2 in human umbilical vein ECs [64]. Antiangiogenic activity of I3C in ECs stimulated with activated macrophages has also been reported [52, 65].

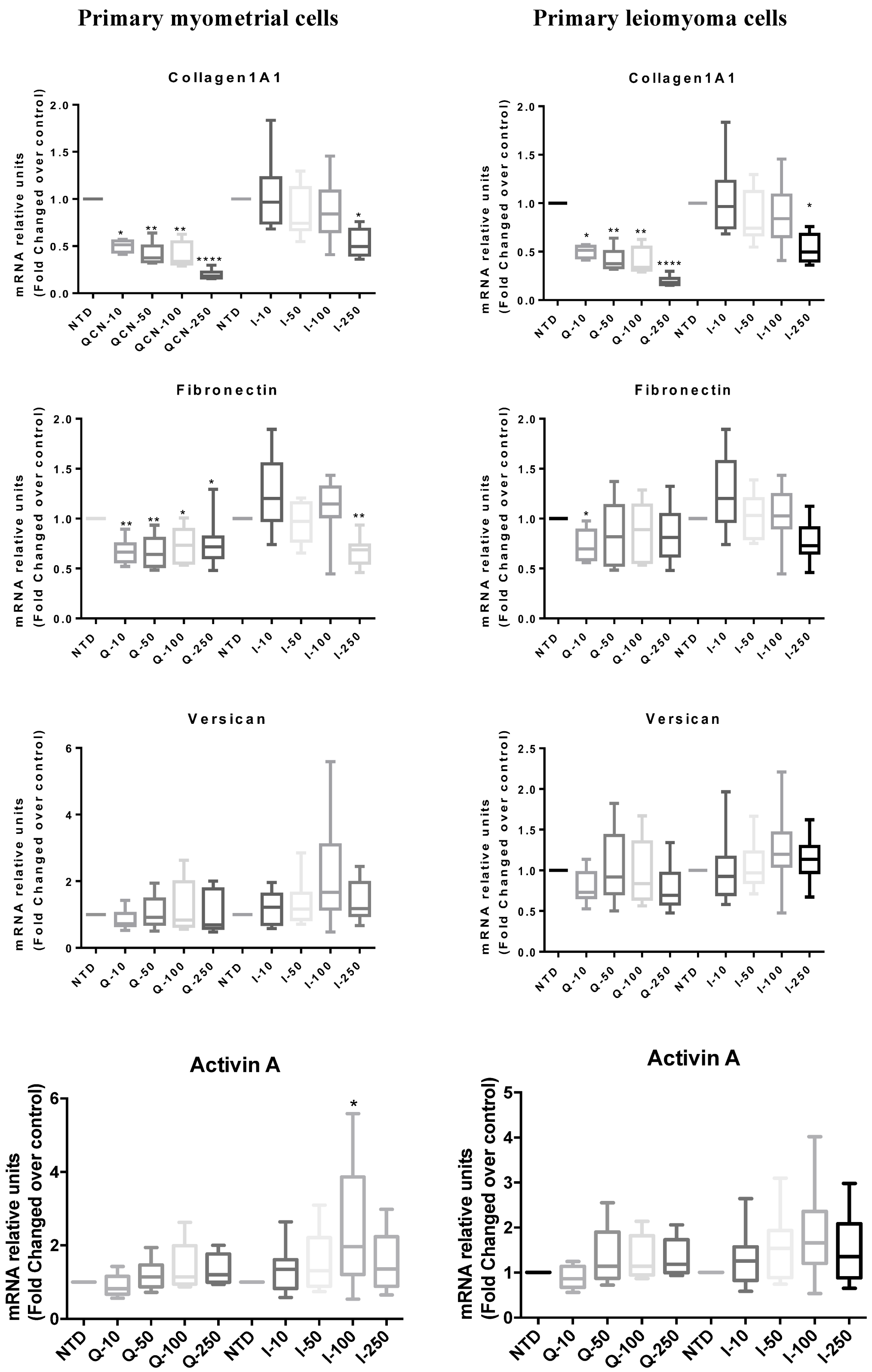


Figure 20. Antifibrotic effect of quercetin and indole-3-carbinol, primary myometrial and leiomyoma cells were treated with both in different concentrations: 10 µg/ml; 50 µg/ml; 100 µg/ml; 250 µg/ml for 48h, and measure mRNA expression by real-time PCR

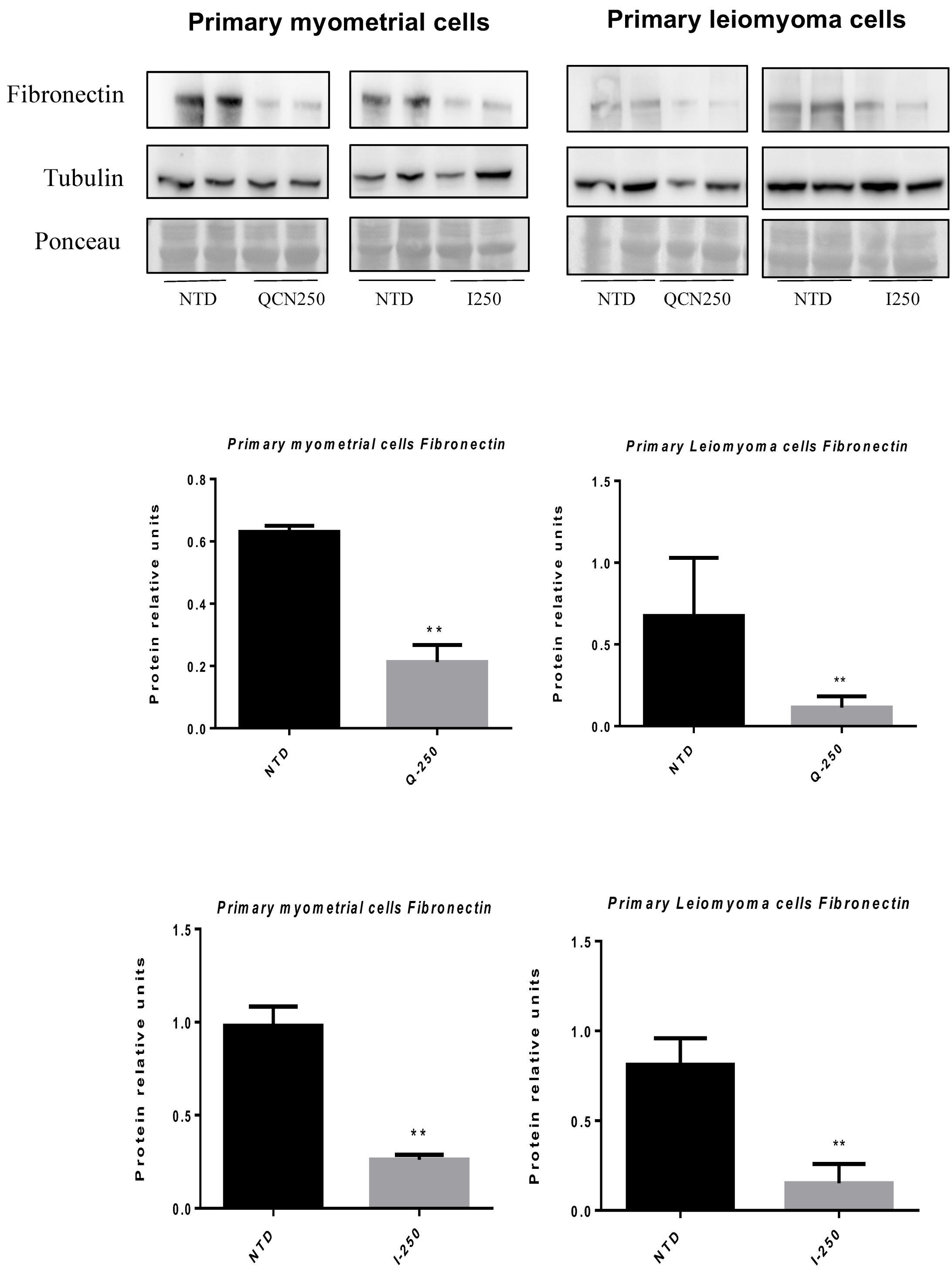


Figure 21. Data from western blotting showed that protein expression of fibronectin was reduced after treatments with quercetin 250 µg/ml (Q-250) and indole-3-carbinol 250 µg/ml (I-250), in primary leiomyoma and myometrial cells compared to untreated controls

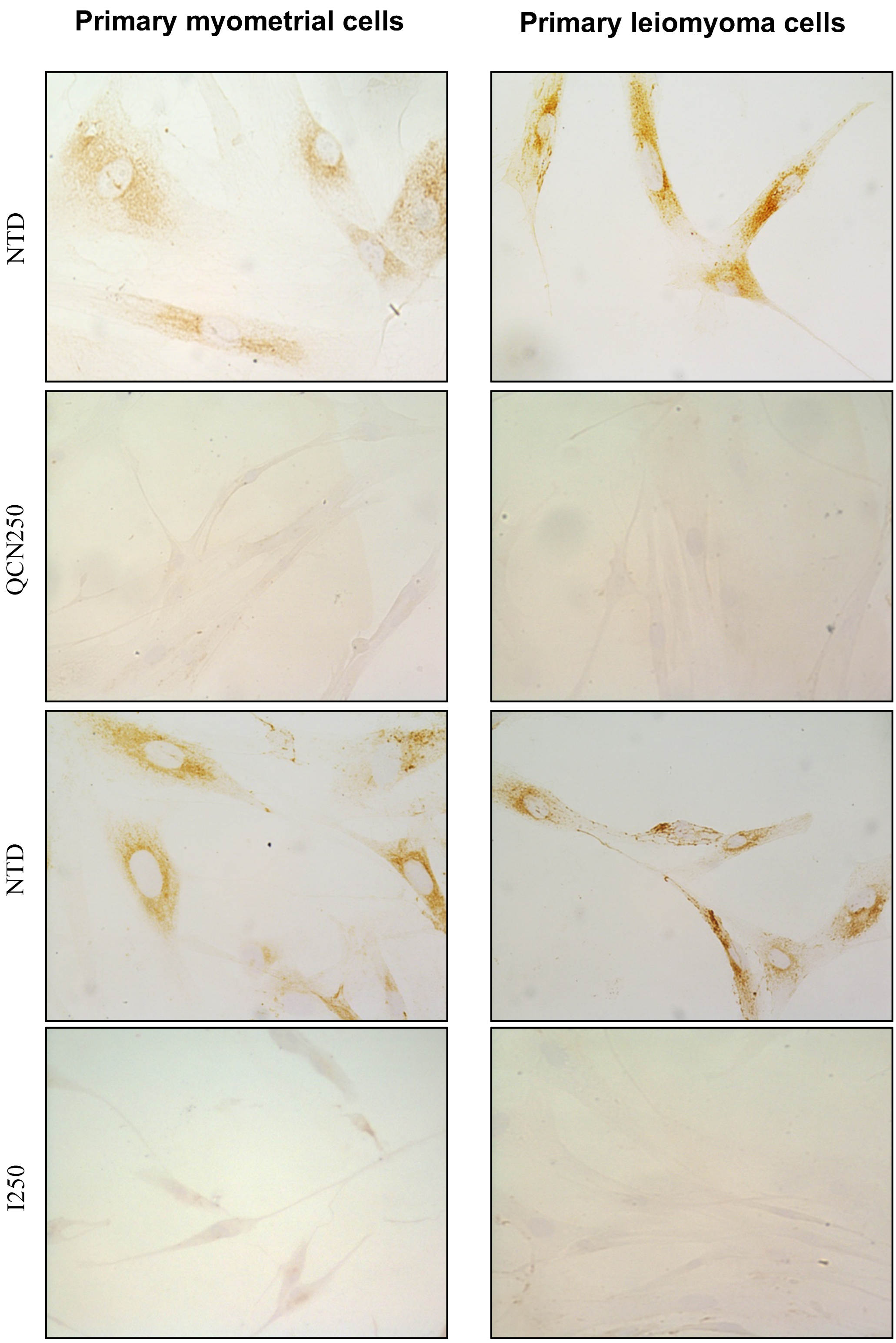
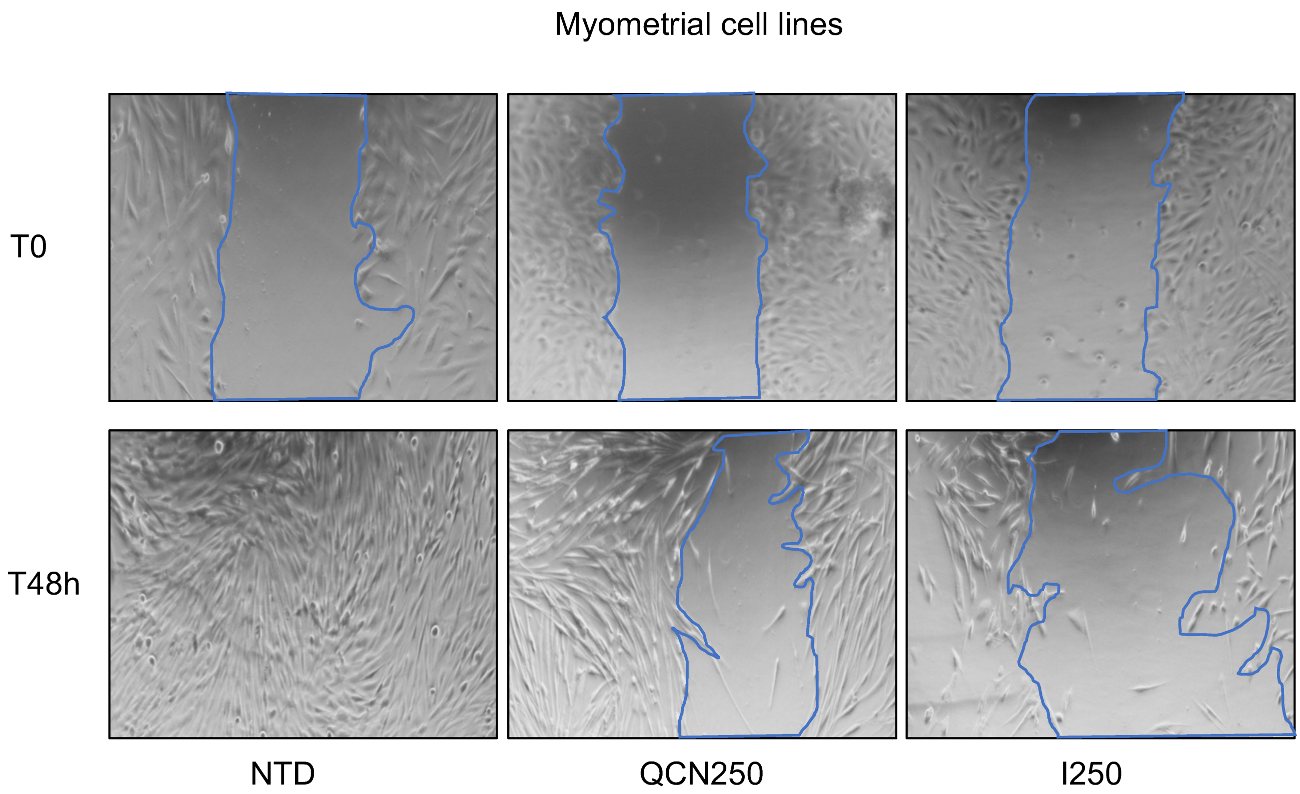


Figure 22. Data from immunocytochemistry also showed decreased the expression of fibronectin in primary leiomyoma and myometrial cells after treatments of quercetin 250 μg/ml (Q-250) and indole-3-carbinol 250 μg/ml (I-250) compared with negative control (untreated cells).



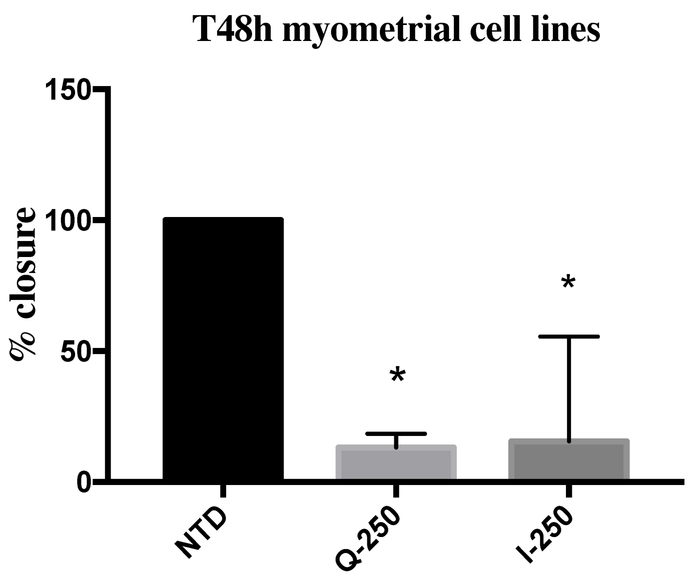
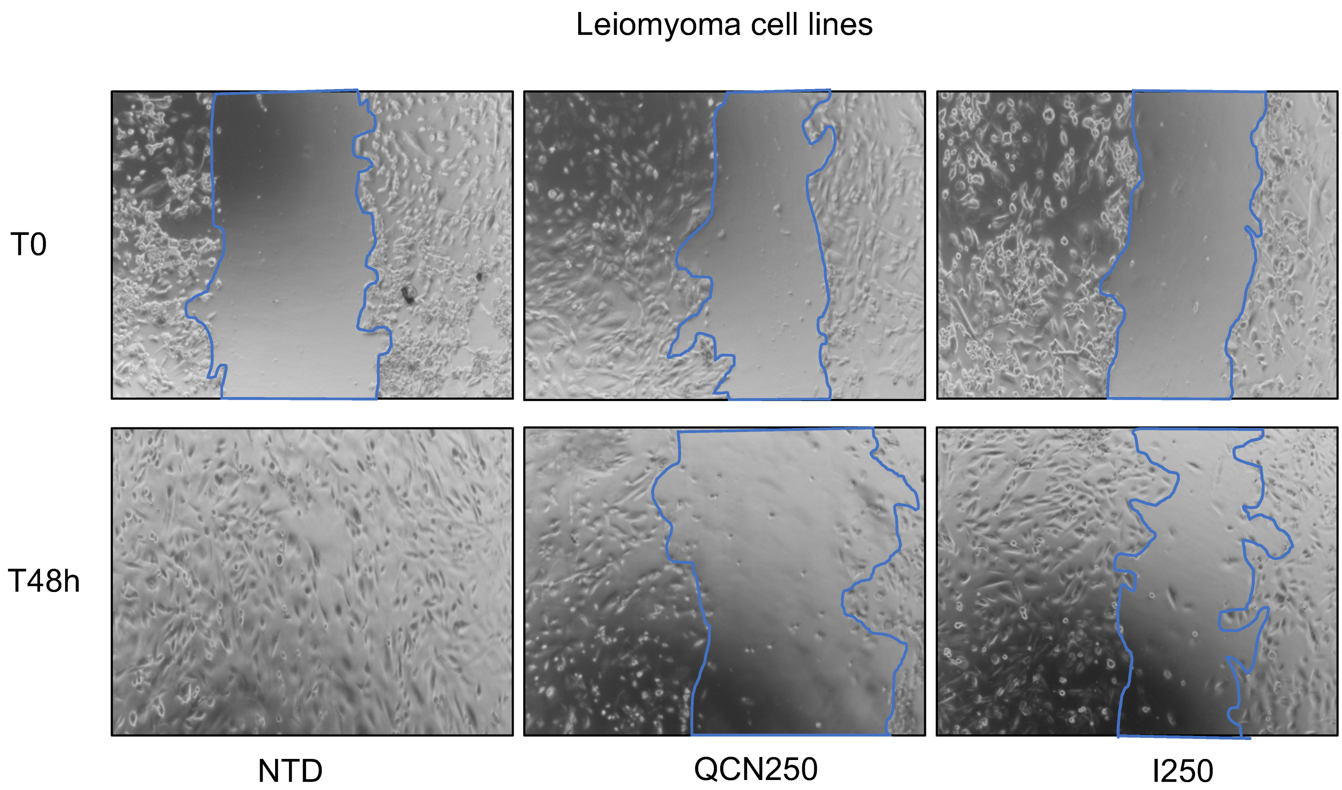


Figure 23. Wound closure experiments. Representative photographs, and graphs showing the effects of quercetin (250 µg/ml) and indole-3-carbinol (250 µg/ml) treatments that enhances wound closure; both treatments significantly inhibits wound closure in myometrial cell lines.



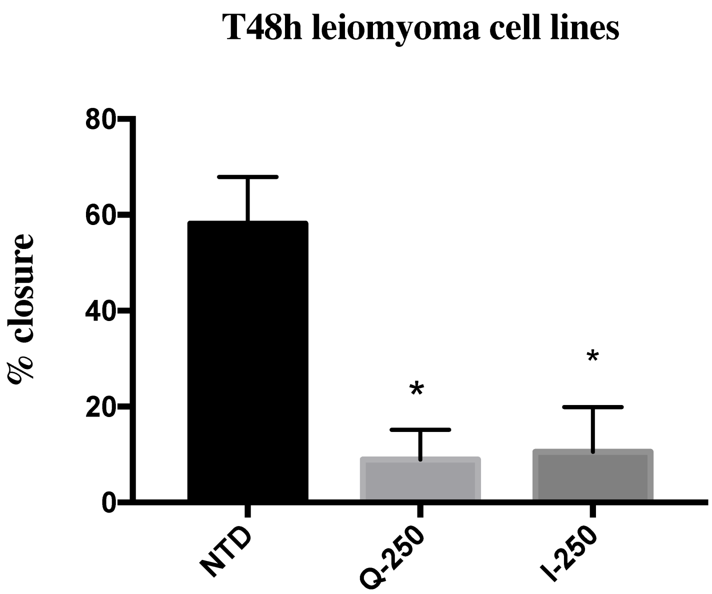


Figure 24. Wound closure experiments. Representative photographs, and graphs showing the effects of quercetin (250 µg/ml) and indole-3-carbinol (250 µg/ml) treatments that enhances wound closure; both treatments significantly inhibit wound closure in leiomyoma cell lines.

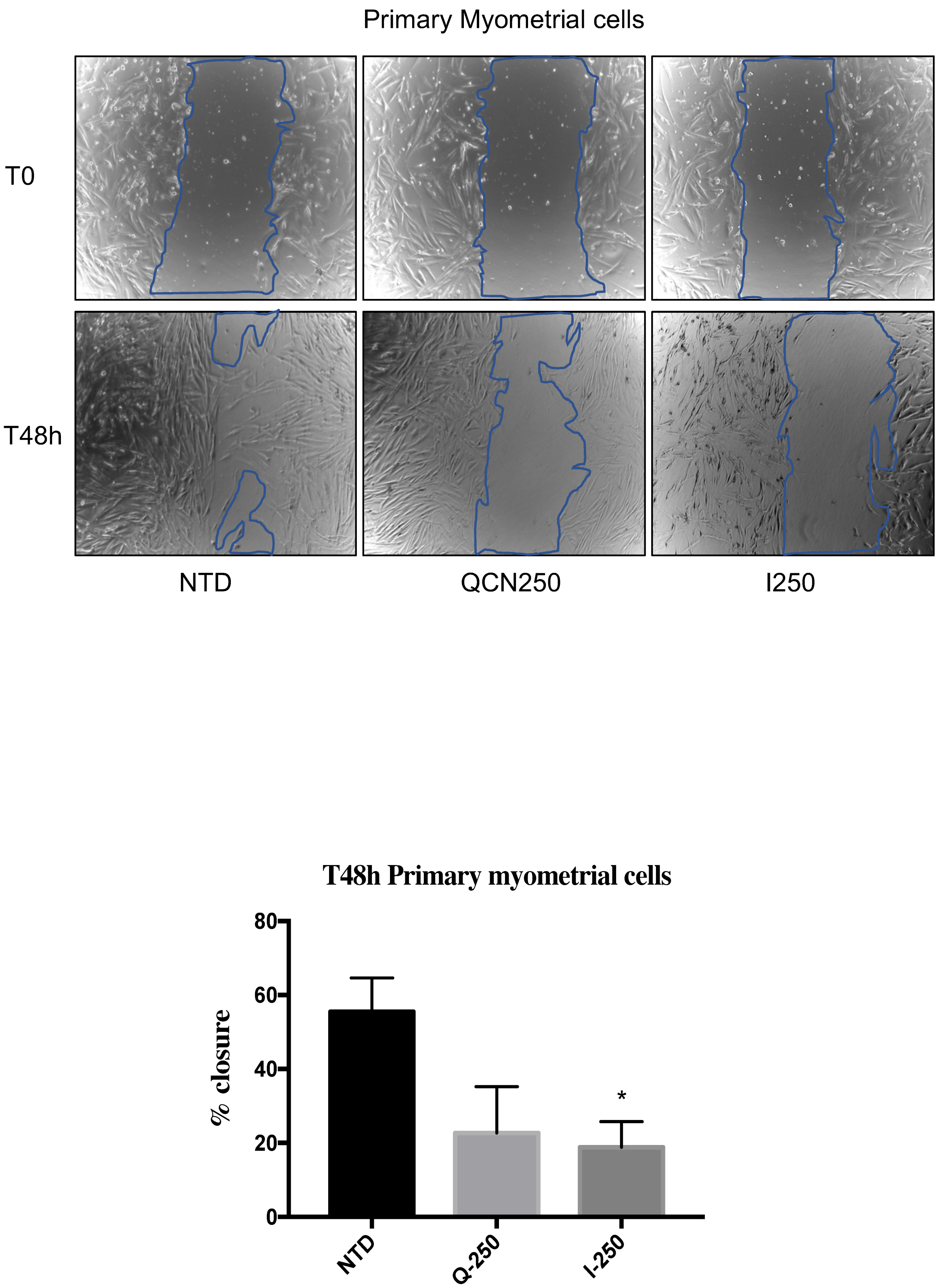
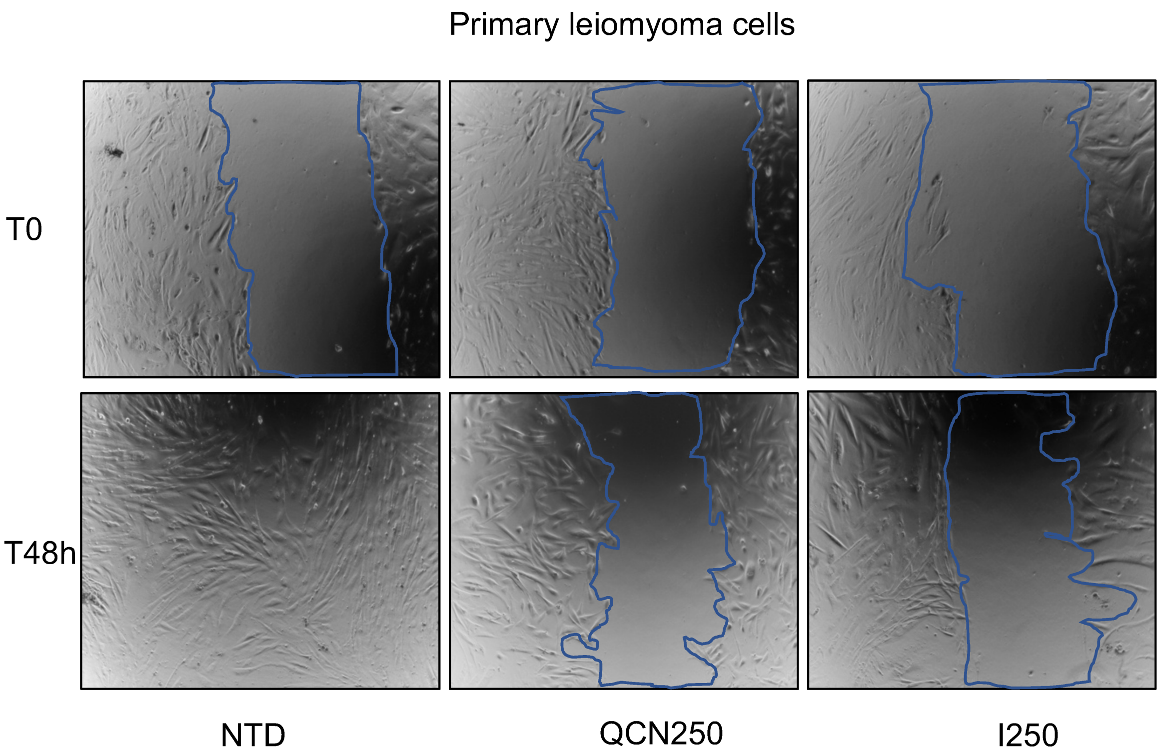


Figure 25 Wound closure experiments. Representative photographs, and graphs showing the effects of quercetin (250 µg/ml) and indole-3-carbinol (250 µg/ml) treatments that enhances wound closure; both treatments significantly inhibits wound closure in primary myometrial cells.



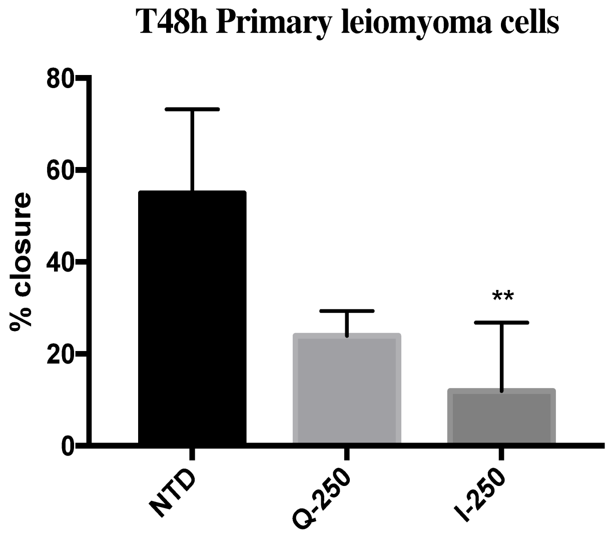
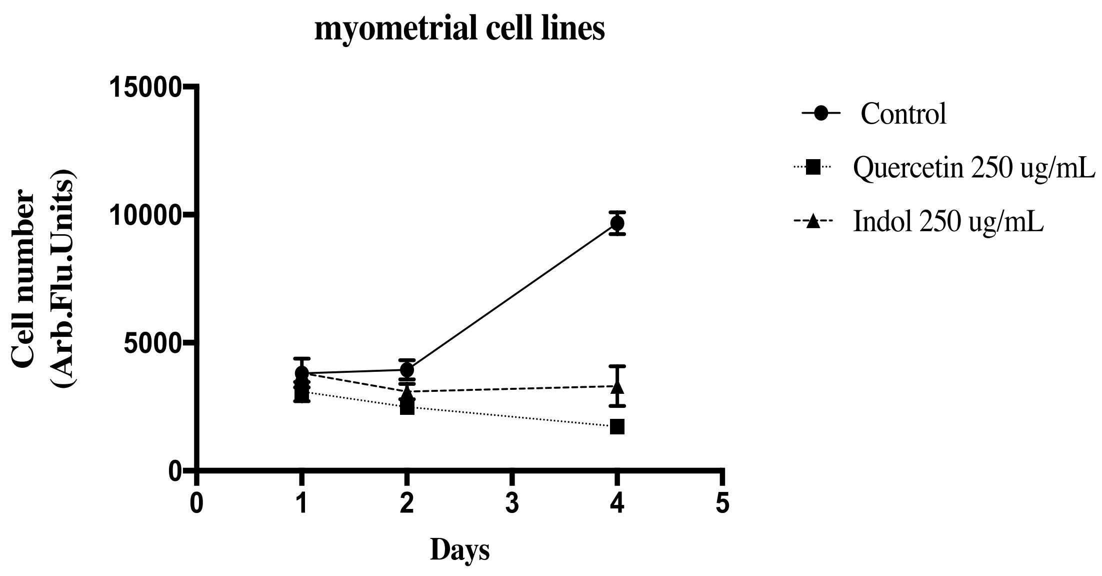


Figure 26 Wound closure experiments. Representative photographs, and graphs showing the effects of quercetin (250 µg/ml) and indole-3-carbinol (250 µg/ml) treatments that enhances wound closure; both treatments significantly inhibit wound closure in primary leiomyoma cells.



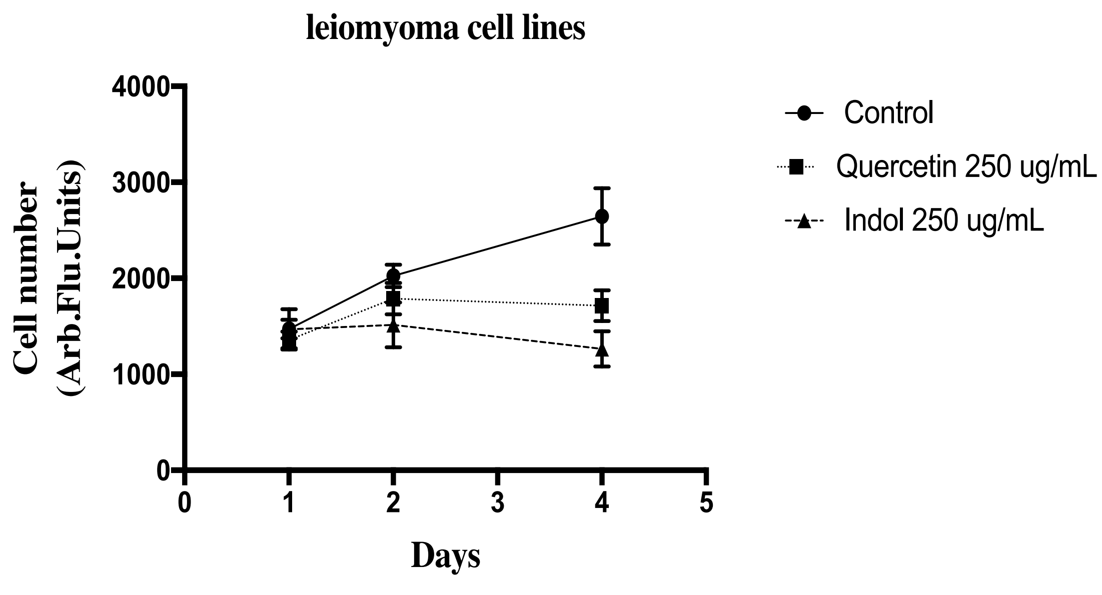
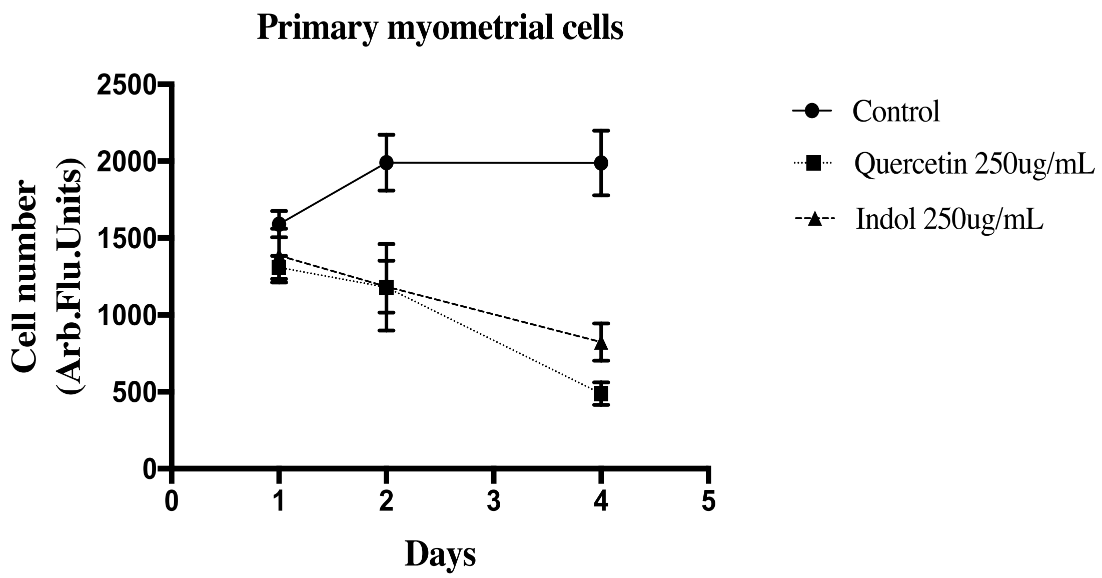


Figure 27. Proliferation assay. Representative graphs showing the effects of quercetin (250 µg/ml) and indole-3-carbinol (250 µg/ml) treatments; both treatments significantly inhibits proliferation in leiomyoma and myometrial cell lines.



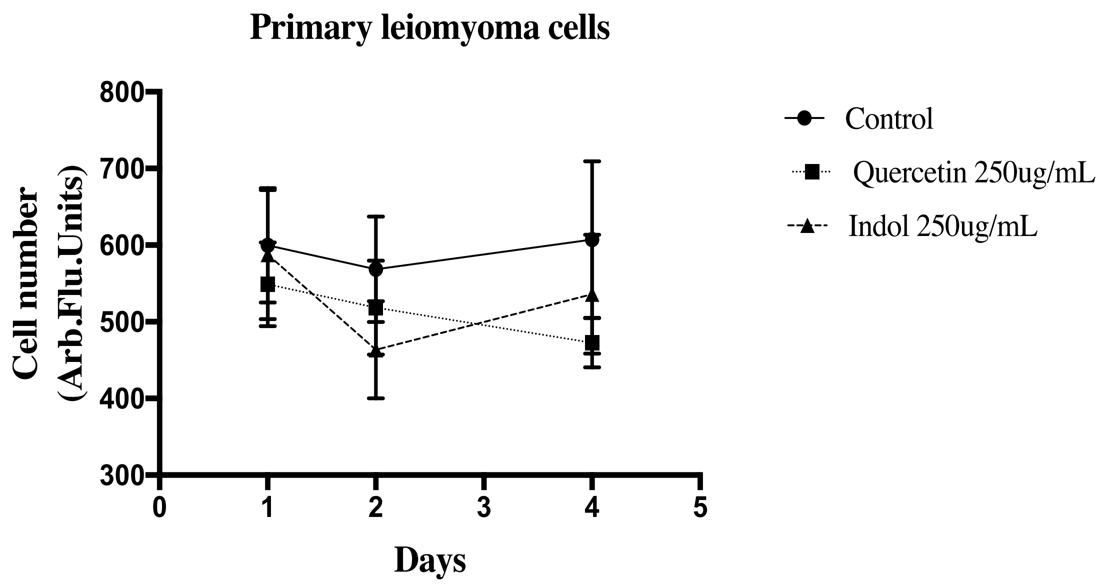


Figure 28. Proliferation assay. Representative graphs showing the effects of quercetin (250 μg/ml) and indole-3-carbinol (250 μg/ml) treatments; both treatments significantly inhibits proliferation in primary leiomyoma and myometrial cells.

4 Conclusions

Briefly, we found that strawberry cultivar of Alba, Romina and its anthocyanin enriched fractions (ACY) of Romina, as well as the quercetin and indol-3-carbinol reduce the gene expression of the extracellular matrix, which is considered one of the key roles in the fibrotic process, in leiomyoma cells.

In addition, quercetin and indol-3-carbinol significantly reduces cellular proliferation and migration.

In conclusion, this study shows the in *vitro* effects of some phytochemical compounds and lays the scientific bases for the development of new therapeutic and/or preventive agents for the uterine leiomyomas.

Appendix: Ultrastructure of a healty myometrium and usual leiomyoma

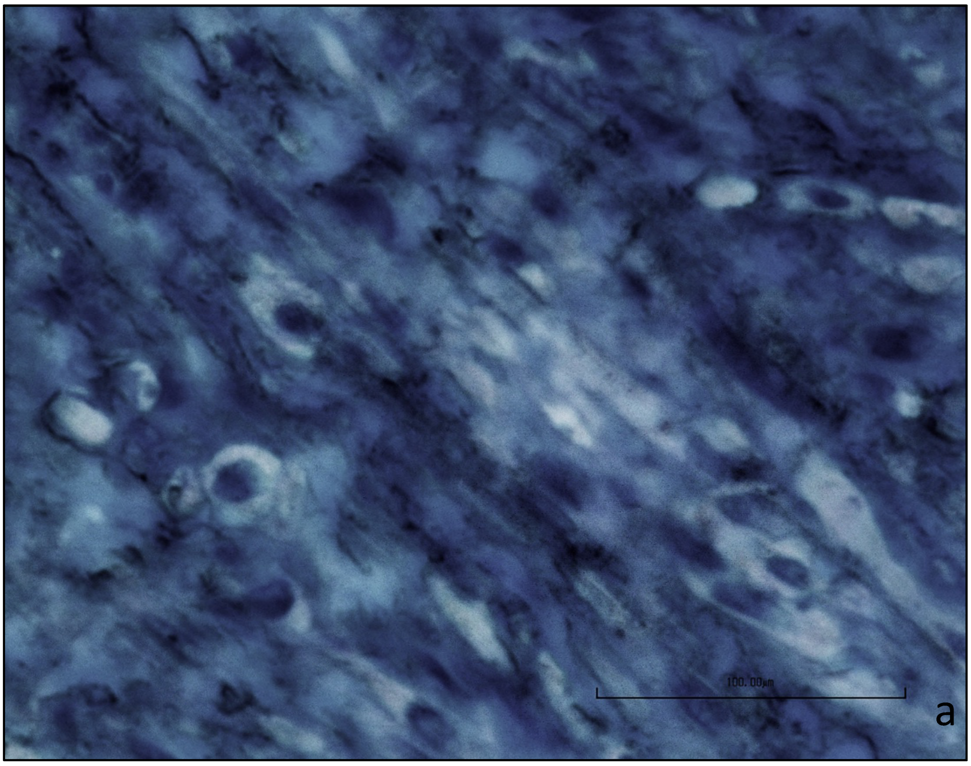
1.1. Preparation of samples for electron microscopy

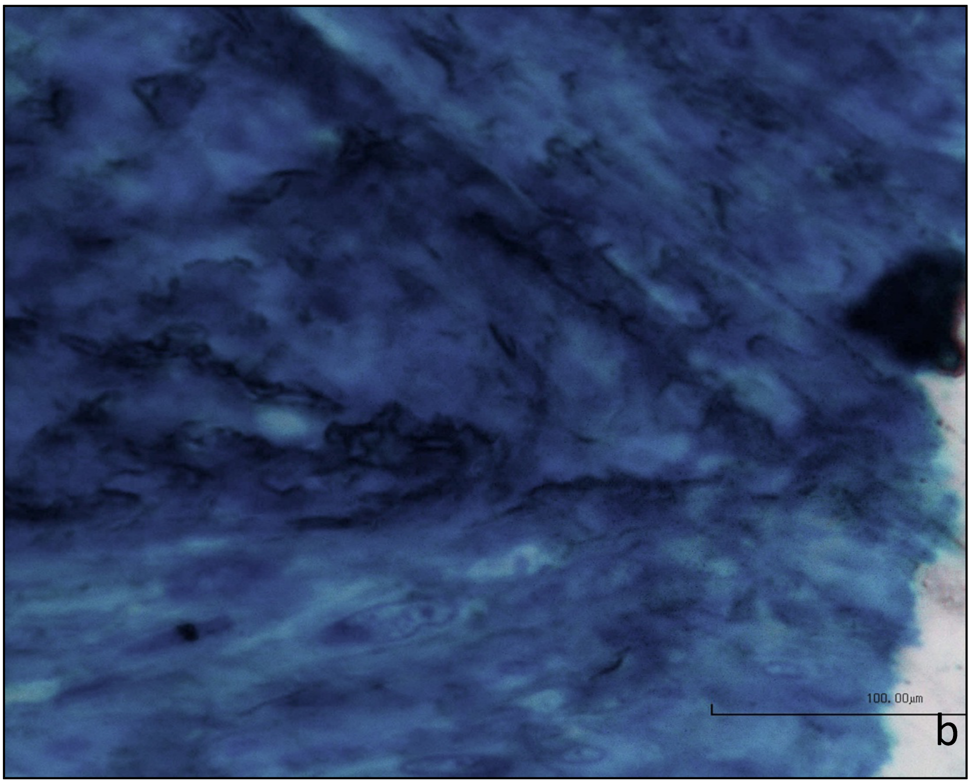
Myometrium and leiomyoma tissues from women undergoing hysterectomy for fibroids used to electron microscopy. Tissue fragments measuring about 1 mm^3 were immersed in a fixative consisting of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/l PB, pH 7.4, over night. They were then washed with phosphate buffer, postfixed in osmium tetroxide OsO_4 1% for 60 min at 4 °C, dehydrated in acetone and embedded in an Epon resin. Thin sections were obtained with an MTX ultra-microtome (RMC, Tucson, AZ, USA), eighty nanometer sections were contrasted with uranyl acetate and lead citrate and examined with a Philips CM 10 transmission electron microscope (TEM)(Philips, Eindhoven, The Netherlands) operating at 100 kV. Electron microscopy pictures displaying myometrium and leiomyoma tissues [80].

1.2. TEM and observation

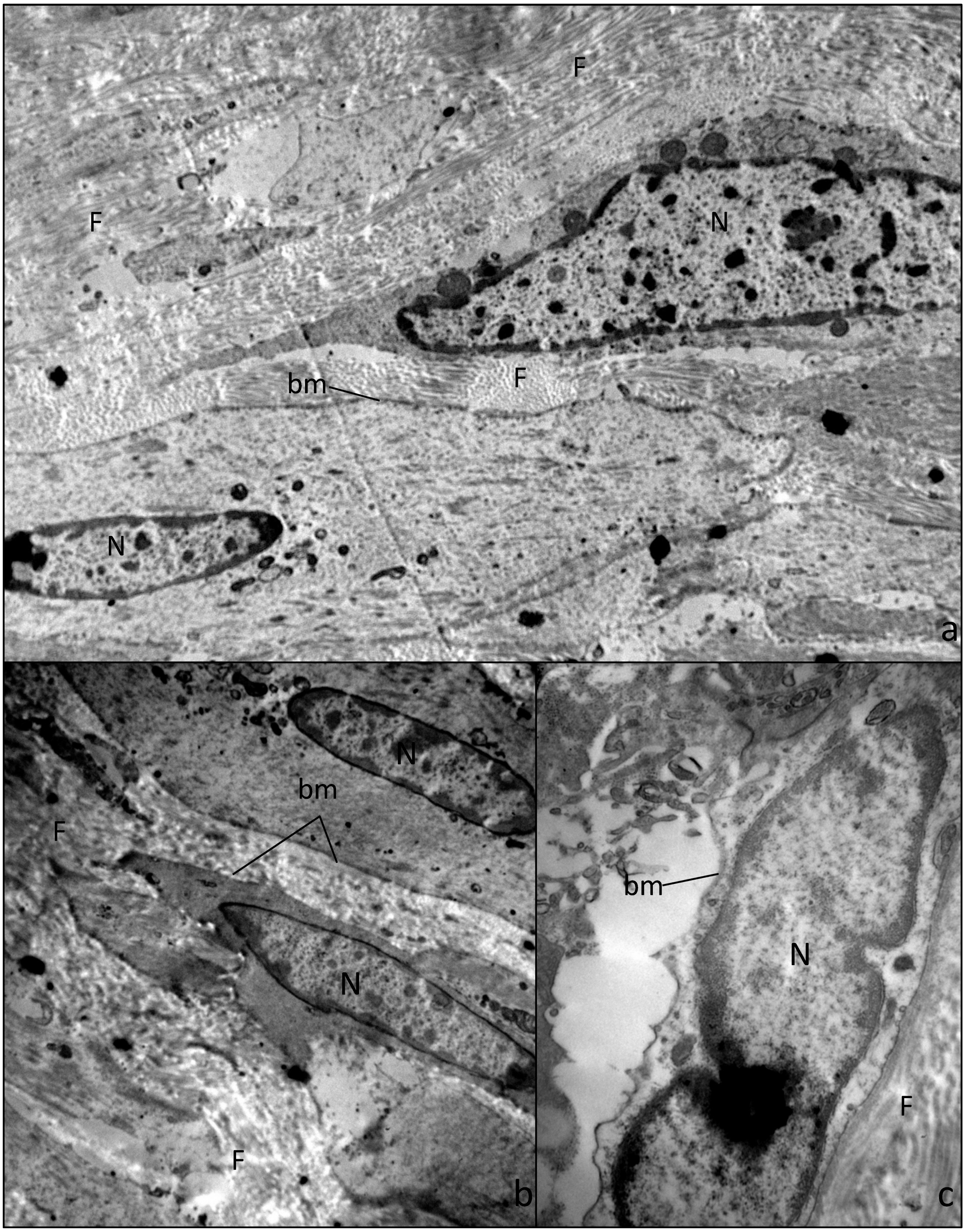
From the previous discussion and previous studies, we have seen how the extracellular matrix plays a key role in the presence and development of uterine fibroma. For this reason, our studies are carried out not only at the molecular level but also at the ultrastructural level. By means of these images of electronic microscopy, it emerged that at the ultrastructural level there is a different organization of the two tissues.

In healthy myometrium, smooth muscle cells have a regular shape, with the typical "serrated" nucleus, and with little presence of cellular organelles. Furthermore, the same extracellular matrix is low and appears organized in a regular pattern. In fact, we can see from the ultrastructural images of the healthy myometrium, the periodicity and the regularity of the bundles of the collagen fibers parallel one to the other.



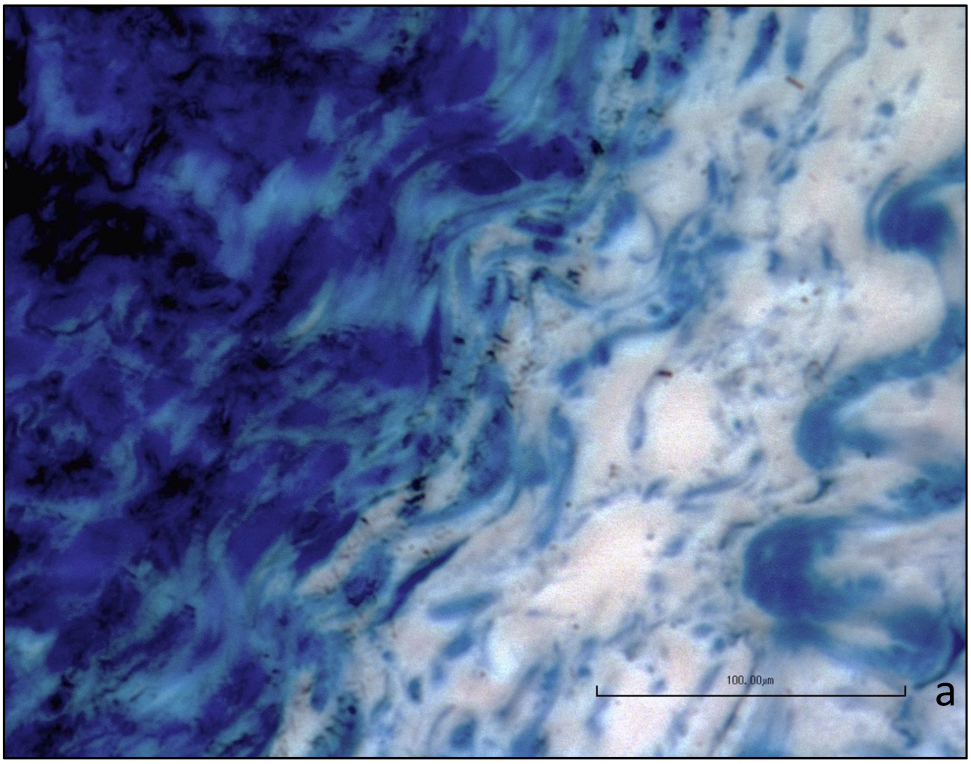


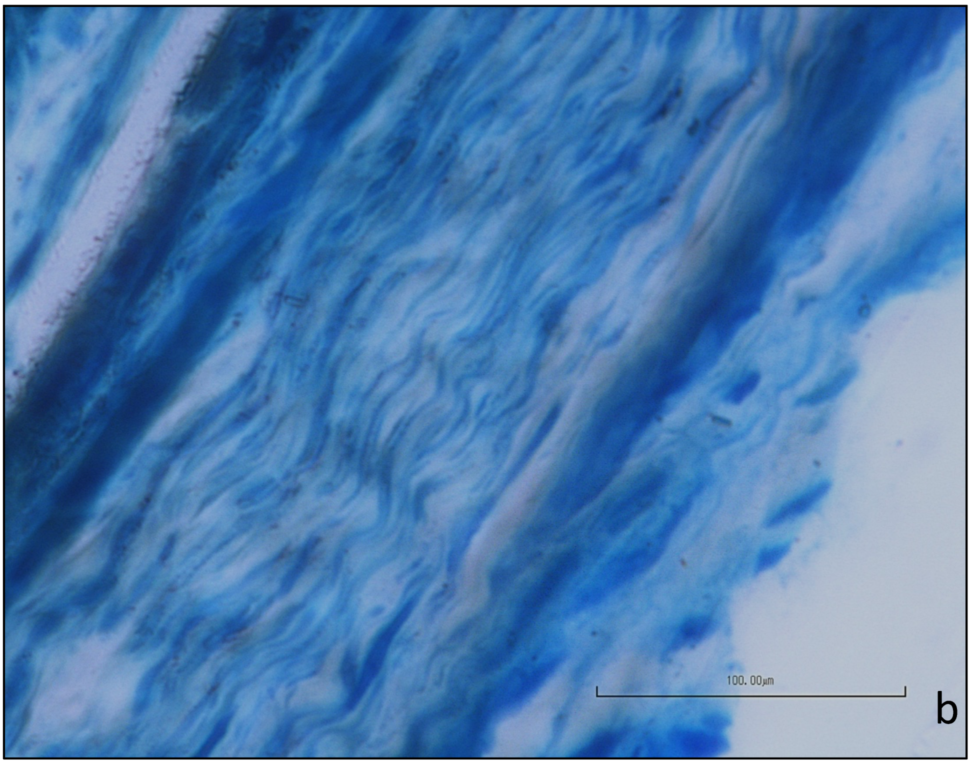
Appendix 1. Semi-thin. Toluidine blue stained Resin-embedded tissues (100X, oil immersion). ***Myometrial tissue.*** **a) in this image, it is possible to see the order of smooth muscle tissue organization with minimal intercellular space, b) the image shows a cross between three different orientations of the muscle bundle.**



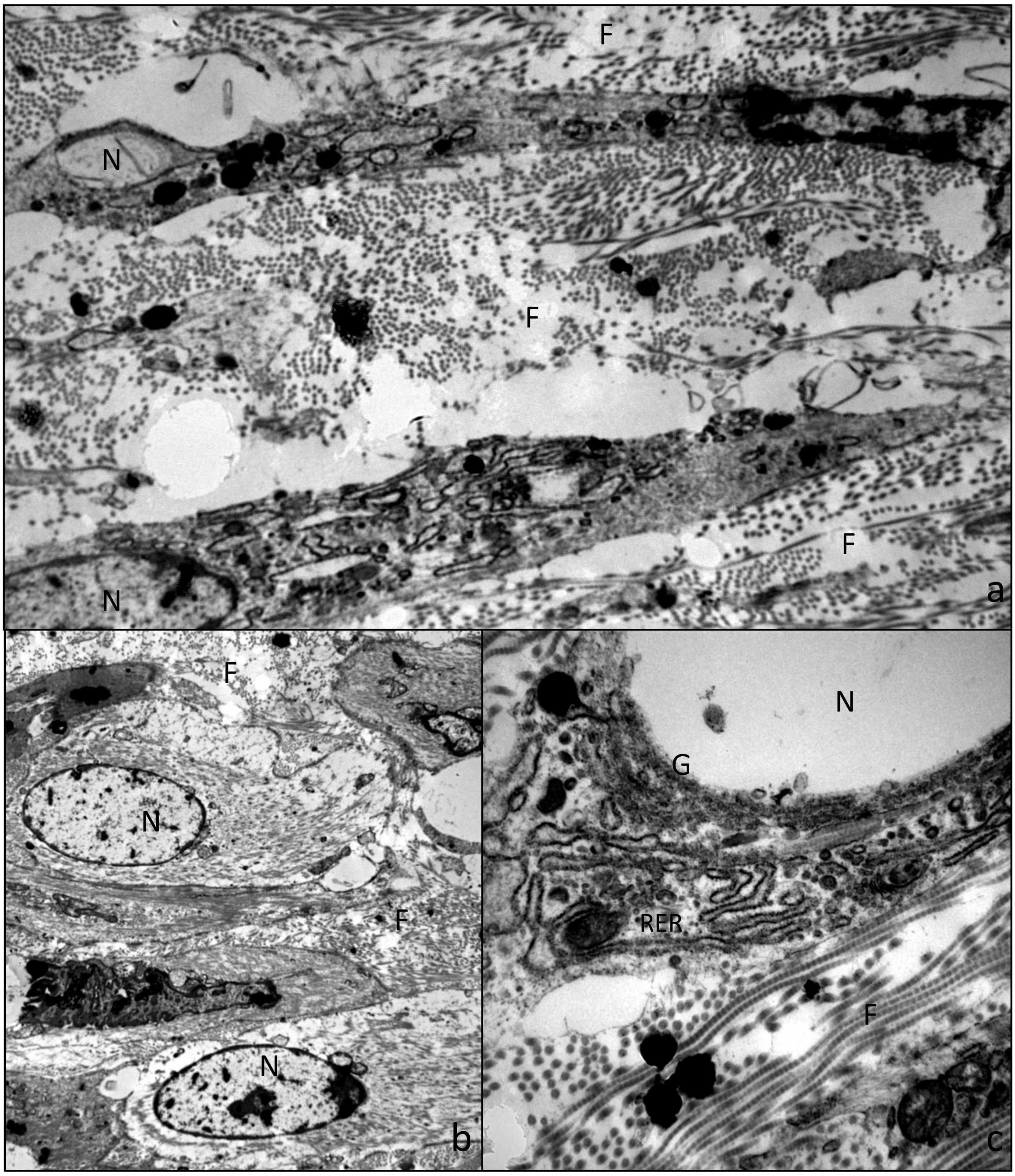
Appendix 2. Representative electron micrograph showing the myometrium tissue. a) This image shows two smooth muscle cells, with nucleus (N) regular cytoplasm and a poor intercellular space, with the presence of collagen fibers (F) with a regular pattern b) The second image shows two cells of smooth muscle, where it is possible to observe the regularity of the basal membrane (bm) and the scarce presence of cellular organelles in the cytoplasm. c) Detail of a smooth muscle cell where the particular "indentation" of the nucleus can be observed. The magnification shown are a) x4,600 b) x7,900 and c) x19,000

In leiomyoma, instead, we can see how the ultrastructure is completely different. Smooth muscle cells have irregular cell membranes and take the fibroblast-like shape. furthermore, there is an increase in the presence of cellular organelles used to produce the extracellular matrix, including the Golgi apparatus and the rough endoplasmic reticulum. In fact, the intercellular space between the cells is much more abundant than in the healthy myometrium, with an arrangement and periodicity of the completely irregular collagen fiber.

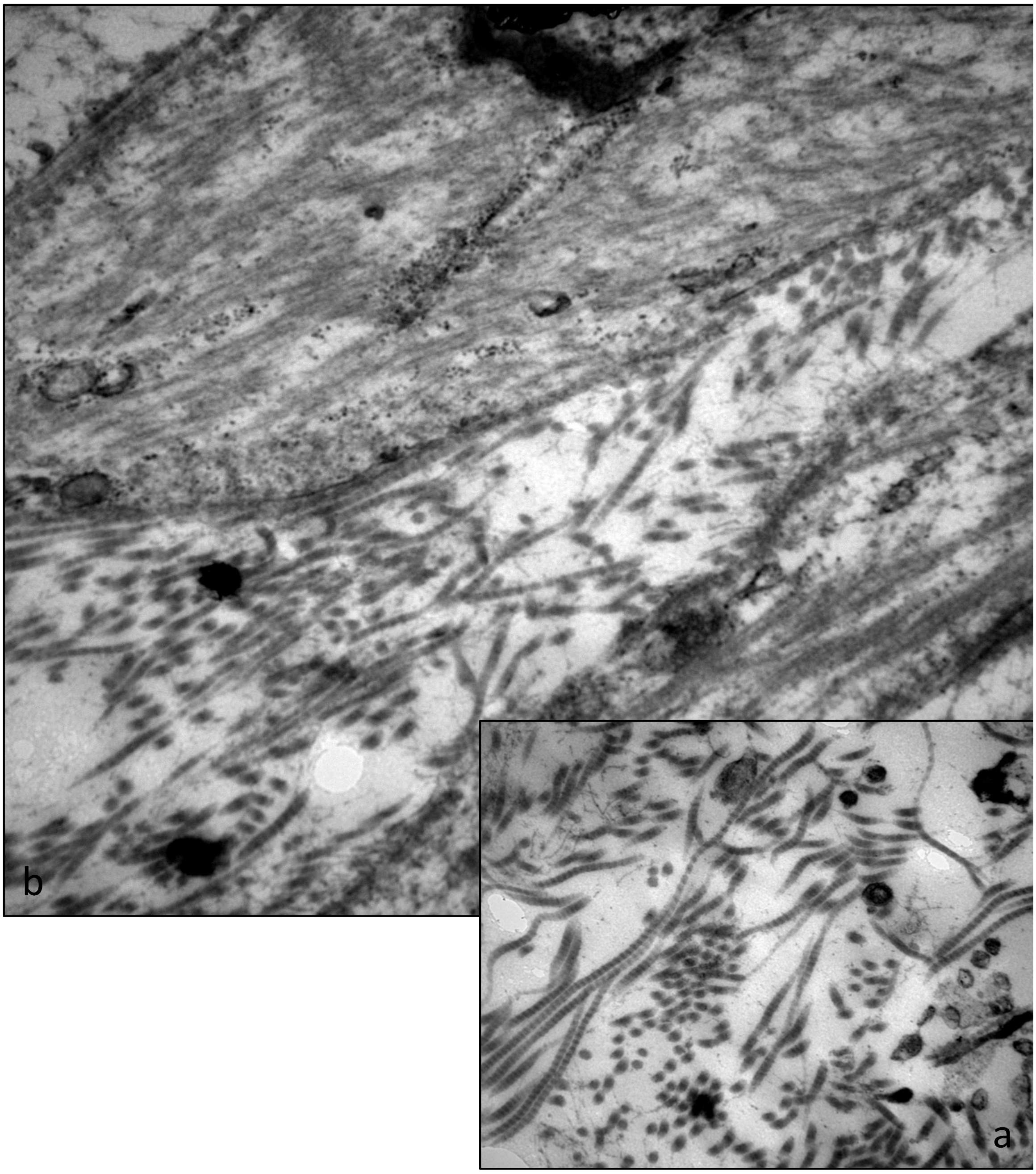




Appendix 3 Semi-thin. Toluidine blue stained Resin-embedded tissues (100X, oil immersion). ***Leiomyoma tissue.*** **a-b) in these images, it is possible to see the large amount of collagen fibers inside the tissue.**



Appendix 4. Representative electron micrograph showing the leiomyoma tissue. a) This image shows two fibroblast cells, with nucleus (N) irregular cytoplasm and an abundance presents of intercellular space, with the presence of collagen fibers (F) with an irregular pattern b) The second image shows two fibro-like cells, where it is possible to observe the abundance presence of extracellular matrix c) Detail of a fibroblast cell where it is possible to observe the organelles in the cytoplasm, in particular apparatus of Golgi (G) and rough endoplasmic reticulum(RER). The magnification shown are a) x5,800 b) x5,800 and c) x25,000.



Appendix 5. Representative electron micrograph showing the disorder of extracellular matrix in leiomyoma tissue. The magnification shown are a) x34,000 b) x25,000

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