UNIVERSITA' POLITECNICA DELLE MARCHE

Faculty of Medicine and Surgery

Ph.D. in Biomedical Sciences

Leiomyoma and leiomyosarcoma two different pathologies with the same origin: identification of a possible new marker and therapeutic target through characterization of Raf kinase inhibitor protein (RKIP)

Tutor:

Professor Andrea Ciavattini

Ph.D. Student:

Doctor Alessandro Zannotti

XXXIV Cycle

Three-year Academic Year 2018-2021

A mamma e papà

che hanno visto dove io non avevo ancora guardato,

che hanno guardato dove solo io vedevo.

Spero, ovunque vada, di guardarmi e vedere in me anche solo una piccola parte di voi.

Para mãe e pai

que viram onde eu ainda não tinha olhado,

que olharam onde só eu via.

Espero, onde quer que eu vá, olhar para mim e ver pelo menos uma pequeninha parte de vocês em mim.

To mum and dad

that saw where I had not looked at yet,

that looked where I only was seeing.

I hope, wherever I will go, I look at myself and see in me even just a little part of you.

Abstract: Malign leiomyosarcoma and benign leiomyoma represent the two counterparts of the myometrium transformation. Nevertheless, the differential diagnosis to discriminate benign from malign lesions represents a remarkable problem. So, it is important to identify new markers making the differential diagnosis more accurate. The pleiotropic RKIP role on leiomyosarcoma is still unclear. In this study, the tendency of five different histological variants of benign lesions to be positive for RKIP and, conversely, the tendency of malign lesions to be negative for RKIP were found by immunohistochemistry. This was particularly evident comparing the malign leiomyosarcoma to the leiomyoma with bizarre nuclei that, although benign, shows intermediate features between benign lesions and malign ones. To understand if RKIP plays a biological role in leiomyosarcoma, *in vitro* knockout of RKIP and *in vitro* overexpression of RKIP in leiomyosarcoma cells were performed. It was found that about cells viability overtime and migratory capability both RKIP knockout cells and RKIP overexpressing cells show a trend similar to that of their respective negative controls. On the other hand, it was observed that RKIP knockout cells show a greater ability to form colonies when compared with negative control where the expression of RKIP was not modified. In conclusion, the obtained results suggest that there could be a RKIP loss in the malign leiomyosarcoma and this may favor the clonogenicity. Altogether these results suggest that RKIP may be a candidate to be considered as an additional marker for the differential analysis to discriminate if a preparation similar to the benign leiomyoma with bizarre nuclei, is not, actually a malign lesion. In light of the rarity of the studied pathologies, these results constitute a starting point for further researches in order to consider RKIP a reliable marker in all respects.

Keywords: leiomyoma; leiomyosarcoma; RKIP-knockout; RKIP-overexpression

1. INTRODUCTION

1.1 Myometrium: the intermediate muscularis layer of the uterus

The uterus is a hollow organ and it represents, together with the Fallopian tubes, the ovaries and the vagina, the female reproductive apparatus.
The uterus functions consist on the receivement and implantation of the product of sexual fertilization. In addition, the uterus allows the growth of the fetus until the childbirth.
In particular, at the moment of the childbirth, for the needed contractions it is fundamental the role played by the myometrium that is the intermediate muscularis layer of the uterus and it is placed between the perimetrium (the more external layer) and the endometrium (the more internal layer). The myometrium which represents the muscular coat of the uterus, has the typical appearance of the smooth muscle tissue and it has a thickness of about 1 cm. In detail, the myometrium is composed of three layers of fibers, all classified as smooth muscle. Precisely, the following layers can be distinguished: the subvascular layer (submucosal layer) which is the inner layer and, in fact, it is also called internal layer and it has no clear boundaries with the endometrium**;** the vascular layer which is the intermediate layer and, in fact, it is also called middle layer and it is well-perfused; the supravascular layer which is the more external layer and, in fact, it is also called external layer.
The subvascular layer is composed by a 3D network of myocellules and vessels, it is quite thin and its main role is played during the menstrual cycle within the separation of the endometrium. The vascular layer is very thick and rather strong. It is composed by circular or oblique myocellules bundles, that together with the vascular branches of which this layer is rich, form a net for the perfusion of the tissue [1-3]. The vascular layer plays a fundamental role during the partum within the complex mechanism that regulates the uterine contractions [4]. The supravascular layer which is composed of a complex of crossing muscle fibers gathered in both circular and longitudinal bundles stabilizes the uterine wall [1-3].
The myometrium myocellules are about 40-60 μm long and about 3-4 μm thick. They exhibit transitional forms with fibroblasts (myofibroblasts) and they are able to produce all the molecules of the extracellular matrix (ECM) [3]. These molecules can be classified as amorphous component and fibrillar component. The first includes glycosaminoglycans (GAG), proteoglycans and glycoproteins (such as fibronectin), the second includes macromolecules (such as collagen) variously organized in filamentous supramolecular structures that confer resistance to tension to the myometrium [3, 5]. The whole ECM helps

to give the myometrium resistance to pressure and traction forces, contributing to the plastic architecture of the tissue [3, 5].

In addition to the native cellular component (fixed cells) that synthesizes the ECM and guarantees its turnover, it can be identified a non-indigenous cellular component (migrating cells) that includes, for example, macrophages [5].

So, the well-perfused myometrium, composed by myocellules and ECM, looks like a set of smooth muscle cells with a dense vascular network, all supported by a fine weave of collagenic matrix [1-3, 6].

The configuration of the myometrium that gives it contractile and endurance capabilities, is particularly useful during menstrual cycle. In fact, during the desquamative phase, contractions albeit slow (0.7 / min) but very powerful (up to 90 mmHg) afflict the myometrium. During the proliferative and the secretive phases, contractions albeit less powerful (10 - 20 mmHg), but faster (2 - 4 / min) than the ones in the desquamative phase afflict the myometrium. In addition to this, even at the moment of childbirth the myometrium must be able to support the contractions stimulated by oxytocin and which are necessary for the expulsion of the fetus [3].

About the physiology and the molecules acting on myometrium, it is noteworthy to highlight that the sexual hormones exert a cyclic action also on this tissue. In particular, estrogens (EST) cause an increase in contractile activity, on the contrary, the progesterone (PRO) cause a decrease in contractile activity [3].

1.2 Uterine tumors

The whole uterus is frequently afflicted by tumors. These tumors can be benign or malign [7].

Benign tumors include polyps, adenomyomas and leiomyomas. Malign tumors include several types of tumors such as endometrial carcinoma and uterine sarcoma [7]. The endometrial carcinoma is common, but it can often be cured, on the other hand, uterine sarcoma is very rare (about 8% of all uterine cancers) (MSD manual version for professionals - *https://www.msdmanuals.com/it-it/professionale/ginecologia-e-ostetricia/tumori-ginecologici/sarcomi-uterini*), but it is often more aggressive and harder to treat (NIH National Cancer Institute - *https://www.cancer.gov/types/uterine*). It is important to note that both leiomyoma which is the most common benign tumor of the uterus [8] and leiomyosarcoma which is the most common uterine sarcoma subtype (leiomyosarcoma

63%, endometrial stromal sarcoma 21%, undifferentiated uterine sarcoma 16%) (MSD manual version for professionals - https://www.msdmanuals.com/it-it/professionale/ginecologia-e-ostetricia/tumori-ginecologici/sarcomi-uterini) and which, above all, shows a high mortality [9] afflict myometrium. Thus, it is evident how important it is to study this tissue and, in particular, these two pathologies that originate from it.

1.3 Malign leiomyosarcoma and benign leiomyoma: the two counterparts of the myometrium transformation

Myometrium can transform itself into leiomyosarcoma, a malign tumor which, although very rare, on the other hand, shows a very poor prognosis [10].
Myometrium can transform itself also into leiomyoma, a benign pathology.

1.4 Leiomyoma epidemiology

Leiomyoma, (uterine leiomyoma, myoma, uterine fibroid or fibroid) since depending on the ethnicity, shows a frequency equal to 70–80% reaching its peak in women of African descent, it is considered the most common female pelvic benign tumor [8, 11]. These percentages refer to women aged 50 [11]. Anyway, leiomyoma tends to increase with age through the reproductive years and decline in the postmenopausal years [12].

1.5 Leiomyoma symptomatology

Although benign, leiomyoma worsens women lifestyle in several ways. In fact, it presents a very heavy symptomatology. Abnormal uterine bleeding, that, in turn, often leads to anemia, pelvic pain and pressure, pain at the level of the back of the legs, a pressure sensation at the level of the lower part of abdomen, back pain, urinary frequency, constipation and pain during sexual intercourse are some of the most relevant symptoms associated with leiomyomas [11]. In addition to these symptoms, leiomyoma also presents other important troubles. Among them it is notable to mention infertility and recurrent miscarriage. In addition to the latter two, leiomyoma can also lead to others poor obstetrical outcomes such as increased risk of preterm labor, cesarean delivery, antepartum bleeding, fetal malpresentation [13], and it can also cause problems for the fetus, in particular, growth restriction [13].

Furthermore, many of the leiomyoma symptoms debilitate not only on a physical level, but also on a psychological level, since they could, in turn, represent a risk factor for emotional stress, depression and anxiety [11, 14].

1.6 Leiomyoma risk factors

Nowadays, it is still debated whether some factors represent risk factors or protective factors for uterine leiomyoma developmet. Among them, early menarche, late age at menopause, nulliparity, late reproductive years, heritability, high blood pressure, obesity, diabetes, polycystic ovary syndrome (PCOS), some lifestyle habits, endogenous or exogenous hormones and stress [12, 15-17] are the most important ones. In addition to this, since black women have a high incidence rate of uterine leiomyomas [18], ethnicity may also be considered as a potential risk factor for this pathology.

Several studies showed that an earlier age at menarche enhances the risk of developing leiomyoma [19-27]. This may be due to a longer history of menstrual cycling. On the other hand, there are not evidences that late age at menopause increases risk for leiomyoma [12]. In addition to this, it was demonstrated that the risk of developing leiomyoma decreases after menopause [20, 22, 28-31].

Several studies highlighted that the risk of developing leiomyoma is reduced by having a child [21, 23, 24, 26, 31-33]. In addition, having a higher number of children seems to be an additional factor contributing to diminish the risk of developing leiomyoma [21, 23, 26, 28, 31, 33]. In particular, it was reported that women with infertility have a higher probability of having leiomyoma [20, 23, 33].

Late reproductive years were associated with a lower risk of developing leiomyoma [20, 22, 24, 26], but not in all studies [21, 28, 33, 34]. Similarly, also the question if the time since the last birth could represent a risk factor of developing leiomyoma [20, 21, 23, 24, 26, 28, 32] is debated [24].

About heritability, it was reported that having first-degree affected relatives represents a risk of developing uterine leiomyoma [35, 36].

Several studies defined high blood pressure as a risk factor for uterine leiomyoma development [20, 37-44].

Many studies agree that body mass index (BMI, kg/m^2) slightly increases the risk of developing leiomyoma [20, 21, 28, 31, 33, 39, 45-47]. In detail, most positive studies suggested that risk rises through the overweight categories, while it starts to decrease

among the heaviest or obese women [21, 29, 46-48]. Anyway, the question about BMI and its correlation with the risk of developing leiomyoma is still debated since there are studies with different results [22, 30, 32].

Since leiomyoma is less vascularized than normal myometrium [49], it was hypothesized that diabetes and hyperinsulinemia may protect against leiomyoma risk via localized vascular dysfunction leading to the inhibition of tumor development [12].

On the contrary, it was suggested that PCOS, despite its association with hyperinsulinemia, may increased the risk of developing leiomyoma [50].

In addition to all this, also some lifestyle habits may represent risk factors of developing leiomyoma. In fact, physical activity seems to play a protective effect [12].

Cigarette smoking seems to play a protective effect [51].

It was reported that there is a positive association between the risk of developing uterine leiomyoma and current consumption of alcohol [52]. This was confirmed in a later study which demonstrated that a higher prevalence of uterine fibroids is associated with a greater alcohol intake [53].

The fact that caffeine is not associated with the risk of developing leiomyoma [54] was disproved by a later study which demonstrated that the heavy consume of coffee (≥3 cups/day) and caffeine (≥500 mg/day) aged <35 years in subjects enhances the risk of developing uterine leiomyoma [52].

On the other hand, higher intakes of fruits and vegetables seems to reduce the risk of developing uterine leiomyoma [55].

In addition to this, the secretion of some endogenous hormones and the use of some exogenous hormones were studied as potential risk or protective factors for leiomyoma development.

In detail, it was shown that the use of exogenous hormones (estrogens only or a combination of estrogens and progestogens) after menopause represents a risk factor for developing leiomyoma [32, 56-58].

On the other hand, it was reported that depot medroxyprogesterone acetate (DMPA) decreases the risk of developing leiomyoma [21,24,59].

On the contrary, it was demonstrated that the risk of developing leiomyoma rises in relation to high levels of luteinizing hormone (LH) [60] in particular, this association is valid for larger tumors.

About oral contraceptives [20-22, 24, 26, 28, 30-32, 61, 62, 65] and prenatal exposure to diethylstilbestrol (DES) [12], the data reported in literature are conflicting [12].

Environmental exposures may act on the risk of developing uterine leiomyoma via several mechanisms, including endocrine disruption, any way the results actually available seem to need to be strengthened [12].
Similarly, stress may exert conflicting effects on the risk of developing leiomyoma because it can act on different pathways with different following effects [64-66].
Thus, it can be understand that, since some of the epidemiological data about the identification of potential risk and protective factors for leiomyoma development are conflicting, further studies about this matter are needed [67].

1.7 Leiomyoma classification

Regarding the classification of uterine leiomyoma, among the many available ones in the literature, the most adopted one is the International Federation of Gynecology and Obstetrics (FIGO) classification (2011) [11]. It is the most recent classification and it identifies eight categories of uterine leiomyoma, based on the location of the tumor and on its degree of intramural/intracavitary extension [68]. In short, according to this classification, depending on their anatomical location, uterine leiomyoma can be described as submucosal leiomyoma, intramural leiomyoma and subserosal leiomyoma [69].

1.8 Leiomyosarcoma epidemiology

Leiomyosarcoma is a malign tumor that can arise from myometrium.
Leiomyosarcoma is an uterine sarcoma subtype. In detail, leiomyosarcoma has been indicated to represent the 63% (with endometrial stromal sarcoma representing the 21%, and undifferentiated uterine sarcoma representing almost 16%) (MSD manual version for professionals - *https://www.msdmanuals.com/it-it/professionale/ginecologia-e-ostetricia/tumori-ginecologici/sarcomi-uterini*) or, more recently, the 30% (with carcinosarcomas of the the endometrium, representing the 40-50% and endometrial stromal sarcoma representing the 15%) (NIH National Cancer Institute - *https://www.cancer.gov/types/uterine*) of all uterine sarcomas depending on the classifications. Other rare forms of uterine sarcomas (mixed endometrial stromal and smooth muscle tumors, adenosarcomas, embryonal botryoides or rhabdomyosarcomas and perivascular epithelial-cell tumor referred to as PEComa) can be found under the World Health Organization (WHO) classification of mesenchymal and mixed tumors of the uterus

(NIH National Cancer Institute - *https://www.cancer.gov/types/uterine*). Anyway, the most important thing is that leiomyosarcoma is one of the most recurrent subtype of the very rare uterine sarcomas that, all together, comprise only the 2-5% of all uterine malignancies and less than 1% of all gynecologic malignancies (NIH National Cancer Institute - *https://www.cancer.gov/types/uterine*).

Women affected by leiomyosarcoma may vary in age [10], but the highest frequency can be associated to the pre- and peri-menopause periods [9] with a median age of 50 years [70] at which a peak incidence occurs (NIH National Cancer Institute - *https://www.cancer.gov/types/uterine*).

1.9 Leiomyosarcoma simptomatology

Leiomyosarcoma presents a symptomatology vague and that can be compared to other benign uterine diseases [10]. In fact, similarly to benign leiomyoma, also the malign leiomyosarcoma may cause hemorrhage and vaginal or abdominal pressure [9] and frequent urination (MSD manual version for professionals - *https://www.msdmanuals.com/it-it/professionale/ginecologia-e-ostetricia/tumori-ginecologici/sarcomi-uterini*).

The most important matter about leiomyosarcoma is that it is an intractable tumor that metastasizes and repeatedly recurs [71, 72] in particular because of leiomyosarcoma stem-like cells that were demonstrated to have a stronger capacity to lead tumor angiogenesis and hematogenous metastasis if compared to normal human leiomyosarcoma cells [73]. Usually the tumor recurs locally, in the abdomen, or in the lungs (MSD manual version for professionals - *https://www.msdmanuals.com/it-it/professionale/ginecologia-e-ostetricia/tumori-ginecologici/sarcomi-uterini*). In addition to this, leiomyosarcoma is an extremely aggressive tumor and it shows a poor overall prognosis [9, 10]. In particular, at the same stage, the prognosis for leiomyosarcoma is usually worse than for endometrial cancer with leiomyosarcoma that shows a mean 5-year survival rate equal to 51% for the first stage and that decreases to only 3% for the major stage (MSD manual version for professionals - *https://www.msdmanuals.com/it-it/professionale/ginecologia-e-ostetricia/tumori-ginecologici/sarcomi-uterini*).

The prognosis associated to leiomyosarcoma, first of all, depends on the extent of disease at the time of diagnosis (NIH National Cancer Institute - *https://www.cancer.gov/types/uterine*). It was reported that if the disease has already spread

beyond the uterus survival is usually low (MSD manual version for professionals - https://www.msdmanuals.com/it-it/professionale/ginecologia-e-ostetricia/tumori-ginecologici/sarcomi-uterini).

1.10 Leiomyosarcoma risk factors

Preventive pelvic radiotherapy can be mentioned among the risk factors for uterine sarcomas in general and therefore also for leiomyosarcoma (MSD manual version for professionals - https://www.msdmanuals.com/it-it/professionale/ginecologia-e-ostetricia/tumori-ginecologici/sarcomi-uterini). In fact, it was seen that from 5 to 25 years after that this treatment had been administered for benign uterine bleeding, uterine sarcomas occured. Prior pelvic radiation therapy has been documented in 10-25% of cases as the only cause of the subsequent onset of these malignant tumors (NIH National Cancer Institute - https://www.cancer.gov/types/uterine).
In addition to this, the use of tamoxifen (MSD manual version for professionals - https://www.msdmanuals.com/it-it/professionale/ginecologia-e-ostetricia/tumori-ginecologici/sarcomi-uterini) may represent another risk factor for uterine sarcomas in general and therefore also for leiomyosarcoma. This selective estrogen receptor modulator was indicated as a risk factor for these malignancies as an increased incidence was noted both when tamoxifen had been used in women with breast cancer and when it had been used in women at high risk of developing breast cancer. Maybe this is due to a possible estrogenic effect that tamoxifen exerts on the uterus (NIH National Cancer Institute - https://www.cancer.gov/types/uterine).

1.11 Leiomyosarcoma classification

Regarding the classification of leiomyosarcoma, among the available ones in the literature, the most adopted is the one established by FIGO and the American Joint Committee on Cancer (AJCC). This classification was designed on carcinoma and it also applies to uterine sarcomas that, as previously said, include leiomyosarcoma. According to this classification, four stages can be identified, each of which may in turn comprise one or more sub-stages [74].
In detail, the stages and the corresponding features are indicated below.
In the stage I the tumor is confined to the corpus uteri.

Stage I includes sub-stages IA and IB.
Sub-stage IA is characterized by an absent or equal to less than half myometrium invasion.
Sub-stage IB is characterized by an invasion equal to or greater than half of the myometrium.
In the stage II, although the tumor invades cervical stroma, it does not extend beyond the uterus.
In the stage III the tumor spreads locally and/or regionally
Stage III includes sub-stages IIIA, IIIB, IIIC, IIIC1, IIIC2.
Sub-stages IIIA is characterized by the fact that the tumor invades the serosa of the corpus uteri and/or adnexae.
Sub-stages IIIB is characterized by the vaginal and/or parametrial involvement.
Sub-stages IIIC is characterized by the presence of metastases in pelvic and/or para-aortic lymph nodes.
Sub-stages IIIC1 is characterized by positive pelvic nodes.
Sub-stages IIIC2 is characterized by positive para-aortic lymph nodes with or without positive pelvic lymph nodes.
In the stage IV the tumor invades bladder and/or bowel mucosa and, in addition or alternatively, it can present distant metastases.
Stage IV includes sub-stages IVA and IVB.
Sub-stage IVA is characterized by the fact that the tumor invades bladder and/or bowel mucosa.
Sub-stage IVB is characterized by the presence of distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes [74].

1.12 Leiomyoma and leiomyosarcoma: a morphological point of view

From a morphological point of view, about the macroscopic aspect, benign leiomyoma shows itself as a white or tan-white, whorled, firm, bulging mass [75] due to the overexpression of the ECM proteins. Thus, leiomyoma can be described as a nodule that, in addition, is typically well circumscribed but nonencapsulated [75] with, instead, a pseudocapsule of compressed smooth muscle fiber cells that separate the leiomyoma mass from the normal myometrium [6]. Calcification is another feature that can be found in leiomyoma. In addition to this, in the largest leiomyoma masses, hemorrhage and infarction can be present [75]. Leiomyoma is often multiple [75] and, although the size is variable, this mass can often reach large dimensions, up to over 15 cm in diameter [6].

From a morphological point of view, about the macroscopic aspect, malign leiomyosarcoma shows itself as a bulky, fleshy tumor invading into myometrium wall or as a polypoid tumor projecting into lumen. Leiomyosarcoma grossly appears invasive / infiltrative. Hemorrhage and necrosis are often present [76]. Leiomyosarcoma is typically solitary [6] and very large with a diameter that can reach an average of 10 cm [76].

About the microscopic aspect, from a histologic point of view, some different types of benign leiomyoma were individuated. Among them, usual leiomyoma, cellular leiomyoma, lipoleiomyoma, apoplectic leiomyoma and bizarre leiomyoma were analyzed in the present study.

There are some recognized histopathological criterions that describe each variant of benign leiomyoma.

The usual leiomyoma (conventional leiomyoma, or commonly referred to as leiomyoma since it is the most common histological variant) exhibits fascicular pattern of typical smooth muscle bundles that appear very similar to normal myometrium musculature [6]. Thus, usual leiomyoma shows a normocellular aspect [75]. Anyway, the usual leiomyoma has defined borders [75]. In addition, the smooth muscle bundles are immersed in abundant ECM. It was reported in literature that usual leiomyoma exhibits almost a quantity of ECM that exceeds the corresponding myometrium by 50% [77-82]. For this reason, usual leiomyoma is the variant considered a fibrotic disorder [83, 84]. Furthermore, usual leiomyoma is characterized by intersecting fascicles of monotonous spindle cells showing indistinct borders, eosinophilic cytoplasm, cigar shaped nuclei (with tapered ends) and small nucleoli. Usual leiomyoma shows thick walls blood vessels. This histological variant displays absent or mild atypia, rare mitoses (in general < 5/10 high power fields). Infarct type necrosis, hyalinization, calcification and cystic change may or may not be encountered [75].

All the other histological variants are rarer than the usual leiomyoma [85, 86].

The cellular leiomyoma exhibits an increased cellularity when compared to background myometrium with which it may present irregular borders [75]. This histological variant was proposed as the first step in the myometrium transformation into benign leiomyoma. In fact, the cellular leiomyoma exhibits low levels of ECM proteins, higher levels of CD68 positive macrophages and a greater number of leukocytes and mast cells if compared to usual leiomyoma. Maybe these features are the result in response to an inflammatory stimulus following which cells of the cellular leiomyoma differentiate into myofibroblasts leading to the upregulation of the ECM proteins that characterize usual leiomyoma hystotype. Therefore, the usual leiomyoma could be defined as the late-step of the myometrium transformation

into benign leiomyoma [17, 81, 87].
The cells belonging to cellular leiomyoma are characterized by scant cytoplasm, they do not have an intese mitotic activity and they do not exhibit atypia [75].
The lipoleiomyoma is composed, similarly to leiomyoma, of smooth muscle cells that are, in addition, mixed with mature adipocytes in variable quantity [75] with benign behavior [88].
The apoplectic leiomyoma is characterized by a central zone of hemorrhage and necrosis. The periphery of this histological variant shows increased mitotic activity and also myxoid changes can be observed (zonation phenomenon). The apoplectic leiomyoma shows an usual appearance away from the central necrosis [75].
The leiomyoma with bizarre nuclei owes its name to the fact that it exhibits bizarrely shaped, hyperchromatic, multilobulated nuclei with nuclear pseudoinclusions [75].
It is also referred to as atypical leiomyoma, or symplastic leiomyoma [89].
The leiomyoma with bizarre nuclei appears arranged in a multifocal to diffuse distribution in a background of a typical leiomyoma [90-94]. In addition to this, the leiomyoma with bizarre nuclei exhibits a variable vasculature pattern such as thick walled vessels, staghorn vessels, luminal vascular obliteration and fibrinoid necrosis. It is also characterized by alveolar edema.
This histological variant displays low mitotic activity (< 5 mitoses/10 high power fields) and absence of tumor cell necrosis [75].
This histological variant is benign, but it shows intermediate features between the leiomyoma and the leiomyosarcoma that represents the malign counterpart of the benign leiomyoma.
In fact, the malign leiomyosarcoma is a cellular tumor characterized by spindled / fascicular cells with moderate to severe pleomorphism [76]. This malign tumor often exhibits a typically high mitotic index [76], higher than 10 mitoses/10 high power fields [95] and the mitoses that can be seen are often atypical [76]. Furthermore, cells appear multinucleated [76].
The leiomyosarcoma is also characterized by a focal or, more commonly, extensive tumor cell necrosis [95].
The malign leiomyosarcoma often exhibits an infiltrative border [76].
Ultimately, benign leiomyoma and malign leiomyosarcoma can be defined as the two counterparts of the myometrium transformation. They represent two different pathologies with the same origin.
The epidemiology observations supported by hysterectomy preparations account for the fact that the usual leiomyoma can reach a diffusion equal to 96% of the patients [85, 86].

Among the other variants, the lipoleiomyoma is the most relatively common one (almost 2.3%) [86]. Therefore, the other benign variants are very rare. On the other hand, the malign leiomyosarcoma occurs in only 0,088% of the women undergone to hysterectomy for benign gynecologic indications [96]. Thus, it turns out even more clearly how much this pathology is rare [93]. Nevertheless, leiomyosarcoma represents the most common sarcoma of the female reproductive tract [97] and it is very aggressive, it can often return and it is characterized by a poor prognosis [98].

1.13 Benign leiomyoma and malign leiomyosarcoma: the problem of the differential diagnosis

In view of the whole foregoing, it is clear how important it is to carry out the differential diagnosis [99, 100] in order to discriminate between the benign leiomyoma and the malign leiomyosarcoma so that it is possible, in turn, to establish the prognosis and the treatment and operative plan according to the patient's actual state of health and to their needs.
The differential diagnosis mainly makes use of histological criteria.
In fact, first of all, the evaluation of tissue architecture by topographic haematoxylin and eosin (H&E) staining is performed [97, 101]. In detail, for the differentiation of uterine smooth muscle tumors, a histological preparation that exhibits severe cell atypia, tumor cell necrosis with island-like aspect and several mitotic figures (≥5 mitoses/10 high power fields) is classified as a malign lesion [102].
On the other hand, a histological preparation that appears well-demarcated, uniform in shape and size with the typical aspect of the smooth muscle cells and that exhibits neither cell atypia, nor tumor cell necrosis (with the exception of ischemic necrosis), nor intravascular components and that exhibits only a low mitotic index (<5 mitoses/10 high power fields) is classified as a benign lesion [102].
Nevertheless, the differential diagnosis aimed at discriminating benign from malign lesions still represents a remarkable problem since sometimes it is hard to discriminate for sure if a histological preparation is benign or malign and the diagnosis is not answered [97, 103]. In these cases the histological preparation is considered as a so called smooth muscle tumor of uncertain malignant potential (STUMP). Kempson first used this term in 1973 [104]. Currently, the WHO uses it within the classification of smooth muscle tumors to indicate a uterine smooth muscle tumor that can not be diagnosed unequivocally as benign or malign [102, 105].

All this implies an increased risk of under or over diagnosis that, in turn, affects the correct choice of treatment for the patient [106].

In particular, problems of certain diagnosis can be found with regard to the histological preparations which appear to exhibit the characteristics of the benign leiomyoma with bizarre nuclei that, as mentioned above, shows intermediate features between benign leiomyoma and malign leiomyosarcoma so that it owns both histological aspects which typically belong to benign lesions, and histological aspects which typically belong to malign lesions.

In particular, both the benign leiomyoma with bizarre nuclei and the malign leiomyosarcoma exhibit severe nuclear atypia [95]. Even if, according to some authors, in the benign leiomyoma with bizarre nuclei this would be only the result of a degenerative process [107], some other authors do not agree and, in fact, the certain discrimination based on nuclear atypia is not always possible [108].

In addition to this, these two histologically similar entities also share the expression pattern of some molecules [93] and, therefore, contrary to other pathologies, they can not be used as biomarkers.

In fact, it was demonstrated that the leiomyoma with bizarre nuclei and the leiomyosarcoma exhibit a similar miRNAs signature [93]. In particular, among the miRNAs similarly expressed by the leiomyoma with bizarre nuclei and the leiomyosarcoma, it is noteworthy to mention the miR-34 that was demonstrated to be significantly downregulated in both leiomyoma with bizarre nuclei and the leiomyosarcoma if compared to the usual leiomyoma [93].

The importance of the sharing of the miR-34 expression pattern by the leiomyoma with bizarre nuclei and the leiomyosarcoma lies in the fact that miR-34 is regulated by the protein product of the tumor protein p53 gene (TP53) [109] which in turn shows mutations in both the leiomyoma with bizarre nuclei and the leiomyosarcoma [93].

Furthermore, this is not the only alteration at the genomic level that confirms the similarity between leiomyoma with bizarre nuclei and the leiomyosarcoma. In fact, the mutations of the mediator complex subunit 12 gene (MED12) were found to be significantly less common in both leiomyoma with bizarre nuclei and leiomyosarcoma than in usual leiomyoma in which, instead, the MED12 mutations are highly frequent [93].

It was also shown that both leiomyoma with bizarre nuclei and leiomyosarcoma exhibit a statically significative higher deletion of the phosphatase and tensin homolog gene (PTEN) if compared with usual leiomyoma [93].

In addition to all this, about genome-wide copy number alterations (CNAs) that were defined as typical hallmarks of the leiomyosarcoma [110-114], up to 37 CNAs shared by the

leiomyoma with bizarre nuclei and the leiomyosarcoma were identified [95]. In detail 8 gains and 29 losses [95]. Besides, the fact that a single sample is characterized by a great number of CNAs (especially copy number losses) is a further feature that brings together the leiomyoma with bizarre nuclei and the leiomyosarcoma [95].

In light of these numerous and conspicuous similarities between the leiomyoma with bizarre nuclei and the leiomyosarcoma, it is understood that the well-described diagnostic markers that are currently available, such as Ki-67 proliferation antigen (Ki-67), p53 protein (p53), estrogens receptors (ER) and progesterone receptors (PR) [97, 115] are not entirely suitable or are not always sufficient for a conclusive and certain differential diagnosis [102].

In addition to those just mentioned, there are several other potential markers that could be taken into consideration.

Among these, it is noteworthy to mention p16 tumor suppressor protein (p16) [116-118], cyclin D1 [119, 120], Bcl-2 [121] and proliferating cell nuclear antigen (PCNA) [122] that are all proteins that control the cell cycle and influence the dynamics of proliferation; vimentin, desmin, nestin and keratin [123, 124] that are all cytoskeletal proteins; mucins [125] and galectins [125, 126] that are all proteins involved in intercellular interactions; caldesmon [127, 128], calponin [120, 127] and α-smooth muscle actin [87, 129] that are all proteins involved in muscle cell contraction.

In addition to these, it is important to mention transforming growth factors (TGFs) [130], epidermal growth factor (EGF) [126], vascular endothelial growth factor (VEGF) [131] and insulin-like growth factors (IGFs) [132] that are considered some of the most important growth factors involved in the mediation of the estrogens and progesterone action within the benign leiomyoma physiology [17, 133-136].

Nevertheless, the diagnostic applicability of all these potential markers has yet to be determined and they are currently being tested [102].

Furthermore, the search for more reliable markers is further complicated by the fact that the exact etiopathogenesis of both the benign leiomyoma, in all its variants, and its malignant counterpart, the leiomyosarcoma has not yet been clarified at all [71, 72, 81, 87, 95].

From what has been said so far, it is clear how complicated it is, but extremely useful, given the similarity of the benign leiomyoma with bizarre nuclei and the malign leiomyosarcoma, to identify new potential markers that help to discriminate with greater certainty whether a histological preparation that seems to exhibit the features of a benign leiomyoma with bizarre nuclei, is not, actually a malign lesion.

In this regard, Raf kinase inhibitor protein (RKIP) could turn out to be another particularly interesting molecule to study.

1.14 RKIP: a pleiotropic molecule that can be also an independent prognostic marker

RKIP, also known as phosphatidylethanolamine-binding protein 1 (PEBP1) and originally purified from bovine brain, is a cytosolic protein. It is a small protein (only 23 kDa). The human RKIP mRNA consists of 1434 base pairs and it just encodes a 187 amino acid long protein. Of these 187 amino acids, 186 overlap with those of bovine RKIP protein (21 kDa) and all 187 amino acids of the human RKIP protein overlap with those of rat RKIP protein (23 kDa). In fact, RKIP is considered a highly conserved protein. On the other hand, RKIP does not share a significant similarity with other proteins [137, 138].

RKIP can be defined, in all respects, a pleiotropic protein. In fact, from a functional point of view, it is known to be involved in the modulation of several signaling pathways that contribute to maintain the biological balance of many cellular processes [139, 140].

In detail, among the signaling cascades modulated by RKIP the best known ones are described below and they are represented in Panel A.

In nuclear factor kappa B (NFκB) pathway, it was demonstrated that RKIP acts as negative modulator. RKIP binds to upstream kinases NIK, TAK, IKKα, and IKKβ so that IkappaBα (IκBα) phosphorylation is inhibited and this, in turn, impairs NF-κB translocation to the nucleus and the consequent transcription of several anti-apoptotic genes [141, 142].

In addition, RKIP was identified as a negative modulator of neurogenic locus notch homolog protein 1 (NOTCH1) pathway. NOTCH1 full-length proteolytic cleavage is impaired by RKIP association with it and this, in turn, avoids NOTCH1 intracellular domain (NICD) release preventing the consequent transcription of Vimentin, N-cadherin and zinc finger protein SNAI1 (SNAIL) that are all epithelial-mesenchymal transition (EMT)-related proteins [143].

Furthermore, within the mitogen-activated protein (MAP) kinase (MAPK) cascades where Raf acts as MAP3 kinase (MAP3K), MAPK/ extracellular signal-regulated kinase (ERK) kinase (MEK) acts as MAPK kinase (MAPKK) and ERK is the MAPK, forming the Ras-Raf-MEK-ERK pathway [144], it was demonstrated that RKIP acts as an endogenous inhibitor of Raf–MEK–ERK pathway. In fact, the RKIP binding to Raf-1 kinase promotes the dissociation of the Raf-1/MEK complex so that MEK can not be phosphorylated [145-147].

In addition to this, RKIP can be phosphorylated by protein kinase C (PKC) and as a result, phosphorylated RKIP (pRKIP) dissociates from Raf-1 and binds to G protein-coupled receptor kinase 2 (GRK2) that is an inhibitor of G-protein coupled receptors (GPCR) [148] that is, in turn, an upstream activator of Raf-1. Thus, the binding between pRKIP and GRK2 inhibits GRK2 and, consequently, activates GPCR. At the same time, the association

between pRKIP and GRK2 increases MAPK activation also due to the fact that Raf-1 is no longer inhibited by RKIP. All this allows the activation of the downstream targets. In this way, RKIP carries out its action of modulation of cellular processes in response to growth factors stimuli [145].

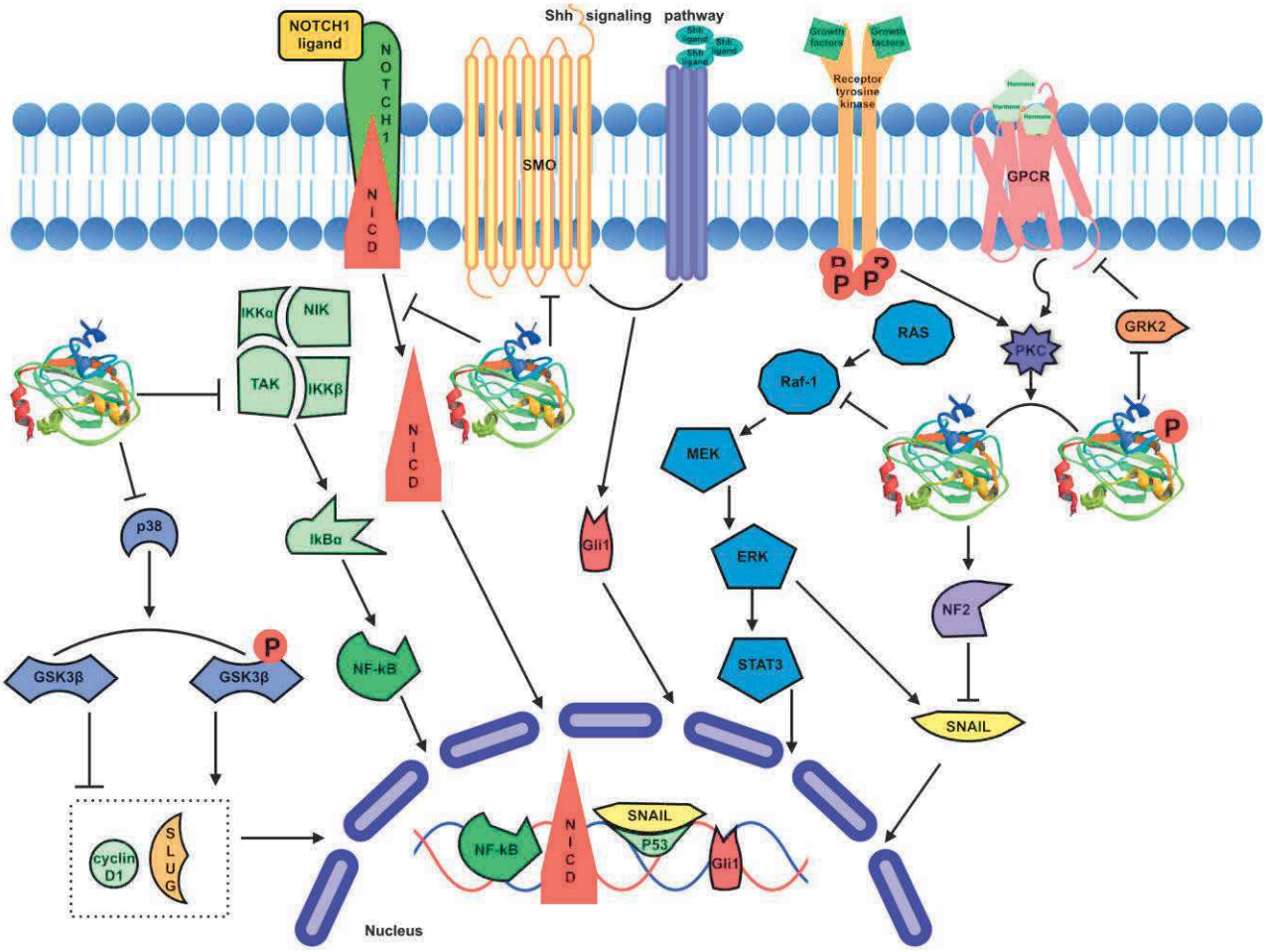
Moreover, it was demonstrated that RKIP action as an endogenous inhibitor of Raf–MEK–ERK pathway also affects the negative modulation of signal transducer and activator of transcription 3 (STAT3) that is a transcription factor involved in the regulation of the transcription of genes related with cell growth, apoptosis, survival and differentiation [149]. In detail, it was reported that RKIP impairs STAT3 activation through the inhibition of Raf and other molecules [150, 151].

In addition to this, studies conducted on malign pleural mesothelioma (MPM) revealed that, in particular in this type of tumor, RKIP acts as a modulator of p53 [152] that is a transcription factor involved in the regulation of tumor suppression genes [153]. In detail, it was shown that in MPM and non-small cell lung carcinoma (NSCLC) cell lines treated with silica the consequent RKIP depletion implies an increase in ERK activation and the inactivation of neurofibromatosis 2 (NF2) protein. This, in turn, leads to SNAIL expression with the consequent p53 inhibition. In this way the tumor can progress [152]. Moreover, SNAIL expression also inhibits E-cadherin. In this way, invasion and metastasis are promoted [152].

Furthermore, RKIP was described as a negative modulator of the Sonic hedgehog (Shh) signaling pathway that is involved in the regulation of proliferation, differentiation, ECM interactions and cancer stem cells (CSCs) activation. In fact, it was shown that when RKIP binds to the signal transducer Smoothened (SMO), the latter is kept inactive and, consequently, the zinc-finger transcription factor Gli1 that is a transcriptional effector at the end of the Shh signaling pathway is not activated [154]. Thus, RKIP impairs the activity of Gli1 [154] whose aberrant activation was described to be involved, in some cancer types, in the promotion of proliferation, survival, angiogenesis, metastasis, metabolic rewiring, and chemotherapeutic resistance [155].

In addition to this, RKIP was demonstrated to activate the glycogen synthase kinase 3 beta (GSK3β) signaling pathway that was shown to play a fundamental role in cell division, proliferation, differentiation, apoptosis, adhesion and motility [156]. In fact, RKIP impairs p38 MAPK-mediated phosphorylation of GSK3β (pGSK3β) and, thus, it stabilizes GSK3β that, in the not phosphorylated form, can inhibit several substrates such as zinc finger protein SNAI2 (SLUG) and cyclin D1, inducing their degradation and ubiquitination by phosphorylation. In this way, when GSK3β is not phosphorylated and, thus, active, SLUG

can not promote EMT and cyclin D1 can not promote cell cycle progression [156].
Moreover, it was reported that the RKIP interactions with several signaling pathways may affect also inflammatory processes [157]. In these, depending on the pathway and the molecules (proteins and likely lipids) with which it interacts, RKIP may exert a anti- or a pro-inflammatory role [157].



Panel A. RKIP: a pleiotropic molecule. The best known signaling cascade pathways modulated by RKIP. The RKIP molecule was obtained by the Swiss PDB viewer according to PDB code 2LZW.

Therefore, since the important modulation action that the RKIP carries out in many different intracellular signaling pathways that, in turn, control several cellular processes, it is evident how a dysregulation in the RKIP expression is implicated in several diseases [138, 158].
In this regard, RKIP was demonstrated to be involved in several inflammatory diseases, such as rheumatoid arthritis [159], primary Sjögren's syndrome (pSS) [160], liver injury [161], diabetic nephropathy [162], lung inflammation [163], chronic intestinal inflammation [164], experimental autoimmune encephalomyelitis (EAE) that is an experimental model for

multiple sclerosis [165], systemic inflammatory response syndrome (SIRS) [166] and colitis [164].

In the light of what has been said, it is evident that RKIP could play an important role in the benign leiomyoma of which the most widespread histological variant, the usual leiomyoma, was described as a typical fibrotic pathology [167]. In fact, it was demonstrated that it exhibits a great deal of ECM [167] maybe due to an exaggerated fibrotic response [83] that arises by means of inflammatory signals [81].

In addition to this, it was demonstrated that a dysregulation in the RKIP expression is involved also in cancer [139, 140].

In fact, in carcinogenesis, it was shown that RKIP plays a modulating role in cell viability [168, 169], migration [170, 171], EMT [172] and invasion [173].

Moreover, it was demonstrated that the loss of RKIP expression is associated with poor prognosis and so, RKIP can be considered as an independent prognostic marker in several tumoral types such as esophageal cancer [174-176], gastric cancer [177, 178], colorectal cancer [179-181], Gastrointestinal stromal tumors (GISTs) [182], prostate [183], hepatocellular carcinoma [184], pancreatic ductal adenocarcinoma [185-187], gliomas [188, 189].

In the light of what has been said, it is evident that RKIP could play an important role in the malign leiomyosarcoma that, although very rare, shows a very poor prognosis [10]. The research group within which this doctorate has taken place, previously published that the RKIP is expressed in greater quantities in the benign usual leiomyoma when compared to the corresponding myometrium [190]. On the other hand, the expression of RKIP has never been investigated, neither in the other histological variants of benign leiomyoma nor in the malign leiomyosarcoma.

Therefore, it is very important to study RKIP within benign leiomyoma and malign leiomyosarcoma that are two different pathologies with the same origin since they both arise from myometrium and whose etiology has not yet been clarified at all [71, 72, 81, 87, 95].

1.15 Aim of the thesis

As previously mentioned, given the similarity of the benign leiomyoma with bizarre nuclei and the malign leiomyosarcoma and, because of this, the absence of certain markers, it is clear how complicated it is, but extremely useful, to identify new potential markers for a certain differential diagnosis in order to solve the so called STUMP.

By virtue of the several cellular processes reported above, in which RKIP is involved, the focus of this thesis was the study of the pleiotropic molecule RKIP in benign leiomyoma and malign leiomyosarcoma.

The aim was to identify any differences in the expression of RKIP between benign leiomyoma, in particular in the histological variant leiomyoma with bizarre nuclei, and malign leiomyosarcoma and then, to characterize the possible biological role of RKIP in malign leiomyosarcoma.

All this with the final aim of identifying the RKIP as a possible new reliable marker for the differential diagnosis of benign leiomyoma with bizarre nuclei and malign leiomyosarcoma even in doubtful cases.

2. MATERIALS AND METHODS

2.1 Patients

Formalin-fixed paraffin-embedded blocks from 27 hysterectomies performed due to benign leiomyoma were retrieved from the pathology archives of Fondazione Policlinico Universitario A. Gemelli, IRCCS, Roma, Italy and formalin-fixed paraffin-embedded blocks from 12 hysterectomies performed due to malign leiomyosarcoma were retrieved from the pathology archives of Azienda Ospedaliera Ospedali Riuniti di Ancona, Università Politecnica delle Marche (UNIVPM), Ancona, Italy.
The samples were classified by pathological anatomy specialists (Fondazione Policlinico Universitario A. Gemelli, IRCCS, Roma, Italy and Azienda Ospedaliera Ospedali Riuniti di Ancona, Università Politecnica delle Marche (UNIVPM), Ancona, Italy). Thus, in detail, the 27 benign leiomyomas include the following histological variants: usual leiomyoma (N=7), cellular leiomyoma (N=9), lipoleiomyoma (N=3), apoplectic leiomyoma (N=4) and leiomyoma with bizarre nuclei (N=4), in addition to the malign leiomyosarcoma (N=12).
In total, the 39 samples were collected from 2015 to 2018.

2.2 Cell lines and cell culture procedures

In the present study the SK-LMS-1 cell line was used as leiomyosarcoma cell line.
The SK-LMS-1 cell line was obtained from ATCC (American Type Culture Collection (Manassas, VA, USA).
This cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM 1X, High Glucose; Gibco, Invitrogen, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, invitrogen) and 1% penicillin/streptomycin (P/S; Gibco, invitrogen), at 37°C and 5% CO_2.

2.3 Haematoxylin and eosin (H&E) staining

Representative 3 mm-thick tissue sections were used for H&E staining. The deparaffinised and rehydrated slides were stained with Mayer's haematoxylin (incubation 2 minutes; Bio-Optica, Milan, Italy). After this, the slides were washed in tap water. Subsequently, the slides were stained with eosin (incubation 2 minutes; Bio-Optica). After this, the slides were washed in tap water.

2.4 Immunohistochemistry analysis of RKIP

3 mm-thick tissue sections were used for immunohistochemical analysis according to the streptavidin-biotin peroxidase complex system (VECTASTAIN® ABC-HRP Kit; Vector Laboratories, Burlingame, CA, USA).

The deparaffinised and rehydrated slides were submitted to heat-induced antigen retrieval for 20 minutes at 100°C with 1 mM EDTA buffer (pH 8.0). Then, the slides were submitted to the inhibition of the endogenous peroxidase activity for 1 hour at room temperature (RT) with 3% hydrogen peroxide in methanol. Subsequently, the slides were washed for 10 minutes for 3 times in phosphate buffered saline (PBS) homemade (pH 7.2). Then, the slides were incubated with normal goat serum (NGS, dilution 1:75 in 1% bovine serum albumin - BSA; Sigma-Aldrich, St. Louis, MO, USA-, incubation 20 minutes at RT; Vector Laboratories). After this, the slides were incubated with the primary polyclonal rabbit antibody raised against RKIP (dilution 1:500, incubation overnight at 4°C; Merck-Millipore, Burlington, MA, USA). Afterwards, the slides were washed for 10 minutes for 3 times in PBS homemade (pH 7.2). Then, the slides were incubated with the secondary biotinylated goat anti-rabbit antibody (dilution 1:200, incubation 30 minutes at RT; Vector Laboratories). After this, the slides were washed for 10 minutes for 3 times in PBS homemade (pH 7.2). Subsequently, the slides were incubated with the streptavidin-biotin peroxidase complex (dilution 1:100, incubation 1H at RT; Vector Laboratories). Afterwards, the slides were washed for 10 minutes for 3 times in PBS homemade (pH 7.2). The immune reaction was visualized by 3,3'-Diamonobenzidine hydrochlorides (DAB, incubation 2 minutes at RT; Sigma-Aldrich) as a chromogen. All sections were counterstained with Mayer's haematoxylin (few seconds; Bio-Optica). For negative controls, primary antibody was omitted.

Sections were scored by three independent assessors for cytoplasmic expression. The score was assigned according to the staining intensity (-, negative; +/-, moderate expression; +, high expression; ++, very high expression).

2.5 Immunohistochemistry analysis of CD68

For the immunohistochemistry analysis of CD68, it was used the same protocol that was previously described about the immunohistochemistry analysis of RKIP with the following variations: the deparaffinised and rehydrated slides were not submitted to antigen retrieval;

the inhibition of the endogenous peroxidase activity was performed for 5 minutes at RT with 3% hydrogen peroxide in distilled water (dH_2O) and after this, the slides were washed for 5 minutes in dH_2O; the slides were incubated with normal horse serum (NHS, dilution 2% in PBS homemade, incubation 20 minutes at RT; Vector Laboratories); the primary monoclonal mouse antibody raised against CD68 clone KP1 (dilution 1:200, incubation overnight at 4°C; DAKO, Copenaghen, Denmark) was used; the secondary biotinylated horse anti-mouse antibody (dilution 1:200, incubation 30 minutes at RT; Vector Laboratories) was used; DAB (incubation minute at RT; Vector Laboratories) was used; sections were not scored for CD68 staining because it was performed a qualitative analysis.

2.6 Immunocytochemistry analysis of RKIP

For RKIP immunocytochemical analysis of leiomyosarcoma cell lines, the cells were plated on 8-chamber slides at a density of 50 or 100 cells per well, allowed to adhere overnight and then they were cultured to at least 95% of confluence in the complete culture medium as specified previously. Subsequently, the cells were washed for few seconds in Dulbecco's PBS w/o Calcium & Magnesium (EuroClone, Milan, Italy). Then the cells were fixed in methanol for 20 minutes. After this, the cells were washed for few seconds for 3 times in Dulbecco's PBS w/o Calcium & Magnesium (EuroClone), followed by permeabilization with 0.2% Triton X-100 in Dulbecco's PBS w/o Calcium & Magnesium (EuroClone) for 5 minutes at RT. Afterwards, the fixed cells were washed for few seconds for 3 times in Dulbecco's PBS w/o Calcium & Magnesium (EuroClone). After that, starting from the inhibition of the endogenous peroxidase activity onwards, for immunocytochemical analysis of RKIP, it was used the same protocol that was previously described about the immunohistochemistry analysis of RKIP with the following variations: the inhibition of the endogenous peroxidase activity was performed for 10 minutes at RT with 0,1% hydrogen peroxide in dH_2O; the primary polyclonal rabbit antibody raised against RKIP (dilution 1:500, incubation 1H at RT; Merck-Millipore); the washes were carried out in the same phases but using Dulbecco's PBS w/o Calcium & Magnesium (EuroClone) for few seconds for 3 times. It was performed a qualitative analysis.

2.7 Generation of stable RKIP knockout SK-LMS-1 cell line and stable RKIP overexpressing SK-LMS-1 cell line

For generation of stable RKIP knockout SK-LMS-1 cell line, the SK-LMS-1 cell line was knocked out using the CRISPR/Cas9 technology. For that, the CRISPR/Cas9 knockout kit (CRISPR/Cas9 KO Plasmid - sc-401270-KO-2 and HDR Plasmid – sc-401270-HDR-2; Santa Cruz Biotechnology, Dallas, TX, USA) was used. For RKIP knockout, cells were transfected with both plasmids. As control, other cells were transfected only with HDR plasmid.

For generation of stable RKIP overexpressing SK-LMS-1 cell line, a pcDNA3 vector (kindly provided by Dr. Evan Keller, University of Michigan) containing the full cDNA of RKIP was used in the SK-LMS-1 cell line. The cells were transfected with the empty vector as control and with RKIP full cDNA containing vector to overexpress it.

For both transfections, the cells were plated into 6-well plates at a density of $5x10^5$ cells per well, allowed to adhere overnight and transfected in serum free Opti-MEM medium (Gibco, Invitrogen) for 24 hours. The transfection was done using the FUGENE HD reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol, with 2µg of plasmid at a ratio of 6:2 (reagent:plasmid). Then, the stable transfectants were selected with 1µg/ml puromycin (Gibco, Invitrogen) for knockout transfection and 1000 µg/ml geneticin (G418; Santa Cruz Biotechnology) for overexpression transfection.

2.8 Western Blot analysis

The cells (wilde type SK-LMS-1 cells, stable RKIP knockout SK-LMS-1 cells and the corresponding negative controls cells, stable RKIP overexpressing SK-LMS-1 cells and the corresponding negative controls cells) were seeded in 6-well plates at a density of $1x10^6$ cells per well and allowed to adhere overnight in DMEM (Gibco, Invitrogen) 10% FBS (Gibco, Invitrogen) 1% P/S (Gibco, Invitrogen). In the next day, the cells were serum starved for 2 hours before protein isolation. The cells were scraped in cold PBS (Gibco; Invitrogen) and lysed in buffer containing 50 mM Tris pH 7.6–8, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 10 mM NaPyrophosphate, 1% NP-40 and 1/7 of Protease cocktail inhibitors (Roche). Western blotting was done using 10% SDS-PAGE gel, loading 40 µg of total protein per lane, with detection by chemiluminescence (WesternBright Sirius HRP substrate, Advansta, San Jose, CA, USA) using Sapphire Biomolecular Imager (Azure Biosystems, Dublin, CA, USA).

The expression of the analyzed proteins was assessed using the antibodies that are shown in the table below (Table A) that includes the names, the characteristics, the dilutions and the manufacturers of each primary antibody (Ab I) and the information about each corresponding secondary antibody (Ab II). All the primary antibodies were incubated overnight at 4°C. α-Tubulin was used as a housekeeping gene.

Protein	Ab I	Dilution	Monoclonal or polyclonal	Manufacturer	Ab II	Dilution	Manufacturer
RKIP	D42F3	1:1000	Monoclonal	Cell Signaling (Danvers, MA, USA)	Anti-rabbit IgG, HRP-linked Antibody 7074	1:2500	Cell Signaling (Danvers, MA, USA)
α-Tubulin	α Tubulin Antibody (B-7): sc-5286	1:5000	Monoclonal	Santa Cruz Biotechnology (Dallas, TX, USA)	m-IgGκ BP-HRP: sc-516102	1:2500	Santa Cruz Biotechnology (Dallas, TX, USA)

Table A. Antibodies used for western blot analysis

2.9 Cell viability assay

The cells were seeded in 48-well plates in triplicate at a density of $2x10^4$ cells per well and allowed to adhere overnight in DMEM (Gibco, Invitrogen) 10% FBS (Gibco, Invitrogen) 1% P/S (Gibco, Invitrogen). In the next day, the cells were submitted to two different medium conditions – DMEM (Gibco, Invitrogen) 0.5% FBS (Gibco, Invitrogen) 1% P/S (Gibco, Invitrogen) or DMEM (Gibco, Invitrogen) 10% FBS (Gibco, Invitrogen) 1% P/S (Gibco, Invitrogen)- and incubated for 24, 48 and 72 hours. The day of conditions imposition was considered the 0h time point. The viable cells were quantified overtime by Sulforhodamine B (SRB) assay (Sigma-Aldrich).

The results were calibrated to the starting value (time 0h, considered as 100% of viability) and expressed as the mean ± SD. The assays were done in triplicate for four times.

2.10 Wound healing migration assay

The cells were seeded in 6-well plates, allowed to adhere overnight and cultured to at least 95% of confluence in DMEM (Gibco, Invitrogen) 10% FBS (Gibco, Invitrogen) 1% P/S (Gibco, Invitrogen). Monolayer cells were washed with PBS (Gibco, Invitrogen), scrapped with a plastic 1000 µl pipette tip and then incubated with fresh DMEM (Gibco, Invitrogen) medium 1% P/S (Gibco, Invitrogen) with either 0.5% FBS (Gibco, Invitrogen) or 10% FBS

(Gibco, Invitrogen). The “wounded” areas were photographed by phase contrast microscopy at specific time points: 0, 12, 24, 36, 48 and 72 hours. The relative migration distance was calculated by the following formula: percentage of wound closure (%) = 100 (A-B)/A, where A is the width of the cell wounds before incubation (0h) and B is the width of the cell wounds after incubation. The results are expressed as the mean ± SD. The assay was done in quadruplicate for three times.

2.11 Clonogenicity assay

The cells were seeded in 12-well plates at a density of 700 cells per well and they were allowed to adhere overnight in DMEM (Gibco, Invitrogen) 10% FBS (Gibco, Invitrogen) 1% P/S (Gibco, Invitrogen). Then, they were left to grow for 9-11 days -DMEM (Gibco, Invitrogen) 10% FBS (Gibco, Invitrogen) 1% P/S (Gibco, Invitrogen)- with medium renewal after 3 days. The colonies were stained with 0,5% Crystal Violet (Sigma-Aldrich) for 30 minutes and manually counted. Results were expressed as the mean colonies ± SD. The assays were done in triplicate at least two times.

2.12 Statistical analysis

For *in vitro* assays, single comparisons between the different conditions studied were done using Student’s t test. Statistical analysis was performed using GraphPad Prism 6 version. The level of significance in all the statistical analysis was set at $p<0.05$.

3. RESULTS

3.1 Characterization of RKIP expression in five different histological variants of benign leiomyoma and in malign leiomyosarcoma

In the present study, 39 myometrium lesions belonging to five different histological variants of benign leiomyoma and to malign leiomyosarcoma were studied for RKIP immunohistochemical expression. In detail, 27 lesions had been classified as benign leiomyoma whose 7 as usual leiomyoma, 9 as cellular leiomyoma, 3 as lipoleiomyoma, 4 as apoplectic leiomyoma and 4 as leiomyoma with bizarre nuclei. The remaining 12 lesions had been classified as malign leiomyosarcoma (Table 1).

In general, RKIP expression was found in 64,1% (25/39) of all the lesions. Specifically in 81,5% (22/27) of all the benign lesions and in 25% (3/12) of all the malign lesions.

It was noticed that RKIP was moderately expressed in 14,3% (1/7) and highly expressed in the remaining 85,7% (6/7) of benign usual leiomyomas. Thus, 100% (7/7) of these samples were classified as positive (Table 1). The positivity was particularly localized in the endothelial cells (Figure 1B, C and D).

It was observed that RKIP was not expressed in 44,4% (4/9) and, on the other hand, it was moderately expressed in the remaining 55,6% (5/9) of benign cellular leiomyomas. Thus, 44,4% (4/9) of these samples were classified as negative, while the remaining 55,6% (5/9) were classified as positive (Table 1). The positivity was particularly localized in the endothelial cells (Figure 1F, G and H).

It was detected that RKIP was highly expressed in 33,3% (1/3) and very highly expressed in the remaining 66,7% (2/3) of benign lipoleiomyomas. Thus, 100% (3/3) of these samples were classified as positive (Table 1). The positivity was particularly localized in adipose cells with inflammation (Figure 1J, K and L). The presence of inflammation in adipose cells was confirmed by immunohistochemistry for CD68 (Figure 2A, B, C and D).

It was seen that RKIP was not expressed in only 25% (1/4) and, on the other hand, it was from moderately expressed to very highly expressed in the remaining 75% (3/4) of benign apoplectic leiomyomas. Thus, 25% (1/4) of these samples were classified as negative, while the remaining 75% (3/4) were classified as positive (Table 1) (Figure 1N, O, and P).

Further images about RKIP expression in benign usual leiomyoma, benign cellular leiomyoma, benign lipoleiomyoma and benign apoplectic leiomyoma are shown in Supplemental Figure 1.

It was noticed that RKIP was moderately expressed in only 25% (1/4) and, on the other hand, it was from highly expressed to very highly expressed in the remaining 75% (3/4) of benign leiomyomas with bizarre nuclei. Thus, 100% (4/4) of these samples were classified as positive (Table 1). The positivity was particularly localized in the atypical nuclei (Figure 1R, S and T).

It was detected that RKIP was from moderately expressed to highly expressed (in the endothelial cells) in only 25% (3/12) and, on the other hand, it was not expressed in the remaining 75% (9/12) of malign leiomyosarcomas. Thus 25% (3/12) of these samples were classified as positive, while the remaining 75% (9/12) were classified as negative (Table 1) (Figure 1V, W and X).

In particular, it was observed that nuclear atypia zones were positive for RKIP in benign leiomyoma with bizarre nuclei, whereas nuclear atypia zones were negative for RKIP in malign leiomyosarcoma (Supplemental Figure 2).

Representative negative controls of the immunohistochemistry analysis in the tested tissues are shown in Supplemental Figure 3.

Table 1. RKIP expression in five different histological variants of benign leiomyoma and in malign leiomyosarcoma.

Histological type	N	Negative (%)	Positive (%)
Benign usual leiomyoma	7	0 (0)	7 (100)
Benign cellular leiomyoma	9	4 (44,4)	5 (55,6)
Benign lipoleiomyoma	3	0 (0)	3 (100)
Benign apoplectic leiomyoma	4	1 (25)	3 (75)
Benign leiomyoma with bizarre nuclei	4	0 (0)	4 (100)
Malign leiomyosarcoma	12	9 (75)	3 (25)

N: Number of cases.

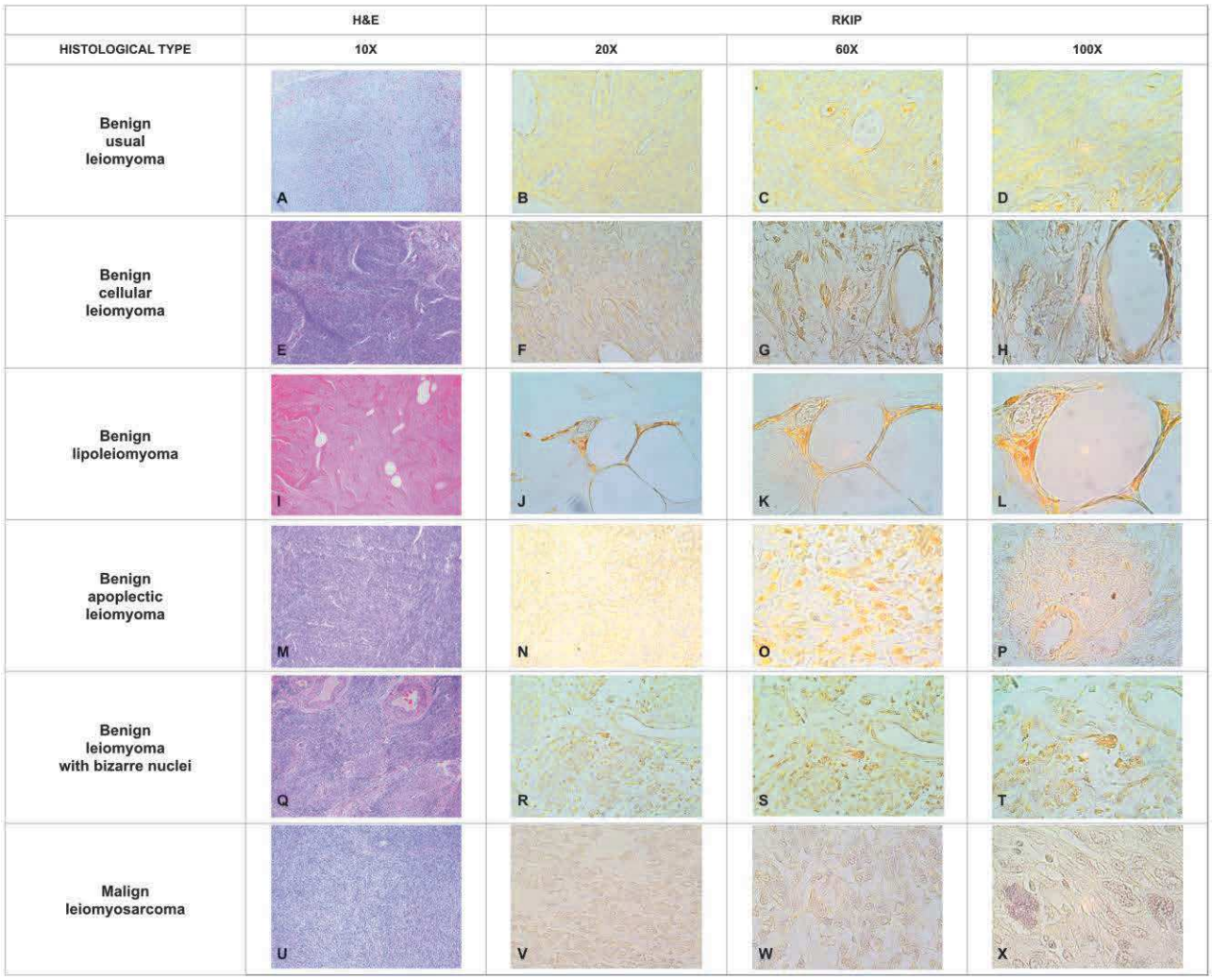


Figure 1. Immunohistochemistry analysis of RKIP in five different histological variants of benign leiomyoma and in malign leiomyosarcoma. Preliminary topographical staining in benign usual leiomyoma **(A)**, in benign cellular leiomyoma **(E)**, in benign lipoleiomyoma **(I)**, in benign apoplectic leiomyoma **(M)**, in benign leiomyoma with bizarre nuclei **(Q)** and in malign leiomyosarcoma **(U)**. RKIP positive expression in benign usual leiomyoma **(B)**, **(C)** and **(D)**. RKIP positive expression in benign cellular leiomyoma **(F)**, **(G)** and **(H)**. RKIP positive expression in benign lipoleiomyoma **(J)**, **(K)** and **(L)**. RKIP positive expression in benign apoplectic leiomyoma **(N)**, **(O)** and **(P)**. RKIP positive expression in benign leiomyoma with bizarre nuclei **(R)**, **(S)** and **(T)**. RKIP negative expression in malign leiomyosarcoma **(V)**, **(W)** and **(X)**. All the pictures were taken with at the magnification specified on the top of each column.

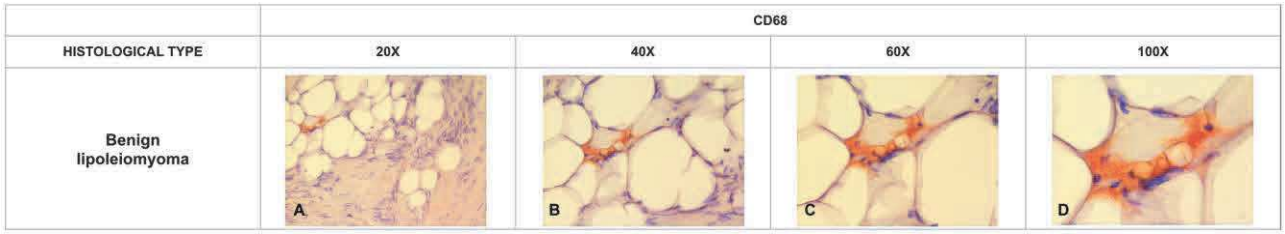
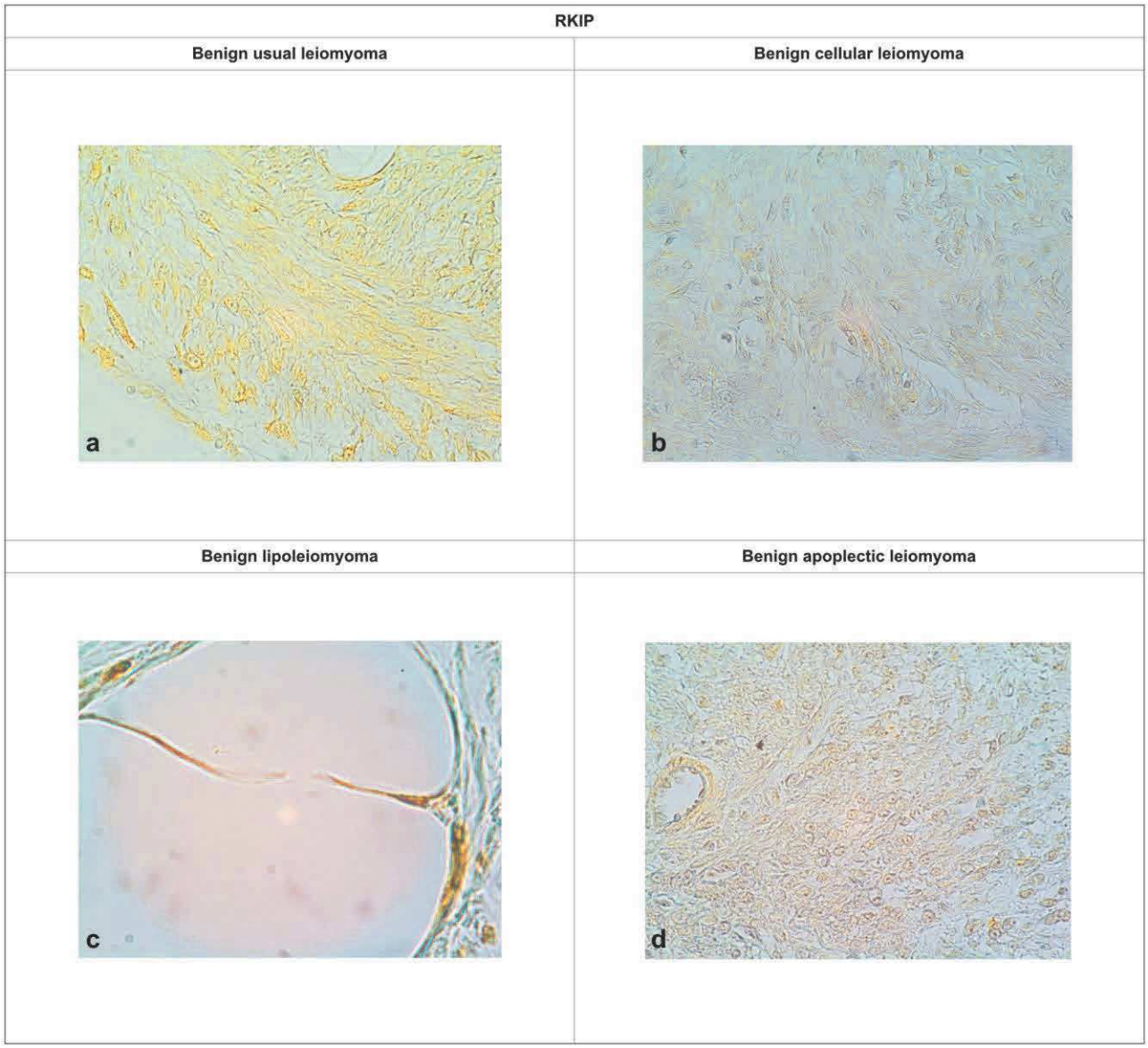
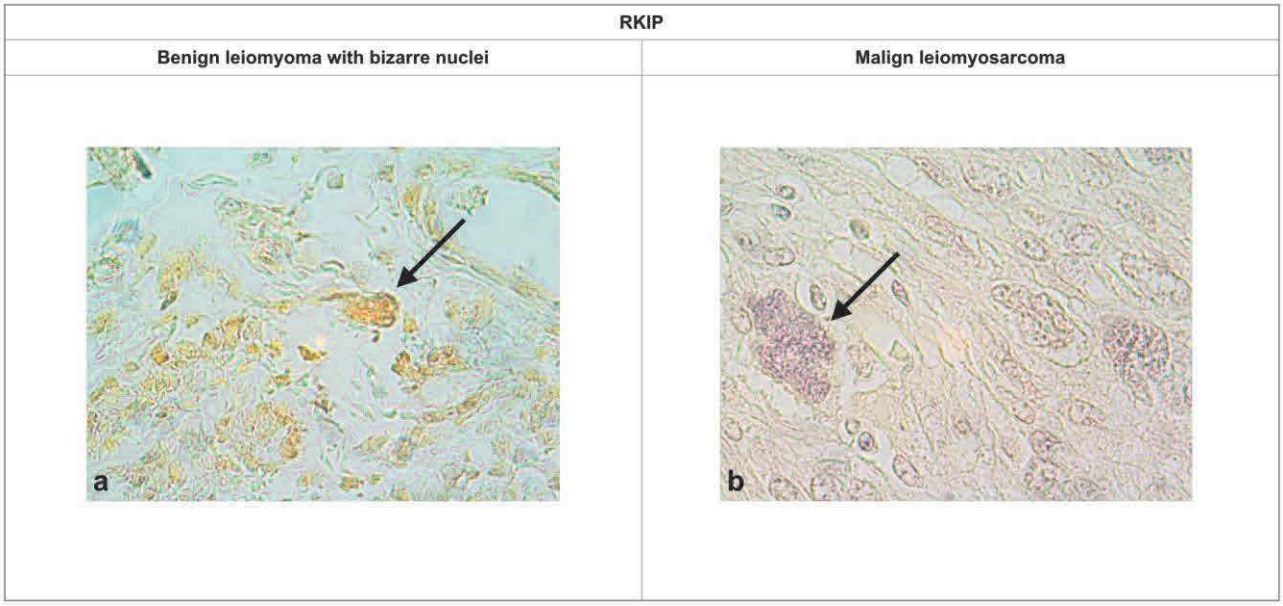


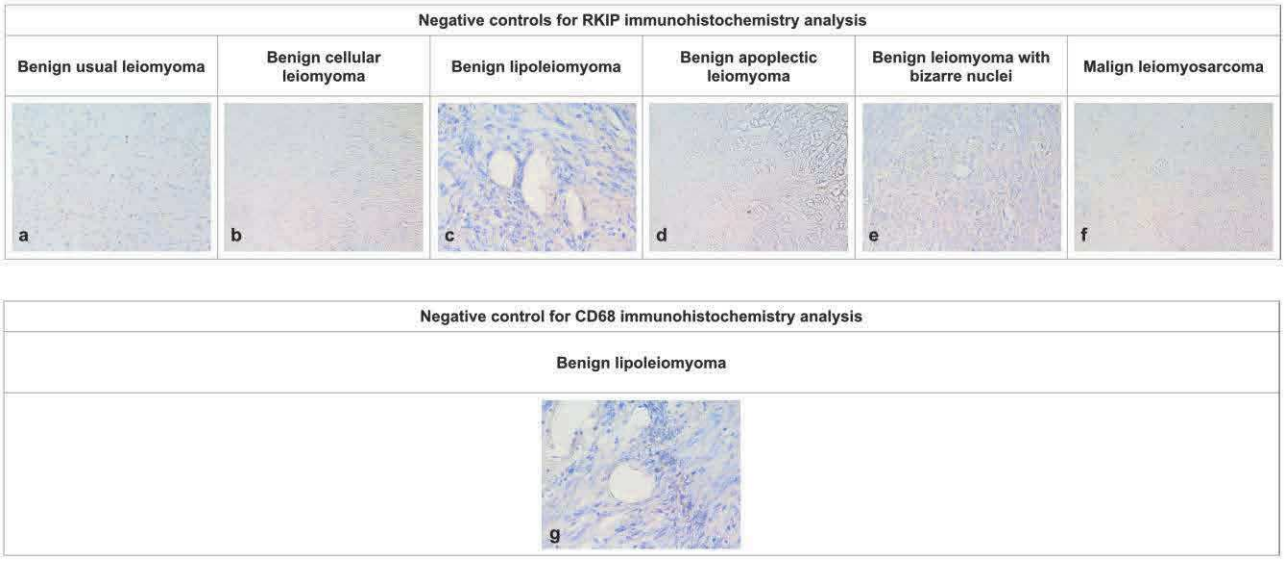
Figure 2. Immunohistochemistry analysis of CD68 in benign lipoleiomyoma. CD68 positive expression in benign lipoleiomyoma showing the typical structures of the inflamed adipose cells, the so called "crown like structures"**(A)**, **(B)**, **(C)** and **(D)**. This result confirmed the presence of inflammation in the adipose cells of benign lipoleiomyoma. All the pictures were taken with at the magnification specified on the top of each column.



Supplemental Figure 1. Additional images about immunohistochemistry analysis of RKIP in benign usual leiomyoma, benign cellular leiomyoma, benign lipoleiomyoma and benign apoplectic leiomyoma. RKIP positive expression in benign usual leiomyoma **(a)**. RKIP positive expression in benign cellular leiomyoma **(b)**. RKIP positive expression in benign lipoleiomyoma **(c)**. RKIP positive expression in benign apoplectic leiomyoma **(d)**. All the pictures were taken with at the magnification 60X.



Supplemental Figure 2. Focus on the comparison between benign leiomyoma with bizarre nuclei and malign leiomyosarcoma regarding the RKIP expression following the immunohistochemistry analysis. RKIP positive expression in benign leiomyoma with bizarre nuclei **(a)** and RKIP negative expression in malign leiomyosarcoma **(b)**. Black arrows indicate nuclear atypia zones positive for RKIP in benign leiomyoma with bizarre nuclei **(a)** and nuclear atypia zones negative for RKIP in malign leiomyosarcoma **(b)**. All the pictures were taken with at the magnification 100X.



Supplemental Figure 3. Negative controls for immunohistochemistry analysis. Representative negative controls of the immunohistochemistry analysis for RKIP in benign usual leiomyoma **(a)**, benign cellular leiomyoma **(b)**, benign lipoleiomyoma **(c)**, benign apoplectic leiomyoma **(d)**, benign leiomyoma with bizarre nuclei **(e)** and malign leiomyosarcoma **(f)**. Representative negative control of the immunohistochemistry analysis for CD68 in benign lipoleiomyoma **(g)**. The negative controls were obtained by omitting the primary antibody. All the pictures were taken with at the magnification 40X.

3.2 Characterization of RKIP expression in leiomyosarcoma cell line

Before further exploring the biological role of RKIP in leiomyosarcoma cells, the expression of RKIP was preliminarily assessed in SK-LMS-1 leiomyosarcoma cell line.
By immunocytochemistry, it was found that RKIP was present in SK-LMS-1 cells (Figure 3). This was confirmed by western blot analysis (data not shown).

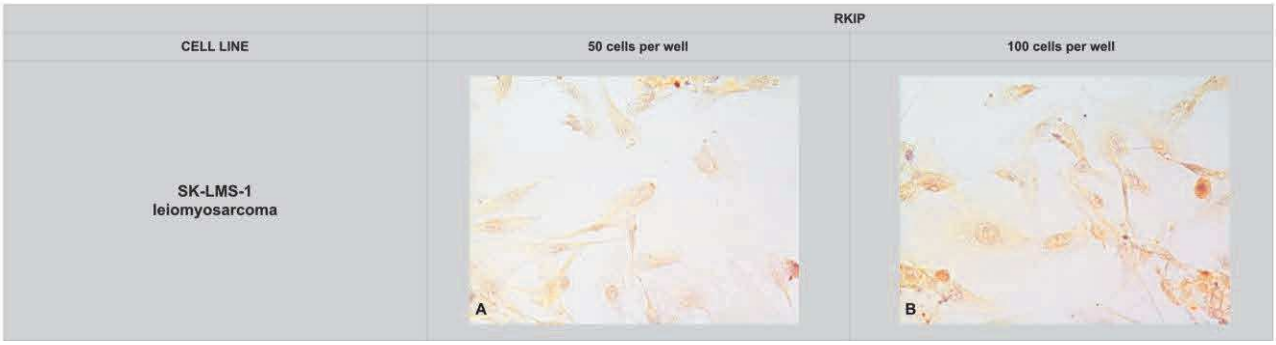


Figure 3. Immunocytochemistry analysis of RKIP in leiomyosarcoma cell line. RKIP positive expression in SK-LMS-1 leiomyosarcoma cell line **(A)** and **(B)**. The seeding density of the cells was specified on the top of each column.

3.3 Effect of RKIP on leiomyosarcoma cell biological behavior *in vitro*

To explore the biological role of RKIP in leiomyosarcoma cells, the *in vitro* knockout of RKIP and also the *in vitro* overexpression of RKIP were performed in the SK-LMS-1 cell line. The knockout of RKIP was carried out by stable transfection using the CRISPR/Cas9 technology. The overexpression of RKIP was carried out by stable transfection using a specific pcDNA3 vector. By western blot analysis (Figure 4), it was confirmed that both the transfections had successfully occurred. In fact, as shown in Figure 4, RKIP protein expression was efficiently impaired in the RKIP knockout SK-LMS-1 cells (KO cells) in comparison with the control SK-LMS-1 cells transfected only with the HDR plasmid (CTR cells). In addition to this, as shown in Figure 4, RKIP protein expression was efficiently increased in the RKIP overexpressing SK-LMS-1 cells (RKIP+ cells) in comparison with the control SK-LMS-1 cells transfected with the empty pcDNA3 vector (Empty cells).

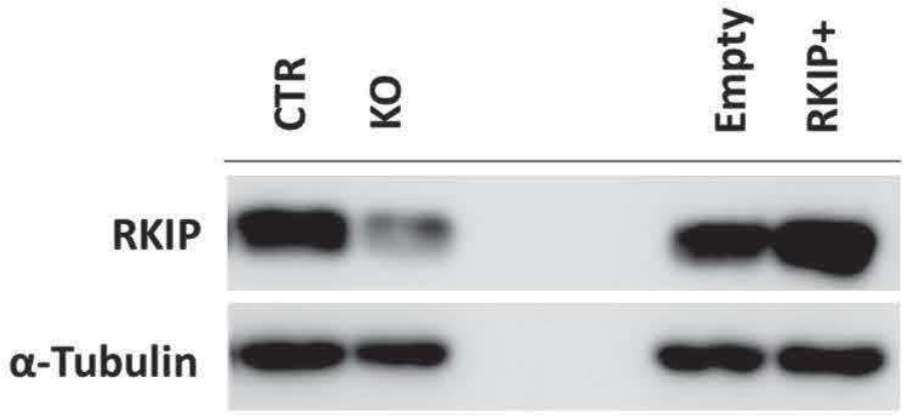


Figure 4. *In vitro* knockout of RKIP and *in vitro* overexpression of RKIP in the SK-LMS-1 cell line. Western blot analysis confirming the transfections success. Stable RKIP knockout SK-LMS-1 cells (KO cells) and stable RKIP overexpressing SK-LMS-1 cells (RKIP+ cells) were generated. RKIP protein expression was efficiently impaired in the KO cells in relation to the control cells transfected only with the HDR plasmid (CTR cells). RKIP protein expression was efficiently increased in the RKIP+ cells in relation to the control cells transfected with the empty pcDNA3 vector (Empty cells). α-Tubulin was used as a housekeeping gene.

Concerning the biological assays, first of all, the effects of RKIP knockout and RKIP overexpression on leiomyosarcoma cells viability over the time were studied (Figure 5A, B, C and D). No statistically significant differences were found in the cell viability of KO cells when compared with CTR cells neither at the 0.5% FBS condition (Figure 5A) nor at the 10% FBS condition (Figure 5B). No statistically significant differences were found in the cell viability of RKIP+ cells when compared with Empty cells neither at the 0.5% FBS condition (Figure 5C) nor at the 10% FBS condition (Figure 5D).

In order to study the effects of RKIP knockout and RKIP overexpression on leiomyosarcoma cellular migration, a wound healing migration assay was performed (Figure 6A, B, C and D). No statistically significant differences were found in the migration of KO cells when compared with CTR cells neither at the 0.5% FBS condition (Figure 6A) nor at the 10% FBS condition (Figure 6B). No statistically significant differences were found in the migration of RKIP+ cells when compared with Empty cells neither at the 0.5% FBS condition (Figure 6C) nor at the 10% FBS condition (Figure 6D).

In addition to this, the effects of RKIP knockout and RKIP overexpression on leiomyosarcoma cells ability to form colonies were evaluated by a clonogenicity assay (Figure 7A and B). It was found that KO cells formed a significantly ($p=0.011$) greater number of colonies than CTR cells (Figure 7A). It was also found that RKIP+ cells seemed to show a trend in which they formed a smaller number of colonies than Empty cells even if the difference was not statistically significant (Figure 7B).

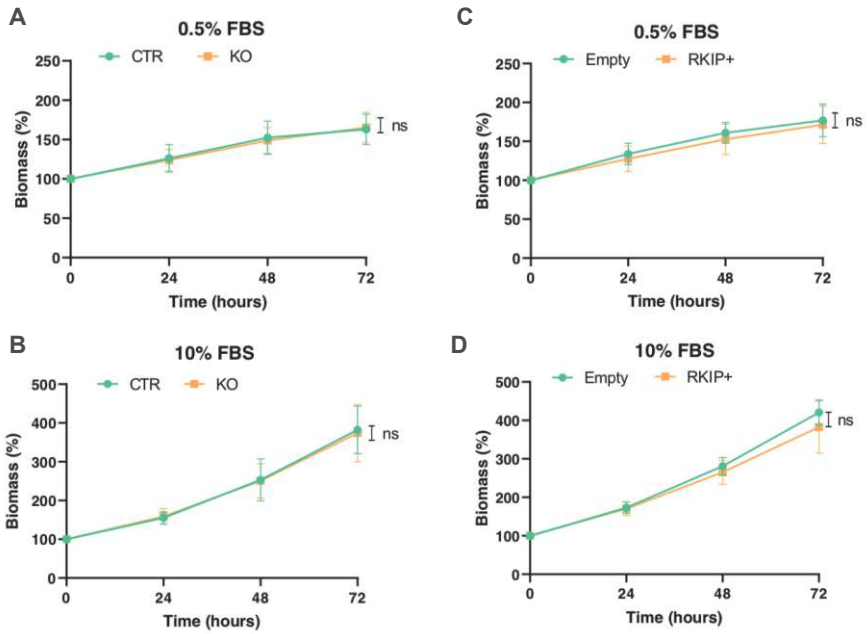


Figure 5. *In vitro* role of RKIP knockout and RKIP overexpression in SK-LMS-1 cells biological behavior: cells viability overtime. Cells viability in two different growth conditions (0.5% FBS and 10% FBS) was measured at 24, 48 and 72 hours by SRB assay. KO cells did not exhibit statistically significant differences in the cell viability overtime when compared with CTR cells neither at the 0.5% FBS condition **(A)** nor at the 10% FBS condition **(B)**. RKIP+ cells did not exhibit statistically significant differences in the cell viability overtime when compared with Empty cells neither at the 0.5% FBS condition **(C)** nor at the 10% FBS condition **(D)**. The experiments were done in triplicate for four times. Results are expressed as the mean ± SD and differences with a $p<0.05$ on the Student's t test were considered statistically significant (indicated as *, while differences not statistically significant are indicated as ns).

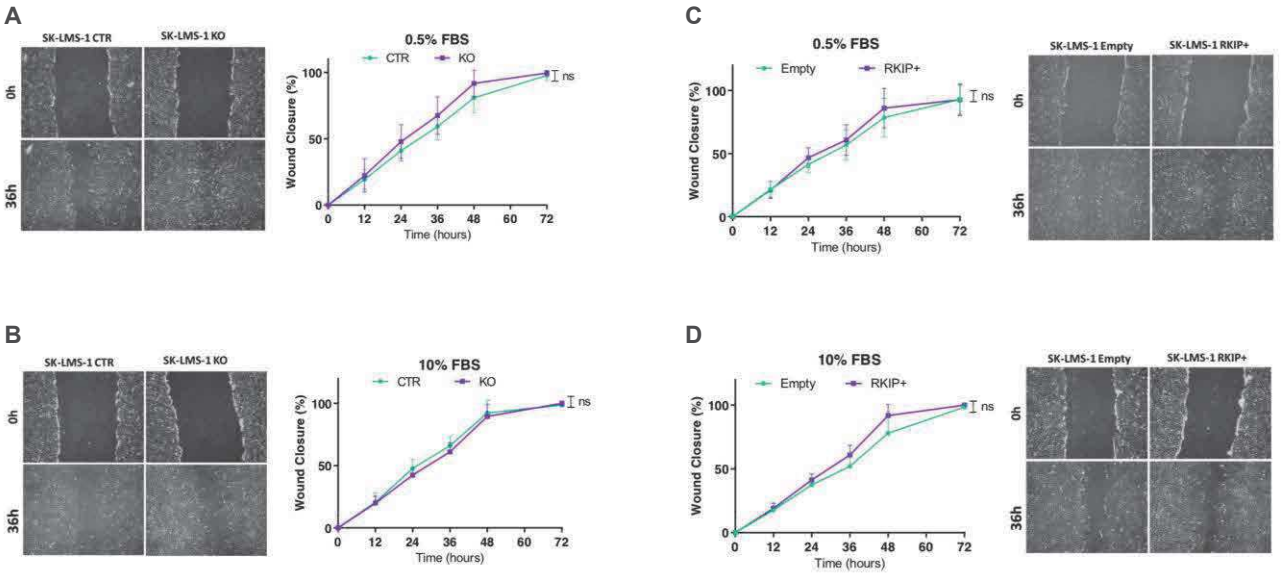


Figure 6. *In vitro* role of RKIP knockout and RKIP overexpression in SK-LMS-1 cells biological behavior: cellular migration overtime. Cellular migration in two different growth conditions (0.5% FBS and 10% FBS) was assessed by wound healing migration assay. A standardized scratch (wound) was applied to monolayer cells and digital images (representative images at 0 and 36 hours are presented alongside the graphs) were taken at several time points (0, 12, 24, 36, 48 and 72 hours). KO cells did not exhibit statistically significant differences in the cellular migration overtime when compared with CTR cells neither at the 0.5% FBS condition **(A)** nor at the 10% FBS condition **(B)**. RKIP+ cells did not exhibit statistically significant differences in the cellular migration overtime when compared with Empty cells neither at the 0.5% FBS condition **(C)** nor at the 10% FBS condition **(D)**. The experiments were done in quadruplicate for three times. Results are expressed as the mean ± SD and differences with a $p<0.05$ on the Student's t test were considered statistically significant (indicated as *, while differences not statistically significant are indicated as ns).

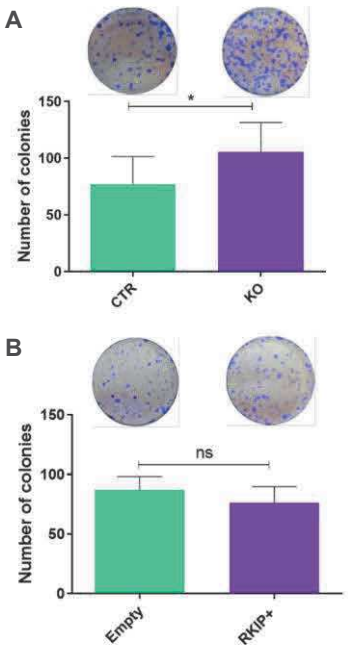


Figure 7. *In vitro* role of RKIP knockout and RKIP overexpression in SK-LMS-1 cells biological behavior: ability to form colonies. Ability to form colonies was assessed by clonogenicity assay manually counting the colonies previously stained with crystal violet (representative images are shown above the graphs). From the point of view of statistical significance, KO cells formed a greater (p=0.011) number of colonies when compared to CTR cells **(A)**. Apparently the RKIP+ cells exhibited a trend to form a smaller number of colonies when compared to Empty cells even if the difference was not statistically significant **(B)**. The experiments were done in triplicate at least two times. Results are expressed as the mean ± SD and differences with a p<0.05 on the Student's t test were considered statistically significant (indicated as *, while differences not statistically significant are indicated as ns).

4. DISCUSSION

Benign and malign tumors can afflict the uterus [7]. It is noteworthy that both leiomyoma which is the most common benign tumor of the uterus [8] and leiomyosarcoma which is the most common malign tumor among the very rare malign uterine sarcomas and which, above all, shows a high mortality [9], afflict myometrium. Although leiomyoma causes a very heavy physical [11] and psychological [11, 14] symptomatology, it is a benign tumor. On the other hand, malign leiomyosarcoma, even if it is very rare, is characterized by a very poor prognosis [10].

Nevertheless, the differential diagnosis aimed at discriminating benign from malign lesions still represents a remarkable problem since sometimes it is hard to discriminate for sure if a histological preparation is benign or malign and the diagnosis is not answered [97, 103].

This problem is particularly encountered for the histological preparations which could be classified as benign leiomyoma with bizarre nuclei since this histological variant shows intermediate features between benign leiomyoma and malign leiomyosarcoma [75, 76].

Therefore, it is important to identify new potential markers that make the differential diagnosis between the benign leiomyoma, in particular, the benign histological variant leiomyoma with bizarre nuclei, and malign leiomyosarcoma more accurate and certain in order to solve the so called STUMP.

Regarding the need to identify new markers for the differential diagnosis, it is noteworthy to mention RKIP. In fact, RKIP is described as a pleiotropic protein since it is known to maintain the biological balance of many cellular processes due to its modulating role in several signaling pathways [139, 140].

In addition to this, since RKIP is downregulated during the metastatic process of several tumors, it is considered to be a metastasis suppressor gene [178, 179, 181, 191, 192].

In the present study, the protein expression level of RKIP was studied in five different histological variants of benign leiomyoma (usual leiomyoma, cellular leiomyoma, lipoleiomyoma, apoplectic leiomyoma and leiomyoma with bizarre nuclei) and in malign leiomyosarcoma. In general, it was found that RKIP is expressed in 81,5% of all the benign lesions and only 25% of the malign lesions stain positively. In particular, 0% of the benign leiomyoma with bizarre nuclei analyzed cases are negative for the RKIP expression, whereas 75% of the malign leiomyosarcoma analyzed cases are negative for the RKIP expression. Moreover, it was noticed that nuclear atypia zones are positive for RKIP in benign leiomyoma with bizzare nuclei, whereas they are negative for RKIP in malign leiomyosarcoma.

These data may represent an important feature that differentiates benign leiomyoma with bizarre nuclei from malign leiomyosarcoma. In fact, both benign leiomyoma with bizarre nuclei and malign leiomyosarcoma show severe nuclear atypia zones [95] and often it is not easy to distinguish with certainty on light microscopic examination [89] malign nuclei from simple degenerative changes in benign cells [107].

Therefore, all these results seem to suggest RKIP as a good candidate to be a marker for the differential diagnosis within benign leiomyoma and malign leiomyosarcoma. However, further studies, analyzing a larger number of cases could validate and extend these findings although it is not easy to find neither many samples of different histological types of benign leiomyoma nor many samples of malign leiomyosarcoma due to their rarity [85, 86, 93, 96].

Martinho *et al* reported results very similar to these analyzing cervical cancer: high RKIP protein levels in benign cervical lesions - cervicitis and squamous intraepithelial lesions (SIL) - and the significant loss of RKIP expression in malign lesions (adenocarcinomas, squamous cell carcinomas and adenosquamous carcinomas) [193]. The results obtained by Martinho *et al*, confirmed the similar findings that had been previously reported by CJ *et al* that had compared RKIP expression in benign SIL with malign cervical cancer lesions [194].

Loss of RKIP protein expression was reported in many studies for several tumor types such as endometrial cancer [195], ovarian cancer in which it was reported also a correlation with differentiation [196], thyroid carcinomas in which it was reported also a correlation with metastasis [197], colorectal cancer in which it was reported also a correlation with metastasis and recurrence of disease in stage II [180], esophageal cancer with [174, 198] or without [175] correlations with tumor stage and presence of matastasis, extrahepatic bile duct carcinoma in which it was reported also a correlation with lymphatic invasion and presence of metastasis [199], gastric cancer with [178, 200-202] or without [177] correlations with tumor size, metastasis, recurrence, clinical stage, histological differentiation, vascular lymphatic and neuronal invasion, and depth of invasion, hepatocellular carcinoma [184, 203] in which it was reported also a correlation with lack of encapsulation, differentiation, tumor size and stage [184], NSCLC in which it was reported also a correlation with metastasis [151], melanoma in which it was reported also a correlation with Clark staging [204], nasopharyngeal carcinoma (NPC) in which it was reported also a correlation with radioresistance and metastasis [205], pancreatic ductal adenocarcinoma in which it was reported also a correlation with stage, presence of metastasis and Kirsten rat sarcoma viral oncogen (KRAS) expression [186, 187] and renal cell carcinoma (RCC) in which it was reported also a correlation with tumor grade and presence of metastasis [206].

Conversely, other tumor types such as bladder cancer [207] and cholangiocarcinoma [208] were reported to be high RKIP expressing tumor types. In particular, acute myeloid leukemia (AML) [209], soft tissue sarcoma [210] and gliomas [189] were reported to show only 10–18% of negative cases.

It is necessary to report that for some tumor types for which multiple studies have been conducted, these did not always show concordant results. For instance, about GIST, Martinho *et al* reported that 8.5% of the tumor tissues demonstrate RKIP negative expression [182], while, Wang *et al* reported a 46% negativity [211]. The reason why conflicting results were obtained may lie in the fact that different antibodies and dilutions were used and also in the fact that different immunohistochemical protocols were adopted. Beyond the reason, the important thing to keep in mind is that conflicting results could contribute to hamper clear conclusions.

In light of all this, it would be very interesting in the future to increase the number of cases so as to reinforce the fact that in this study it was found that the lesions belonging to the histological variants of benign leiomyoma tend to be positive for RKIP and, conversely, the lesions classified as malign leiomyosarcoma tend to be negative for RKIP.

For the time being, the purpose was to relate the loss of RKIP in malign leiomyosarcoma with a possible biological significance behind this event.

In fact, the involvement of RKIP in the balance of many signal transduction pathways that in turn regulate growth, motility, invasion and differentiation in cancer cells [139, 140, 145, 146, 158, 212], highlights the importance of functional studies to evaluate its role in tumors where its expression is downregulated.

To understand the biological role of the loss of RKIP and of the overexpression of RKIP in malign leiomyosarcoma, *in vitro* studies with the SK-LMS-1 leiomyosarcoma cell line were performed. After a preliminary analysis about RKIP protein expression in SK-LMS-1 cells by immunocytochemistry and subsequent western blot which revealed that SK-LMS-1 cells express RKIP, transfection was performed to generate stable RKIP knockout SK-LMS-1 cells (KO cells) and stable RKIP overexpressing SK-LMS-1 cells (RKIP+ cells). It was performed also the overexpression of RKIP in order to be able to study both opposite features (RKIP knockout and RKIP overexpression) even if the not transfected cells already showed a high expression of RKIP. In addition, it must be said that there currently no other suitable leiomyosarcoma cell lines that can be used as a study model.

For knockout CRISPR/Cas9 technology was used and for overexpression a pcDNA3 vector containing the full cDNA of RKIP was used.

The confirmation that the transfection successfully occurred was obtained by western blot.
RKIP protein expression was impaired in KO cells if compared to CTR cells (obtained as already described in the Materials and methods section) that represent the negative control.
On the other hand, RKIP protein expression was increased in RKIP+ cells if compared to Empty cells (obtained as already described in the Materials and methods section) that represent the negative control.
Subsequently, the cell viability, the migration and the ability to form colonies were analyzed in KO cells that do not express RKIP and in RKIP+ cells that, on the contrary, overexpress the RKIP by comparing them to their respective negative controls (CTR cells and Empty cells) in which the expression of the RKIP was not modified.
These analyzes were carried out in order to establish whether RKIP plays a biological role in leiomyosarcoma.
With the analyzes carried out, it was observed that there are no statistically significant differences in the cell viability of KO cells when compared with CTR cells (used as negative control) neither at the 0.5% FBS condition nor at the 10% FBS condition. Similarly, there are no statistically significant differences in the cell viability of RKIP+ cells when compared with Empty cells (used as a negative control) neither at the 0.5% FBS condition nor at the 10% FBS condition.
Both the cells after the RKIP knockout, and the cells after the RKIP overexpression seem to show a behavior similar to that of their respective negative controls (CTR cells and Empty cells) in which the expression of the RKIP was not modified.
These data do not agree with the results obtained by other authors in some tumor types such as cervical cancer [193] and gliomas [189], in which it was observed that the RKIP decrease leads to a viability advantage in cells if compared to cells in which the expression of the RKIP was not modified. In the cervical cancer, this advantage is due to a higher proliferation [193], in gliomas this advantage is not due to proliferation [189].
In the present study, it was also observed that there are no statistically significant differences regarding the migration of KO cells when compared with CTR cells (used as negative control) neither at the 0.5% FBS condition nor at the 10% FBS condition. Similarly, it was observed that there are no statistically significant differences in migration of RKIP+ cells when compared with Empty cells (used as negative control) neither at the 0.5% FBS condition nor at the 10% FBS condition.
Both the cells after RKIP knockout and the cells after RKIP overexpression seem to show a behavior similar to that of their respective negative controls (CTR and EMPTY) in which the

expression of the RKIP was not modified.

These results are in contrast with those obtained by other authors in other tumor types such as cervical cancer [193], gliomas [189, 213], melanoma [214], NSCLC [151], gastric cancer [215] and pancreatic ductal adenocarcinoma [187]. In fact, Martinho *et al* observed that the RKIP knockdown gives the cervical cancer cells a higher migratory capacity [193] in accordance with the results previously obtained also in gliomas cells [189]. About gliomas, also Lei *et al* reported that RKIP knockdown increases cellular migration and that, on the other hand, RKIP overexpression could inhibit cellular migration [213]. The results obtained by Lei *et al,* in turn, are similar to those obtained by Zhou *et al* analyzing the effects of RKIP downregulation and RKIP overexpression in melanoma cells [214]. Wang *et al* observed that RKIP overexpression decreases cellular migration in NSCLC cells [151].

Not only by acting directly on the downregulation or overexpression of RKIP, but also by acting on other molecules that, in turn, are involved in the regulation of the expression of RKIP, similar results have been obtained regarding the cellular migration of some tumors. In fact, Li *et al* reported that acting on the B cell – specific Moloney murine leukemia virus integration site 1 (Bmi-1)/miR-27a and miR-155 axis, because of the Bmi-1 knockdown, the RKIP expression is not inhibited and the cellular migration is inhibited in gastric cancer cells [215]. On the other hand, because of the upregulation of miR-27a and miR-155, the RKIP expression is inhibited and the cellular migration is increased in gastric cancer cells [215]. Yang *et al,* acting on KRAS and RKIP whose expressions are inversely related in PDAC, demonstrated also that KRAS knockdown inhibits the cellular migration and that conversely, RKIP knockdown increases the cellular migration. Moreover, they observed that the RKIP knockdown mitigates the KRAS knockdown and all this leads to an increase of the cellular migration in PDAC [187].

Conversely, in the present study, it was not observed any RKIP effect on the migration of the leiomyosarcoma cells. Anyway, it is necessary to bear in mind that, as testified by the above mentioned studies, there are many molecules and related pathways that may affect cell viability and cell migration beyond the RKIP signaling, also depending on the different cellular type and tumor.

On the other hand, the most interesting result obtained in the present study is that it was shown that there are statistically significant differences in the ability to form colonies exhibited by the cells after RKIP knockout when compared with CTR cells (used as a negative control).

Therefore, RKIP knockout cells seem to show a greater ability to form colonies when compared with their negative control CTR cells in which the expression of RKIP was not modified.

These data are in accordance with those obtained by Martinho *et al* after RKIP knockdown in cervical cancer cell lines [193].

In addition, these data agree with those of Li *et al* who obtained similar results about RKIP effect on the ability to form colonies exhibited by the gastric cancer cell lines after the knockdown of Bmi-1 [215] whose expression was shown to be inversely associated with RKIP expression in gastric cancers [201].

Moreover, in the present study it was also noticed that RKIP+ cells that overexpress the RKIP seem to tend towards a diminished ability to form colonies when compared with the Empty cells (used as negative control) in which the expression of the RKIP was not modified, even if the difference is not statistically significant. Anyway, this result agrees with the studies reported by Wang *et al* who reported that RKIP overexpression inhibits the number and size of colonies in a NSCLC cell line [216]. The particular importance of the results obtained in the present study regarding the ability of leiomyosarcoma cells to form colonies is due precisely to the relevance of the technique used to obtain them: the clonogenicity assay. In fact, this *in vitro* cell survival test assesses the ability of single cells to survive and reproduce to form colonies [217].

The formation of clones is interpreted as a trait of cancer cells with tumor-initiating capabilities. In this way, the clonogenicity assay is a standard tool in cancer research to evaluate cellular growth and it may be fundamental in order to study the malignancy of tumors as well as the mechanisms underlying the onset of the metastasis that is a distinctive feature of the leiomyosarcoma [71, 72].

Hence, the findings regarding the clonogenicity of leiomyosarcoma cells after RKIP knockout and RKIP overexpression suggest that RKIP plays a biological role in leiomyosarcoma cells.

Therefore, considering what has been said and the results of the *in vitro* experiments obtained in the present study, it seems that RKIP does not play a fundamental role in the cellular viability and in the cellular migration in leiomyosarcoma, but, more importantly, it was also observed that RKIP downregulation seems to favor the clonogenicity in the leiomyosarcoma.

5. CONCLUSIONS

In conclusion, the obtained results by immunohistochemistry seem to suggest that there could be a RKIP loss in the malign leiomyosarcoma and that this may have a significance in the malignant progression and in the clinical outcome of leiomyosarcoma.

Although following *in vitro* assays, it was observed that RKIP downregulation does not seem to play a fundamental role in the cellular viability and in the cellular migration in the malign leiomyosarcoma, it was also observed that RKIP downregulation seems to favor the clonogenicity in the malign leiomyosarcoma.

In addition to this, it must be considered that although some malign leiomyosarcoma tissues were found to be positive for RKIP expression, this positivity was detected only at the endothelial level.

Altogether these results suggest that RKIP may be a candidate to be considered as an additional marker for the differential analysis to help discriminating with greater certainty whether a histological preparation that seems to exhibit the features of a benign leiomyoma with bizarre nuclei, is not, actually a malign lesion. Anyway, the data need to be further validated. This, in particular, because it is evident that the number of cases used for RKIP immunohistochemistry is limited to meaningful statistical conclusions. Nevertheless, it is important to bear in mind that the low number of cases that it has been possible to study is due to the fact that, fortunately, both the malign leiomyosarcoma and the histological variants of benign leiomyoma are very rare pathologies.

Therefore, it is necessary to underline that, in the largest series studied so for, the results obtained in this study should be proposed as a starting point for further researches. In fact, since it was observed that, at the morphological level, the benign lesions show the tendency to be positive for RKIP and that, conversely, the malign lesions show the tendency to be negative for RKIP, together with the fact that, *in vitro*, the RKIP loss seems to favor the clonogenicity in the leiomyosarcoma, can lead to important findings in order to be able to consider RKIP a reliable marker in all respects.

REFERENCES

1. Valente, R.; Malesani, M.G. Dizionario Medico; Larousse: Paris, France, 1984; p. 928.

2. Gentile, F. Enciclopedia Italiana; Grolier: New Delhi, India, 1987; Volume 16, p. 271.

3. Anastasi G, Capitani S, Carnazza ML, Cinti S, De Caro R, Donato RF, Ferrario VF, Fonzi L, Franzi AT, Gaudio E, Geremia R, Giordano Lanza G, Grossi CE, Gulisano M, Manzoni FA, Mazzotti G, Michetti F, Miscia S, Mitolo V, Montella A, Orlandini G, Paparelli A, Renda T, Ribatti D, Ruggeri A, Sirigu P, Soscia A, Tredici G, Vitale M, Zaccheo D, Zauli G, Zecchi S. Trattato di anatomia umana; edi-ermes: Milan, Italy, 2012; p. 450-452

4. McEvoy A, Sabir S. Physiology, Pregnancy Contractions; StatPearls: Treasure Island, FL, USA, 2021.

5. Angelini F, Botti D, Colombo R, Gornati R, Monaco G, Odierna G, Olmo E, Ottaviani E, Panzica GC, Rosati F, Sottile L, Stingo V, Venturini G. Biologia dei Tessuti; edi-ermes: Milan, Italy, 2009; p.ß93-111

6. Young B, O'Dowd G, Woodford P. (Italian edition by Billi AM. et al). Wheater Istologia e anatomia microscopica; Edra S.p.A.: Milan, Italy, 2017; p.367

7. Treccani G. Enciclopedia italiana di scienze, lettere ed arti; Istituto della enciclopedia italiana fondata da Giovanni Treccani: Rome, Italy, 2009; Volume 16, p. 541.

8. Mathew RP, Francis S, Jayaram V, Anvarsadath S. Uterine leiomyomas revisited with review of literature. Abdom Radiol (NY). 2021 Oct;46(10):4908-4926. doi: 10.1007/s00261-021-03126-4. Epub 2021 May 31. PMID: 34057564.

9. Juhasz-Böss I, Gabriel L, Bohle RM, Horn LC, Solomayer EF, Breitbach GP. Uterine Leiomyosarcoma. Oncol Res Treat. 2018;41(11):680-686. doi: 10.1159/000494299. Epub 2018 Oct 16. PMID: 30321869.

10. Roberts ME, Aynardi JT, Chu CS. Uterine leiomyosarcoma: A review of the literature and update on management options. Gynecol Oncol. 2018 Dec;151(3):562-572. doi: 10.1016/j.ygyno.2018.09.010. Epub 2018 Sep 21. PMID: 30244960.

11. Giuliani E, As-Sanie S, Marsh EE. Epidemiology and management of uterine fibroids. Int J Gynaecol Obstet. 2020 Apr;149(1):3-9. doi: 10.1002/ijgo.13102. Epub 2020 Feb 17. PMID: 31960950.

12. Wise LA, Laughlin-Tommaso SK. Epidemiology of Uterine Fibroids: From Menarche to Menopause. Clin Obstet Gynecol. 2016 Mar;59(1):2-24. doi: 10.1097/GRF.0000000000000164. PMID: 26744813; PMCID: PMC4733579.

13. Levens ED, Wesley R, Premkumar A, Blocker W, Nieman LK. Magnetic resonance imaging and transvaginal ultrasound for determining fibroid burden: implications for research and clinical care. Am J Obstet Gynecol. 2009 May;200(5):537.e1-7. doi: 10.1016/j.ajog.2008.12.037. Epub 2009 Mar 9. PMID: 19268886; PMCID: PMC2701348.

14. Ghant MS, Sengoba KS, Recht H, Cameron KA, Lawson AK, Marsh EE. Beyond the physical: a qualitative assessment of the burden of symptomatic uterine fibroids on women's emotional and psychosocial health. J Psychosom Res. 2015 May;78(5):499-503. doi: 10.1016/j.jpsychores.2014.12.016. Epub 2015 Feb 2. PMID: 25725565.

15. Okolo, S. Incidence, aetiology and epidemiology of uterine fibroids. Best Pract. Res. Clin. Obstet. Gynaecol. 2008, 22, 571–588.

16. Walker, C.L.; Stewart, E.A. Uterine fibroids: The elephant in the room. Science 2005, 308, 1589–1592.

17. Flake, G.P.; Andersen, J.; Dixon, D. Etiology and pathogenesis of uterine leiomyomas: A review. Environ. Health Perspect. 2003, 111, 1037–1054.

18. Day Baird, D.; Dunson, D.B.; Hill, M.C.; Cousins, D.; Schectman, J.M. High cumulative incidence of uterine leiomyoma in black and white women: Ultrasound evidence. Am. J. Obstet. Gynecol. 2003, 188, 100–107.

19. D'Aloisio AA, et al. Association of intrauterine and early-life exposures with diagnosis of uterine leiomyomata by 35 years of age in the sister study. Environ Health Perspect. 2010; 118(3):375– 381. [PubMed: 20194067]

20. Templeman C, et al. Risk factors for surgically removed fibroids in a large cohort of teachers. Fertil Steril. 2009; 92(4):1436–1446. [PubMed: 19019355]

21. Lumbiganon P, et al. Protective effect of depot-medroxyprogesterone acetate on surgically treated uterine leiomyomas: a multicentre case-control study. Br J Obstet Gynaecol. 1996; 103(9):909– 914. [PubMed: 8813312]

22. Romieu I, Walker AM, Jick S. Determinants of uterine fibroids. Post Mark Surveill. 1991; 5:119– 133.

23. Marshall LM, et al. A prospective study of reproductive factors and oral contraceptive use in relation to the risk of uterine leiomyomata. Fertil Steril. 1998; 70(3):432–439. [PubMed: 9757871]

24. Wise LA, et al. Reproductive factors, hormonal contraception and risk of uterine leiomyomata in African-American women: a prospective study. Am J Epidemiol. 2004; 159(2):113–123. [PubMed: 14718211]

25. Faerstein E, Szklo M, Rosenshein N. Risk factors for uterine leiomyoma: a practice-based case- control study. I. African-American heritage, reproductive history, body size, and smoking. American Journal of Epidemiology. 2001; 153(1):1–10. [PubMed: 11159139]

26. Terry KL, et al. Reproductive characteristics and risk of uterine leiomyomata. Fertil Steril. 2010; 94(7):2703–2707. [PubMed: 20627243]

27. Dragomir AD, et al. Potential risk factors associated with subtypes of uterine leiomyomata. Reprod Sci. 2010; 17(11):1029–1035. [PubMed: 20693498]

28. Ross RK, et al. Risk factors for uterine fibroids: reduced risk associated with oral contraceptives. Br Med J Clin Res Ed. 1986; 293(6543):359–362.

29. Parazzini F, et al. Epidemiologic characteristics of women with uterine fibroids: a case-control study. Obstet Gynecol. 1988; 72(6):853–857. [PubMed: 3186092]

30. Samadi AR, et al. Risk factors for self-reported uterine fibroids: a case-control study. Am J Public Health. 1996; 86(6):858–862. [PubMed: 8659663]

31. Parazzini F. Risk factors for clinically diagnosed uterine fibroids in women around menopause. Maturitas. 2006; 55(2):174–179. [PubMed: 16533580]

32. Chen CR, et al. Risk factors for uterine fibroids among women undergoing tubal sterilization. American Journal of Epidemiology. 2001; 153(1):20–26. [PubMed: 11159141]

33. Parazzini F, et al. Reproductive factors and risk of uterine fibroids. Epidemiology. 1996; 7(4):440– 442. [PubMed: 8793374]

34. Brett KM, Marsh JV, Madans JH. Epidemiology of hysterectomy in the United States: demographic and reproductive factors in a nationally representative sample. J Womens Health. 1997; 6(3):309–316. [PubMed: 9201665]

35. Vikhlyaeva EM, Khodzhaeva ZS, Fantschenko ND. Familial predisposition to uterine leiomyomas. Int J Gynaecol Obstet. 1995; 51(2):127–131.

36. Van Voorhis BJ, Romitti PA, Jones MP. Family history as a risk factor for development of uterine leiomyomas. Results of a pilot study. J Reprod Med. 2002; 47(8):663–669.

37. Luoto R, Rutanen EM, Auvinen A. Fibroids and hypertension - A cross-sectional study of women undergoing hysterectomy. Journal of Reproductive Medicine. 2001; 46(4):359–364. [PubMed: 11354837]

38. Silver MA, et al. Systemic hypertension among women with uterine leiomyomata: potential final common pathways of target end-organ remodeling. J Clin Hypertens (Greenwich). 2005; 7(11): 664–668. [PubMed: 16278524]

39. Takeda T, et al. Relationship between metabolic syndrome and uterine leiomyomas: a case-control study. Gynecol Obstet Invest. 2008; 66(1):14–17. [PubMed: 18230910]

40. Faerstein E, Szklo M, Rosenshein NB. Risk factors for uterine leiomyoma: a practice-based case- control study. II. Atherogenic risk factors and potential sources of uterine irritation. American Journal of Epidemiology. 2001; 153(1):11–19. [PubMed: 11159140]

41. Settnes A, Andreasen AH, Jorgensen T. Hypertension is associated with an increased risk for hysterectomy: a Danish cohort study. Eur J Obstet Gynecol Reprod Biol. 2005; 122(2):218–224. [PubMed: 16219523]

42. Boynton-Jarrett R, et al. A prospective study of hypertension and risk of uterine leiomyomata. American Journal of Epidemiology. 2005; 161(7):628–638. [PubMed: 15781952]

43. Radin RG, et al. Hypertension and risk of uterine leiomyomata in US black women. Hum Reprod. 2012; 27(5):1504–1509. [PubMed: 22371286]

44. Haan YC, et al. Hypertension risk in Dutch women with symptomatic uterine fibroids. Am J Hypertens. 2015; 28(4):487–492. [PubMed: 25241046]

45. Marshall LM, et al. Risk of uterine leiomyomata among premenopausal women in relation to body size and cigarette smoking. Epidemiology. 1998; 9(5):511–517. [PubMed: 9730029]

46. Wise LA, et al. Influence of body size and body fat distribution on risk of uterine leiomyomata in U.S. black women. Epidemiology. 2005; 16(3):346–354. [PubMed: 15824551]

47. Terry KL, et al. Anthropometric Characteristics and Risk of Uterine Leiomyoma. Epidemiology. 2007; 18(6):758–763. [PubMed: 17917603]

48. Baird DD, et al. Association of physical activity with development of uterine leiomyoma. Am J Epidemiol. 2007; 165(2):157–163. [PubMed: 17090618]

49. Walocha JA, Litwin JA, Miodonski AJ. Vascular system of intramural leiomyomata revealed by corrosion casting and scanning electron microscopy. Hum Reprod. 2003; 18(5):1088–1093. [PubMed: 12721189]

50. Wise LA, et al. Polycystic ovary syndrome and risk of uterine leiomyomata. Fertil Steril. 2007; 87(5):1108–1115. [PubMed: 17241625]

51. Parazzini F, Negri E, La Vecchia C, Rabaiotti M, Luchini L, Villa A, Fedele L. Uterine myomas and smoking. Results from an Italian study. J Reprod Med. 1996 May;41(5):316-20. PMID: 8725755.

52. Wise LA, Palmer JR, Harlow BL, Spiegelman D, Stewart EA, Adams-Campbell LL, Rosenberg L. Risk of uterine leiomyomata in relation to tobacco, alcohol and caffeine consumption in the Black Women's Health Study. Hum Reprod. 2004 Aug;19(8):1746-54. doi: 10.1093/humrep/deh309. Epub 2004 Jun 24. PMID: 15218005; PMCID: PMC1876785.

53. Nagata C, Nakamura K, Oba S, Hayashi M, Takeda N, Yasuda K. Association of intakes of fat, dietary fibre, soya isoflavones and alcohol with uterine fibroids in Japanese women. Br J Nutr. 2009 May;101(10):1427-31. doi: 10.1017/s0007114508083566. PMID: 19459228.

54. Chiaffarino F, et al. Diet and uterine myomas. Obstet Gynecol. 1999; 94(3):395–398. [PubMed: 10472866]

55. Wise LA, et al. Intake of fruit, vegetables, and carotenoids in relation to risk of uterine leiomyomata. Am J Clin Nutr. 2011; 94(6):1620–1631. [PubMed: 22071705]

56. Cramer SF, et al. Myometrial hyperplasia in pediatric, adolescent, and young adult uteri. J Pediatr Adolesc Gynecol. 2003; 16(5):301–306. [PubMed: 14597019]

57. Parsanezhad ME, et al. A randomized, controlled clinical trial comparing the effects of aromatase inhibitor (letrozole) and gonadotropin-releasing hormone agonist (triptorelin) on uterine leiomyoma volume and hormonal status. Fertil Steril. 93(1):192–198. [PubMed: 19135657]

58. Bulun SE, et al. Aromatase in endometriosis and uterine leiomyomata. J Steroid Biochem Mol Biol. 2005; 95(1–5):57–62. [PubMed: 16024248]

59. Harmon QE, Baird DD. Use of depot medroxyprogesterone acetate and prevalent leiomyoma in young African American women. Hum Reprod. 2015; 30(6):1499–1504. [PubMed: 25820696]

60. Baird DD, Kesner JS, Dunson DB. Luteinizing hormone in premenopausal women may stimulate uterine leiomyomata development. J Soc Gynecol Investig. 2006; 13(2):130–135.

61. Parazzini F, et al. Oral contraceptive use and risk of uterine fibroids. Obstet Gynecol. 1992; 79(3):430–433. [PubMed: 1738528]

62. Chiaffarino F, et al. Use of oral contraceptives and uterine fibroids: results from a case-control study. Br J Obstet Gynaecol. 1999; 106(8):857–860. [PubMed: 10453838]

63. Ramcharan S, Pelligrin FA, Ray R, et al. The Walnut Creek Contraceptive Drug Study. A prospective study of the side effects of oral contraceptives. Center Popul Res Monogr. 1981:69– 74.

64. Baird D, Wise LA. Childhood abuse and fibroids. Epidemiology. 2011; 22(1):15–17. [PubMed: 21150350]

65. Ferin M. Clinical review 105: Stress and the reproductive cycle. J Clin Endocrin Metab. 1999; 84(6):1768–1774.

66. Puder JJ, et al. Stimulatory effects of stress on gonadotropin secretion in estrogen-treated women. J Clin Endocrin Metab. 2000; 85(6):2184–2188.

67. Pavone D, Clemenza S, Sorbi F, Fambrini M, Petraglia F. Epidemiology and Risk Factors of Uterine Fibroids. Best Pract Res Clin Obstet Gynaecol. 2018 Jan;46:3-11. doi: 10.1016/j.bpobgyn.2017.09.004. Epub 2017 Oct 1. PMID: 29054502.

68. Munro MG, Critchley HOD, Broder MS, Fraser IS. FIGO classification system (PALM-COEIN) for causes of abnormal uterine bleeding in nongravid women of reproductive age; for the FIGO working group on menstrual disorders. Int J Gynecol Obstet. 2011;113:3–13.

69. Zepiridis, L.I.; Grimbizis, G.F.; Tarlatzis, B.C. Infertility and uterine fibroids. Best Pract. Res. Clin. Obstret. Gynaecol. 2016, 34, 66–73

70. Zaloudek CJ, Hendrickson MR, Soslow RA: Mesen- chymal tumors of the uterus; in Kurman RJ, Hedrick Ellenson L, Ronnett BM (eds). Blaustein's Pathology of the Female Genital Tract. New York, Springer, 2011.

71. Wu TI, Chang TC, Hsueh S, Hsu KH, Chou HH, Huang HJ, Lai CH. Prognostic factors and impact of adjuvant chemotherapy for uterine leiomyosarcoma. Gynecol Oncol. 2006 Jan;100(1):166-72. doi: 10.1016/j.ygyno.2005.08.010. Epub 2005 Sep 22. PMID: 16182349.

72. Leitao MM, Soslow RA, Nonaka D, Olshen AB, Aghajanian C, Sabbatini P, Dupont J, Hensley M, Sonoda Y, Barakat RR, Anderson S. Tissue microarray immunohistochemical expression of estrogen, progesterone, and androgen receptors in uterine leiomyomata and leiomyosarcoma. Cancer. 2004 Sep 15;101(6):1455-62. doi: 10.1002/cncr.20521. PMID: 15316901.

73. Hayashi T, Sano K, Ichimura T, Kanai Y, Zharhary D, Aburatani H, Yaegashi N, Konishi I. Characteristics of Leiomyosarcoma: Induction of Hematogenous Metastasis by Isolated Uterine Mesenchymal Tumor Stem-like Cells. Anticancer Res. 2020 Mar;40(3):1255-1265. doi: 10.21873/anticanres.14067. PMID: 32132022.

74. Corpus uteri – carcinoma and carcinosarcoma. In: Amin MB, Edge SB, Greene FL, et al., eds.: AJCC Cancer Staging Manual. 8th ed. Springer; 2017, pp 661-69.

75. Alran L, Rychlik A, Croce S. Leiomyoma-general. PathologyOutlines.com website. https://www.pathologyoutlines.com/topic/uterusleiomyoma.html. Accessed January 27th, 2022.

76. Mhawech-Fauceglia P. Leiomyosarcoma. PathologyOutlines.com website. https://www.pathologyoutlines.com/topic/uteruslms.html. Accessed January 27th, 2022.

77. Fujita, M. Histological and biochemical studies of collagen in human uterine leiomyomas. Hokkaido J. Med. Sci. 1985, 60, 602–615.

78. Arici, A.; Sozen, I. Transforming growth factor-beta3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation. Fertil. Steril. 2000, 73, 1006–1011

79. Norian, J.M.; Malik, M.; Parker, C.Y.; Joseph, D.; Leppert, P.C.; Segars, J.H.; Catherino, W.H. Transforming growth factor beta3 regulates the versican variants in the extracellular matrix-rich uterine leiomyomas. Reprod. Sci. 2009, 16, 1153–1164.

80. Stewart, E.A.; Friedman, A.J.; Peck, K.; Nowak, R.A. Relative overexpression of collagen type I and collagen type III messenger ribonucleic acids by uterine leiomyomas during the proliferative phase of the menstrual cycle. J. Clin. Endocrinol. Metab. 1994, 79, 900–906

81. Zannotti A, Greco S, Pellegrino P, Giantomassi F, Delli Carpini G, Goteri G, Ciavattini A, Ciarmela P. Macrophages and Immune Responses in Uterine Fibroids. Cells. 2021 Apr 22;10(5):982. doi: 10.3390/cells10050982. PMID: 33922329; PMCID: PMC8146588.

82. Giuliani, A.; Greco, S.; Pacile, S.; Zannotti, A.; Delli Carpini, G.; Tromba, G.; Giannubilo, S.R.; Ciavattini, A.; Ciarmela, P. Advanced 3D Imaging of Uterine Leiomyoma's Morphology by Propagation-based Phase-Contrast Microtomography. Sci. Rep. 2019, 9, 10580.

83. Leppert, P.C.; Catherino, W.H.; Segars, J.H. A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays. Am. J. Obstret. Gynecol. 2006, 195, 415–420.

84. Malik, M.; Norian, J.; McCarthy-Keith, D.; Britten, J.; Catherino, W.H. Why leiomyomas are called fibroids: The central role of extracellular matrix in symptomatic women. Semin. Reprod. Med. 2010, 28, 169–179

85. Cramer, S.F.; Patel, A. The frequency of uterine leiomyomas. Am. J. Clin. Pathol. 1990, 94, 435–438

86. Oi, Y.; Katayama, K.; Hirata, G.; Ishidera, Y.; Yoshida, H.; Shigeta, H. Significance of postmenopausal uterine leiomyomas: Focus on variants. J. Obstet. Gynaecol. Res. 2018, 44, 1445–1450.

87. Protic, O.; Toti, P.; Islam, M.S.; Occhini, R.; Giannubilo, S.R.; Catherino, W.H.; Cinti, S.; Petraglia, F.; Ciavattini, A.; Castellucci, M.; et al. Possible involvement of inflammatory/reparative processes in the development of uterine fibroids. Cell Tissue Res. 2016, 364, 415–427.

88. Wang X, Kumar D, Seidman JD. Uterine lipoleiomyomas: a clinicopathologic study of 50 cases. Int J Gynecol Pathol. 2006 Jul;25(3):239-42. doi: 10.1097/01.pgp.0000192273.66931.29. PMID: 16810060.

89. Dastranj Tabrizi A, Ghojazadeh M, Thagizadeh Anvar H, Vahedi A, Naji S, Mostafidi E, Berenjian S. Immunohistochemical Profile of Uterine Leiomyoma With Bizarre Nuclei; Comparison With Conventional Leiomyoma, Smooth Muscle Tumors of Uncertain Malignant Potential and Leiomyosarcoma. Adv Pharm Bull. 2015 Dec;5(Suppl 1):683-7. doi: 10.15171/apb.2015.093. Epub 2015 Dec 31. PMID: 26793616; PMCID: PMC4708041.

90. Croce S, Young RH, Oliva E. Uterine leiomyomas with bizarre nuclei: a clinicopathologic study of 59 cases. Am J Surg Pathol. 2014 Oct;38(10):1330-9. doi: 10.1097/PAS.0000000000000249. PMID: 25140893.

91. Bennett JA, Weigelt B, Chiang S, Selenica P, Chen YB, Bialik A, Bi R, Schultheis AM, Lim RS, Ng CKY, Morales-Oyarvide V, Young RH, Reuter VE, Soslow RA, Oliva E. Leiomyoma with bizarre nuclei: a morphological, immunohistochemical and molecular analysis of 31 cases. Mod Pathol. 2017 Oct;30(10):1476-1488. doi: 10.1038/modpathol.2017.56. Epub 2017 Jun 30. PMID: 28664937; PMCID: PMC5626591.

92. Ubago JM, Zhang Q, Kim JJ, Kong B, Wei JJ. Two Subtypes of Atypical Leiomyoma: Clinical, Histologic, and Molecular Analysis. Am J Surg Pathol. 2016 Jul;40(7):923-33. doi: 10.1097/PAS.0000000000000646. PMID: 27015034; PMCID: PMC5777155.

93. Zhang Q, Ubago J, Li L, Guo H, Liu Y, Qiang W, Kim JJ, Kong B, Wei JJ. Molecular analyses of 6 different types of uterine smooth muscle tumors: Emphasis in atypical leiomyoma. Cancer. 2014 Oct 15;120(20):3165-77. doi: 10.1002/cncr.28900. Epub 2014 Jul 1. PMID: 24986214.

94. Downes KA, Hart WR. Bizarre leiomyomas of the uterus: a comprehensive pathologic study of 24 cases with long-term follow-up. Am J Surg Pathol. 1997 Nov;21(11):1261-70. doi: 10.1097/00000478-199711000-00001. PMID: 9351564

95. Gao T, Finkelman BS, Ban Y, Li Y, Yin P, Bulun SE, Lu X, Ha C, Wei JJ. Integrated histologic and molecular analysis of uterine leiomyosarcoma and 2 benign variants with nuclear atypia. Cancer Sci. 2021 May;112(5):2046-2059. doi: 10.1111/cas.14775. Epub 2021 Mar 22. PMID: 33338329; PMCID: PMC8088951.

96. Kho, K.A.; Lin, K.; Hechanova, M.; Richardson, D.L. Risk of occult uterine sarcoma in women undergoing hysterectomy for benign indications. Obstet. Gynecol. 2016, 127, 468–473.

97. Hanley, K.Z.; Birdsong, G.G.; Mosunjac, M.B. Recent developments in surgical pathology of the uterine corpus. Arch. Pathol. Lab. Med. 2017, 141, 528–541.

98. Mas A, Alonso R, Garrido-Gómez T, et al. The differential diagnoses of uterine leiomyomas and leiomyosarcomas using DNA and RNA sequencing. Am J Obstet Gynecol. 2019;221(4):320.e1-320.e23.

99. Seagle, B.L.; Alexander, A.L.; Strohl, A.E.; Shahabi, S. Discussing sarcoma risks during informed consent for nonhysterectomy management of fibroids: An unmet need. Am. J. Obstet. Gynecol. 2018, 218, 103.e101–103.e105

100. Bi, Q.; Xiao, Z.; Lv, F.; Liu, Y.; Zou, C.; Shen, Y. Utility of clinical parameters and multiparametric mri as predictive factors for differentiating uterine sarcoma from atypical leiomyoma. Acad. Radiol. 2018, 25, 993–1002.

101. Flake, G.P.; Moore, A.B.; Sutton, D.; Kissling, G.E.; Horton, J.; Wicker, B.; Walmer, D.; Robboy, S.J.; Dixon, D. The natural history of uterine leiomyomas: Light and electron microscopic studies of fibroid phases, interstitial ischemia, inanosis, and reclamation. Obstet. Gynecol. Int. 2013, 2013, 528376.

102. Rubisz P, Ciebiera M, Hirnle L, Zgliczyńska M, Łoziński T, Dzięgiel P, Kobierzycki C. The Usefulness of Immunohistochemistry in the Differential Diagnosis of Lesions Originating from the Myometrium. Int J Mol Sci. 2019 Mar 6;20(5):1136. doi: 10.3390/ijms20051136. PMID: 30845657; PMCID: PMC6429074.

103. Gupta, M.; Laury, A.L.; Nucci, M.R.; Quade, B.J. Predictors of adverse outcome in uterine smooth muscle tumours of uncertain malignant potential (STUMP): A clinicopathological analysis of 22 cases with a proposal for the inclusion of additional histological parameters. Histopathology 2018, 73, 284–298.

104. Kempson RL. Sarcomas and related neo- plasms. In: Norris HJ, Hertig AT, Abell MR, editors. The Uterus. Baltimore: Williams & Wilkins; 1973.

105. Tavassoli FA, Devilee P. World Health Organiza- tion Classification of Tumours: Tumours of the Breast and Female Genital Organs. Lyon: Inter- national Agency for Research on Cancer Press; 2003. pp. 236-239.

106. Croce S, Ducoulombier A, Ribeiro A, Lesluyes T, Noel JC, Amant F, Guillou L, Stoeckle E, Devouassoux-Shisheboran M, Penel N, Floquet A, Arnould L, Guyon F, Mishellany F, Chakiba C, Cuppens T, Zikan M, Leroux A, Frouin E, Farre I, Genestie C, Valo I, MacGrogan G, Chibon F. Genome profiling is an efficient tool to avoid the STUMP classification of uterine smooth muscle lesions: a comprehensive array-genomic hybridization analysis of 77 tumors. Mod Pathol. 2018 May;31(5):816-828. doi: 10.1038/modpathol.2017.185. PMID: 29327710.

107. Sun X, Mittal K. MIB-1 (Ki-67), estrogen receptor, progesterone receptor, and p53 expression in atypical cells in uterine symplastic leiomyomas. Int J Gynecol Pathol 2010;29(1):51-4. doi: 10.1097/pgp.0b013e3181b0259b

108. Kefeli M, Caliskan S, Kurtoglu E, Yildiz L, Kokcu A. Leiomyoma with bizarre nuclei: clinical and pathologic features of 30 patients. Int J Gynecol Pathol. 2018;37(4):379-387

109. Chang TC, Wentzel EA, Kent OA, et al. Transactivation of miR- 34a by p53 broadly influences gene expression and promotes apopto- sis. Mol Cell. 2007;26:745-752.

110. Cuppens T, Moisse M, Depreeuw J, et al. Integrated genome anal- ysis of uterine leiomyosarcoma to identify novel driver genes and targetable pathways. Int J Cancer. 2018;142(6):1230-1243.

111. Chudasama P, Mughal SS, Sanders MA, et al. Integrative genomic and transcriptomic analysis of leiomyosarcoma. Nat Commun. 2018;9(1):1-15.

112. Abeshouse A, Adebamowo C, Adebamowo SN, et al. Comprehensive and integrated genomic characterization of adult soft tissue sarco- mas. Cell. 2017;171(4):950-965.

113. Hemming ML, Klega K, Rhoades J, et al. Detection of circulating tumor DNA in patients with leiomyosarcoma with progressive dis- ease. JCO Precis Oncol. 2019;3:1-11

114. Silveira SM, Villacis RAR, Marchi FA, et al. Genomic signatures pre- dict poor outcome in undifferentiated pleomorphic sarcomas and leiomyosarcomas. PLoS One. 2013;8(6):e67643.

115. Chen, L.; Yang, B. Immunohistochemical analysis of p16, p53, and ki-67 expression in uterine smooth muscle tumors. Int. J. Gynecol. Pathol. 2008, 27, 326–332.

116. Demura, T.A.; Revazova, Z.V.; Kogan, E.A.; Adamyan, L.V. [The molecular mechanisms and morphological manifestations of leiomyoma reduction induced by selective progesterone receptor modulators]. Arkh. Patol. 2017, 79, 19–26.

117. Gannon, B.R.; Manduch, M.; Childs, T.J. Differential immunoreactivity of p16 in leiomyosarcomas and leiomyoma variants. Int. J. Gynecol. Pathol. 2008, 27, 68–73.

118. Schaefer, I.M.; Hornick, J.L.; Sholl, L.M.; Quade, B.J.; Nucci, M.R.; Parra-Herran, C. Abnormal p53 and p16 staining patterns distinguish uterine leiomyosarcoma from inflammatory myofibroblastic tumour. Histopathology 2017, 70, 1138–1146.

119. Lee, C.H.; Ali, R.H.; Rouzbahman, M.; Marino-Enriquez, A.; Zhu, M.; Guo, X.; Brunner, A.L.; Chiang, S.; Leung, S.; Nelnyk, N.; et al. Cyclin D1 as a diagnostic immunomarker for endometrial stromal sarcoma with ywhae-fam22 rearrangement. Am. J. Surg. Pathol. 2012, 36, 1562–1570.

120. Buza, N.; Hui, P. Immunohistochemistry in gynecologic pathology: An example-based practical update. Arch. Pathol. Lab. Med. 2017, 141, 1052–1071.

121. Courtoy, G.E.; Donnez, J.; Marbaix, E.; Barreira, M.; Luyckx, M.; Dolmans, M.M. Progesterone receptor isoforms, nuclear corepressor-1 and steroid receptor coactivator-1 and B-cell lymphoma 2 and AKT and AKT phosphorylation status in uterine myomas after ulipristal acetate treatment: A systematic immunohistochemical evaluation. Gynecol. Obstet. Investig. 2018, 83, 443–454.

122. Guan, R.; Zheng, W.; Xu, M. A retrospective analysis of the clinicopathologic characteristics of uterine cellular leiomyomas in China. Int. J. Gynaecol. Obstet. 2012, 118, 52–55.

123. Sarlomo-Rikala, M.; Tsujimura, T.; Lendahl, U.; Miettinen, M. Patterns of nestin and other intermediate filament expression distinguish between gastrointestinal stromal tumors, leiomyomas and schwannomas. APMIS 2002, 110, 499–507

124. Abeler, V.M.; Nenodovic, M. Diagnostic immunohistochemistry in uterine sarcomas: A study of 397 cases. Int. J. Gynecol. Pathol. 2011, 30, 236–243.

125. Weissenbacher, T.; Kuhn, C.; Mayr, D.; Pavlik, R.; Friese, K.; Scholz, C.; Jeschke, U.; Ditsch, N.; Dian, D. Expression of mucin-1, galectin-1 and galectin-3 in human leiomyosarcoma in comparison to leiomyoma and myometrium. Anticancer Res. 2011, 31, 451–457.

126. Soltan, M.M.; Albasry, A.M.; Eldosouky, M.K.; Abdelhamid, H.S. Immunoexpression of progesterone receptor, epithelial growth factor receptor and galectin-3 in uterine smooth muscle tumors. Cell. Mol. Biol. 2018, 64, 7–12.

127. Oliva, E.; Young, R.H.; Amin, M.B.; Clement, P.B. An immunohistochemical analysis of endometrial stromal and smooth muscle tumors of the uterus: A study of 54 cases emphasizing the importance of using a panel because of overlap in immunoreactivity for individual antibodies. Am. J. Surg. Pathol. 2002, 26, 403–412.

128. Garg, G.; Mohanty, S.K. Uterine angioleiomyoma: A rare variant of uterine leiomyoma. Arch. Pathol. Lab. Med. 2014, 138, 1115–1118.

129. Zhu, X.Q.; Shi, Y.F.; Cheng, X.D.; Wu, Y.Z. [The differential diagnosis between uterine leiomyosarcoma and the special subtypes of leiomyoma]. Zhonghua Yi Xue Za Zhi 2003, 83, 1419–1421.

130. Ciebiera, M.; Wlodarczyk, M.; Wrzosek, M.; Meczekalski, B.; Nowicka, G.; Lukaszuk, K.; Ciebiera, M.; Slabuszewska-Jozwiak, A.; Jakiel, G. Role of transforming growth factor beta in uterine fibroid biology. Int. J. Mol. Sci. 2017, 18, 2435

131. Tal, R.; Segars, J.H. The role of angiogenic factors in fibroid pathogenesis: Potential implications for future therapy. Hum. Reprod. Update 2014, 20, 194–216.

132. Dixon, D.; He, H.; Haseman, J.K. Immunohistochemical localization of growth factors and their receptors in uterine leiomyomas and matched myometrium. Environ. Health Perspect. 2000, 108 (Suppl. 5), 795–802.

133. Ciarmela, P.; Bloise, E.; Gray, P.C.; Carrarelli, P.; Islam, M.S.; De Pascalis, F.; Severi, F.M.; Vale, W.; Castellucci, M.; Petraglia, F. Activin-A and myostatin response and steroid regulation in human myometrium: Disruption of their signalling in uterine fibroid. J. Clin. Endocrinol. Metab. 2011, 96, 755–765

134. Sozen, I.; Arici, A. Interactions of cytokines, growth factors, and the extracellular matrix in the cellular biology of uterine leiomyomata. Fertil. Steril. 2002, 78, 1–12

135. Ciarmela, P.; Wiater, E.; Vale, W. Activin-A in myometrium: Characterization of the actions on myometrial cells. Endocrinology 2008, 149, 2506–2516.

136. Ciarmela, P.; Wiater, E.; Smith, S.M.; Vale, W. Presence, actions, and regulation of myostatin in rat uterus and myometrial cells. Endocrinology 2009, 150, 906–914.

137. Al-Mulla, F.; Bitar, M.S.; Taqi, Z.; Yeung, K.C. RKIP: Much more than Raf kinase inhibitory protein. J. Cell Physiol. 2013, 228, 1688–1702.

138. Keller, E.T.; Fu, Z.; Brennan, M. The role of Raf kinase inhibitor protein (RKIP) in health and disease. Biochem. Pharmacol. 2004, 68, 1049–1053

139. Farooqi AA, Li Y, Sarkar FH. The biological complexity of RKIP signaling in human cancers. Exp Mol Med 2015;47:e185.

140. Yesilkanal AE, Rosner MR. Targeting Raf kinase inhibitory protein regulation and function. Cancers (Basel) 2018;10(9):e306.

141. Yeung, K.C.; Rose, D.W.; Dhillon, A.S.; Yaros, D.; Gustafsson, M.; Chatterjee, D.; McFerran, B.; Wyche, J.; Kolch, W.; Sedivy, J.M. Raf kinase inhibitor protein interacts with NF-kappaB-inducing kinase and TAK1 and inhibits NF-kappaB activation. Mol. Cell. Biol. 2001, 21, 7207–7217.

142. Escara-Wilke J, Yeung K, Keller ET. Raf kinase inhibitor protein (RKIP) in cancer. Cancer Metastasis Rev. 2012 Dec;31(3-4):615-20. doi: 10.1007/s10555-012-9365-9. PMID: 22684368.

143. Noh HS, Hah YS, Ha JH, et al. Regulation of the epithelial to mesenchymal transition and metastasis by Raf kinase inhibitory protein-dependent Notch1 activity. Oncotarget 2016;7(4):4632–46.

144. Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis. Exp Ther Med. 2020 Mar;19(3):1997-2007. doi: 10.3892/etm.2020.8454. Epub 2020 Jan 15. PMID: 32104259; PMCID: PMC7027163.

145. Yesilkanal, A.E.; Rosner, M.R. Raf kinase inhibitory protein (RKIP) as a metastasis suppressor: Regulation of signaling networks in cancer. Crit. Rev. Oncog. 2014, 19, 447–454.

146. Vandamme, D.; Herrero, A.; Al-Mulla, F.; Kolch, W. Regulation of the MAPK pathway by raf kinase inhibitory protein. Crit. Rev. Oncog. 2014, 19, 405–415.

147. Yeung, K.; Janosch, P.; McFerran, B.; Rose, D.W.; Mischak, H.; Sedivy, J.M.; Kolch, W. Mechanism of suppression of the Raf/MEK/extracellular signal-regulated kinase pathway by the raf kinase inhibitor protein. Mol. Cell. Biol. 2000, 20, 3079–3085.

148. Lorenz, K.; Lohse, M.J.; Quitterer, U. Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2. Nature 2003, 426, 574–579.

149. Zou S, Tong Q, Liu B, Huang W, Tian Y, Fu X. Targeting STAT3 in Cancer Immunotherapy. Mol Cancer. 2020 Sep 24;19(1):145. doi: 10.1186/s12943-020-01258-7. PMID: 32972405; PMCID: PMC7513516.

150. Touboul R, Baritaki S, Zaravinos A, Bonavida B. RKIP Pleiotropic Activities in Cancer and Inflammatory Diseases: Role in Immunity. Cancers (Basel). 2021 Dec 13;13(24):6247. doi: 10.3390/cancers13246247. PMID: 34944867; PMCID: PMC8699197.

151. Wang A, Duan G, Zhao C, et al. Reduced RKIP expression levels are associated with frequent non-small cell lung cancer metastasis and STAT3 phosphorylation and activation. Oncol Lett 2017;13(5):3039–45.

152. Cho JH, et al. NF2 blocks Snail-mediated p53 suppression in mesothelioma. Oncotarget 2015;6(12):10073–85.

153. Sabapathy K, Lane DP. Understanding p53 functions through p53 antibodies. J Mol Cell Biol. 2019 Apr 1;11(4):317-329. doi: 10.1093/jmcb/mjz010. Erratum in: J Mol Cell Biol. 2019 Dec 19;11(12):1105. PMID: 30907951; PMCID: PMC6487784.

154. Xie SY, Li G, Han C, Yu YY, Li N. RKIP reduction enhances radioresistance by activating the Shh signaling pathway in non-small-cell lung cancer. Onco Targets Ther 2017;10:5605–19.

155. Avery JT, Zhang R, Boohaker RJ. GLI1: A Therapeutic Target for Cancer. Front Oncol. 2021 May 25;11:673154. doi: 10.3389/fonc.2021.673154. PMID: 34113570; PMCID: PMC8186314.

156. Al-Mulla F, Bitar MS, Al-Maghrebi M, Behbehani AI, Al-Ali W, Rath O, Doyle B, Tan KY, Pitt A, Kolch W. Raf kinase inhibitor protein RKIP enhances signaling by glycogen synthase kinase-3β. Cancer Res. 2011 Feb 15;71(4):1334-43. doi: 10.1158/0008-5472.CAN-10-3102. Epub 2011 Feb 8. PMID: 21303975.

157. Zhao, J.; Wenzel, S. Interactions of RKIP with inflammatory signaling pathways. Crit. Rev. Oncog. 2014, 19, 497–504.

158. Gabriela-Freitas M, Pinheiro J, Raquel-Cunha A, Cardoso-Carneiro D, Martinho O. RKIP as an Inflammatory and Immune System Modulator: Implications in Cancer. Biomolecules. 2019 Nov 22;9(12):769. doi: 10.3390/biom9120769. PMID: 31766768; PMCID: PMC6995551.

159. Ahn, J.K.; Hwang, J.W.; Bae, E.K.; Lee, J.; Jeon, C.H.; Koh, E.M.; Cha, H.S. The role of Raf kinase inhibitor protein in rheumatoid fibroblast-like synoviocytes invasiveness and cytokine and matrix metalloproteinase expression. Inflammation 2012, 35, 474–483.

160. Sisto, M.; Lisi, S.; D'Amore, M.; Lofrumento, D.D. Rituximab-mediated Raf kinase inhibitor protein induction modulates NF-kappaB in Sjogren syndrome. Immunology 2014, 143, 42–51.

161. Huang, Q.; Bai, F.; Nie, J.; Lu, S.; Lu, C.; Zhu, X.; Zhuo, L.; Lin, X. Didymin ameliorates hepatic injury through inhibition of MAPK and NF-kappaB pathways by up-regulating RKIP expression. Int. Immunopharmacol. 2017, 42, 130–138.

162. Li, L.; Zhao, Y.W.; Zeng, J.S.; Fan, F.; Wang, X.; Zhou, Y.; Zhu, Z. Rituximab regulates the expression of the Raf kinase inhibitor protein via NF-kappaB in renal tissue of rats with diabetic nephropathy. Genet. Mol. Res. GMR 2013, 12, 2973–2981.

163. Liu, L.; Sun, Q.; Bao, R.; Roth, M.; Zhong, B.; Lan, X.; Tian, J.; He, Q.; Li, D.; Sun, J.; et al. Specific regulation of PRMT1 expression by PIAS1 and RKIP in BEAS-2B epithelia cells and HFL-1 fibroblasts in lung inflammation. Sci. Rep. 2016, 6, 21810.

164. Arthur, S.; Sundaram, U. Protein kinase C-mediated phosphorylation of RKIP regulates inhibition of Na-alanine cotransport by leukotriene D(4) in intestinal epithelial cells. Am. J. Physiol. Cell Physiol. 2014, 307, C1010–C1016.

165. Lin, W.; Wang, N.; Zhou, K.; Su, F.; Jiang, Y.; Shou, J.; Liu, H.; Ma, C.; Qian, Y.; Wang, K.; et al. RKIP mediates autoimmune inflammation by positively regulating IL-17R signaling. EMBO Rep. 2018, 19, e44951.

166. Wright, K.T.; Vella, A.T. RKIP contributes to IFN-gamma synthesis by CD8+ T cells after serial TCR triggering in systemic inflammatory response syndrome. J. Immunol. 2013, 191, 708–716.

167. Gelse, K.; Pöschl, E.; Aigner, T. Collagens-structure, function, and biosynthesis. Adv. Drug Deliv. Rev. 2003, 55, 1531–1546.

168. Akaishi, J.; Onda, M.; Asaka, S.; Okamoto, J.; Miyamoto, S.; Nagahama, M.; Ito, K.; Kawanami, O.; Shimizu, K. Growth-suppressive function of phosphatidylethanolamine-binding protein in anaplastic thyroid cancer. Anticancer Res. 2006, 26, 4437–4442.

169. Zhang, L.; Fu, Z.; Binkley, C.; Giordano, T.; Burant, C.F.; Logsdon, C.D.; Simeone, D.M. Raf kinase inhibitory protein inhibits beta-cell proliferation. Surgery 2004, 136, 708–715.

170. Bement, W.M. A role for RKIP in cell motility. Chem. Biol. 2005, 12, 953–954.

171. Al-Mulla, F.; Bitar, M.S.; Taqi, Z.; Rath, O.; Kolch, W. RAF kinase inhibitory protein (RKIP) modulates cell cycle kinetics and motility. Mol. Biosyst. 2011, 7, 928–941.

172. Baritaki, S.; Chapman, A.; Yeung, K.; Spandidos, D.A.; Palladino, M.; Bonavida, B. Inhibition of epithelial to mesenchymal transition in metastatic prostate cancer cells by the novel proteasome inhibitor, NPI-0052: Pivotal roles of Snail repression and RKIP induction. Oncogene 2009, 28, 3573–3585.

173. Hellmann, J.; Rommelspacher, H.; Muhlbauer, E.; Wernicke, C. Raf kinase inhibitor protein enhances neuronal differentiation in human SH-SY5Y cells. Dev. Neurosci. 2010, 32, 33–46.

174. Birner P, et al. RAF-kinase inhibitor protein (RKIP) downregulation in esophageal cancer and its metastases. Clin Exp Metastasis 2012;29(6):551–9.

175. Gao C, et al. Prognostic value of raf kinase inhibitor protein in esophageal squamous cell carcinoma. Pathol Oncol Res 2012;18(2):471–7.

176. Kim HS, et al. Reduced expression of Raf-1 kinase inhibitory protein predicts regional lymph node metastasis and shorter survival in esophageal squamous cell carcinoma. Pathol Res Pract 2012;208(5):292–9.

177. Chatterjee D, et al. Inverse association between Raf kinase inhibitory protein and signal transducers and activators of transcription 3 expression in gastric adenocarcinoma patients: implications for clinical outcome. Clin Cancer Res 2008;14(10):2994–3001.

178. Martinho O, et al. Absence of RKIP expression is an independent prognostic biomarker for gastric cancer patients. Oncol Rep 2013;29(2):690–6.

179. Al-Mulla F, et al. Raf kinase inhibitor protein expression in a survival analysis of colorectal cancer patients. J Clin Oncol 2006;24(36):5672–9.

180. Wang Y, et al. Effect of Raf kinase inhibitor protein expression on malignant biological behavior and progression of colorectal cancer. Oncol Rep 2015;34(4):2106–14.

181. Minoo P, et al. Loss of raf-1 kinase inhibitor protein expression is associated with tumor progression and metastasis in colorectal cancer. Am J Clin Pathol 2007;127(5):820–7.

182. Martinho O, et al. Loss of RKIP expression is associated with poor survival in GISTs. Virchows Arch 2009;455 (3):277–84.

183. Fu Z, et al. Metastasis suppressor gene Raf kinase inhibitor protein (RKIP) is a novel prognostic marker in prostate cancer. Prostate 2006; 66(3):248–56.

184. Xu YF, et al. PEBP1 downregulation is associated to poor prognosis in HCC related to hepatitis B infection. J Hepatol 2010;53(5):872–9.

185. Song SP, et al. Reduced expression of Raf kinase inhibitor protein correlates with poor prognosis in pancreatic cancer. Clin Transl Oncol 2012;14(11):848–52.

186. Kim HS, et al. Loss of Raf-1 kinase inhibitory protein in pancreatic ductal adenocarcinoma. Pathology 2010;42 (7):655–60.

187. Yang K, Li Y, Lian G, Lin H, Shang C, Zeng L, Chen S, Li J, Huang C, Huang K, Chen Y. KRAS promotes tumor metastasis and chemoresistance by repressing RKIP via the MAPK-ERK pathway in pancreatic cancer. Int J Cancer. 2018 Jun 1;142(11):2323-2334. doi: 10.1002/ijc.31248. Epub 2018 Jan 21. PMID: 29315556.

188. Maresch J, et al. Additive effect on survival of Raf kinase inhibitor protein and signal transducer and activator of transcription 3 in high-grade glioma. Cancer 2011;117(11):2499–504.

189. Martinho O, et al. Downregulation of RKIP is associated with poor outcome and malignant progression in gli- omas. PLoS ONE 2012;7(1):e30769.

190. 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. PMID: 27565262.

191. Fu Z, Smith PC, Zhang L, Rubin MA, Dunn RL, et al. (2003) Effects of raf kinase inhibitor protein expression on suppression of prostate cancer metastasis. J Natl Cancer Inst 95: 878–889.

192. Hagan S, Al-Mulla F, Mallon E, Oien K, Ferrier R, et al. (2005) Reduction of Raf-1 kinase inhibitor protein expression correlates with breast cancer metastasis. Clin Cancer Res 11: 7392–7397. 11/20/7392 [pii];10.1158/1078– 0432.CCR-05–0283 [doi].

193. Martinho O, Pinto F, Granja S, Miranda-Gonçalves V, Moreira MA, Ribeiro LF, di Loreto C, Rosner MR, Longatto-Filho A, Reis RM. RKIP inhibition in cervical cancer is associated with higher tumor aggressive behavior and resistance to cisplatin therapy.

PLoS One. 2013;8(3):e59104. doi: 10.1371/journal.pone.0059104. Epub 2013 Mar 19. PMID: 23527098; PMCID: PMC3602518.

194. Hu CJ, Zhou L, Zhang J, Huang C, Zhang GM (2011) Immunohistochemical detection of Raf kinase inhibitor protein in normal cervical tissue and cervical cancer tissue. J Int Med Res 39: 229–237.

195. Martinho O, et al. Loss of RKIP expression during the carcinogenic evolution of endometrial cancer. J Clin Pathol 2012;65(2):122–8.

196. Li HZ, et al. Effects of raf kinase inhibitor protein expression on metastasis and progression of human epithelial ovarian cancer. Mol Cancer Res 2008;6(6):917–28.

197. Trojanowicz B, et al. Relationship between RKIP protein expression and clinical staging in thyroid carcinoma. Open Endocrinol J 2008;2:16–20.

198. Zhao D, et al. Raf kinase inhibitor protein inhibits esophageal cancer cell invasion through downregulation of matrix metalloproteinase expression. Oncol Rep 2013;30(1):304–12.

199. Kim HS, et al. Expression of Raf-1 kinase inhibitory protein in extrahepatic bile duct carcinoma.Korean J Pathol 2010;44(3):234.

200. Guo W, et al. Aberrant methylation and loss expression of RKIP is associated with tumor progression and poor prognosis in gastric cardia adenocarcinoma. Clin Exp Metastasis 2013;30(3):265–75.

201. Chen Y, et al. Inverse association between Bmi-1 and RKIP affecting clinical outcome of gastric cancer and revealing the potential molecular mechanisms underlying tumor metastasis and chemotherapy resistance. Gastric Cancer 2016;19(2):392–402.

202. Jia B, et al. RKIP expression associated with gastric cancer cell invasion and metastasis. Tumour Biol 2012;33(4):919–25.

203. Schuierer MM, et al. Raf kinase inhibitor protein is downregulated in hepatocellular carcinoma. Oncol Rep 2006;16(3):451–6.

204. Cardile V, et al. Raf kinase inhibitor protein (RKIP) and phospho-RKIP expression in melanomas. Acta Histochem 2013;115(8):795–802.

205. Yuan L, Yi HM, Yi H, et al. Reduced RKIP enhances nasopharyngeal carcinoma radioresistance by increasing ERK and AKT activity. Oncotarget 2016;7(10):11463–77.

206. Hill B, De Melo J, Yan J, et al. Common reduction of the Raf kinase inhibitory protein in clear cell renal cell carcinoma. Oncotarget 2014;5(17):7406–19.

207. Afonso J, et al. Low RKIP expression associates with poor prognosis in bladder cancer patients.Virchows Arch 2013;462(4):445–53.

208. Ma J, Shi J, Zhao D, et al. Raf kinase inhibitor protein inhibits cholangiocarcinoma cell metastasis by downregulating matrix metalloproteinase 9 and upregulating tissue inhibitor of metalloproteinase 4 expres- sion. Oncol Lett 2015;9(1):15–24.

209. Zebisch A, et al. Frequent loss of RAF kinase inhibitor protein expression in acute myeloid leukemia. Leukemia 2012;26(8):1842–9.

210. Martinho O, Campos M, Ribeiro G, Penna V, Curcelli EC, Olivieri MV, Morini S, Scapulatempo C, Abrahao-Machado LF, Reis RM. Raf kinase inhibitor protein expression and prognostic value in soft tissue sarcomas. Pathobiology 2016;83(1):41–6.

211. Wang Y, et al. Clinical and prognostic significance of Raf kinase inhibitory protein expression in gastrointestinal stromal tumors. World J Gastroenterol 2018;24(23):2508–17.

212. Zaravinos, A.; Bonavida, B.; Chatzaki, E.; Baritaki, S. RKIP: A Key Regulator in Tumor Metastasis Initiation and Resistance to Apoptosis: Therapeutic Targeting and Impact. Cancers 2018, 10, E287.

213. Lei X, Chang L, Ye W, Jiang C, Zhang Z. Raf kinase inhibitor protein (RKIP) inhibits the cell migration and invasion in human glioma cell lines in vitro. Int J Clin Exp Pathol. 2015 Nov 1;8(11):14214-20. PMID: 26823735; PMCID: PMC4713521.

214. Zhou K, Zhang YY, Cen Y, Zhu GN, Zhang YG. [Effect of RKIP on proliferation and migration of malignant melanoma cells and potential mechanism]. Zhonghua Yi Xue Za Zhi. 2019 Feb 26;99(8):616-621. Chinese. doi: 10.3760/cma.j.issn.0376-2491.2019.08.010. PMID: 30818932.

215. Li Y, Tian Z, Tan Y, Lian G, Chen S, Chen S, Li J, Li X, Huang K, Chen Y. Bmi-1-induced miR-27a and miR-155 promote tumor metastasis and chemoresistance by targeting RKIP in gastric cancer. Mol Cancer. 2020 Jun 24;19(1):109. doi: 10.1186/s12943-020-01229-y. PMID: 32580736; PMCID: PMC7315508.

216. Wang Q, Li XY, Wan B, Zhang J, Sun R, Zhou CY, Zhan P, Song Y. Overexpression of Raf-1 kinase inhibitor protein inhibits cell invasion and migration in lung cancer cells through suppressing epithelial-mesenchymal transition. Transl Cancer Res. 2019 Oct;8(6):2295-2306. doi: 10.21037/tcr.2019.09.56. PMID: 35116982; PMCID: PMC8797464.

217. Franken, N. A. P., Rodermond, H. M., Stap, J., Haveman, J. & Bree, C. V. Clonogenic assay of cells in vitro. Nature Protocols1, 2315–2319 (2006).

ACKNOWLEDGMENTS

With great pleasure, I want to thank my Tutor Professor Andrea Ciavattini, Professor Pasquapina Ciarmela, the Department of Specialist and Odontostomatological Clinical Sciences, Polytechnic University of Marche, Ancona, Italy and the Department of Experimental and Clinical Medicine, Polytechnic University of Marche, Ancona, Italy for the teachings they gave me during my Ph.D. program.

A special thanks also to Doctor Olga Martinho and all her Team for the help they gave me during the mobility abroad period spent at the University of Minho in Braga, Portugal.

INDEX

APPENDIX

cells

MDPI

Review

Macrophages and Immune Responses in Uterine Fibroids

Alessandro Zannotti [1,2,†], Stefania Greco [2,†], Pamela Pellegrino [2], Federica Giantomassi [3], Giovanni Delli Carpini [1], Gaia Goteri [3], Andrea Ciavattini [1] and Pasquapina Ciarmela [2,*]

1 Department of Specialist and Odontostomatological Clinical Sciences, Università Politecnica delle Marche, 60126 Ancona, Italy; a.zannotti@pm.univpm.it (A.Z.); g.dellicarpini@staff.univpm.it (G.D.C.); a.ciavattini@univpm.it (A.C.)
2 Department of Experimental and Clinical Medicine, Università Politecnica delle Marche, 60126 Ancona, Italy; s.greco@pm.univpm.it (S.G.); p.pellegrino@pm.univpm.it (P.P.)
3 Department of Biomedical Sciences and Public Health, Università Politecnica delle Marche, 60126 Ancona, Italy; f.giantomassi@virgilio.it (F.G.); g.goteri@staff.univpm.it (G.G.)
* Correspondence: p.ciarmela@univpm.it; Tel.:+39-071-220-6270
† These authors contributed equally.

Abstract: Uterine fibroids represent the most common benign tumors of the uterus. They are considered a typical fibrotic disorder. In fact, the extracellular matrix (ECM) proteins—above all, collagen 1A1, fibronectin and versican—are upregulated in this pathology. The uterine fibroids etiology has not yet been clarified, and this represents an important matter about their resolution. A model has been proposed according to which the formation of an altered ECM could be the result of an excessive wound healing, in turn driven by a dysregulated inflammation process. A lot of molecules act in the complex inflammatory response. Macrophages have a great flexibility since they can assume different phenotypes leading to the tissue repair process. The dysregulation of macrophage proliferation, accumulation and infiltration could lead to an uncontrolled tissue repair and to the consequent pathological fibrosis. In addition, molecules such as monocyte chemoattractant protein-1 (MCP-1), granulocyte macrophage-colony-stimulating factor (GM-CSF), transforming growth factor-beta (TGF-β), activin A and tumor necrosis factor-alfa (TNF-α) were demonstrated to play an important role in the macrophage action within the uncontrolled tissue repair that contributes to the pathological fibrosis that represents a typical feature of the uterine fibroids.

Keywords: uterine fibroids; ECM; inflammatory process; tissue repair; macrophages; pathological fibrosis

check for updates

Citation: Zannotti, A.; Greco, S.; Pellegrino, P.; Giantomassi, F.; Delli Carpini, G.; Goteri, G.; Ciavattini, A.; Ciarmela, P. Macrophages and Immune Responses in Uterine Fibroids. *Cells* **2021**, *10*, 982. https://doi.org/10.3390/cells10050982

Academic Editors: Maciej Lech and Stefanie Steiger

Received: 25 March 2021
Accepted: 20 April 2021
Published: 22 April 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Uterine Leiomyoma: A Typical Fibrotic Pathology

Uterine leiomyomas (leiomyomas, myomas, uterine fibroids, fibroids) are the most common benign tumors of the uterus. The perimetrium constitutes the more external layer of the uterus; it equals the peritoneum and is surrounded by a thin connective tissue layer. The perimetrium resembles a typical serosa/adventitia layer. The endometrium constitutes the more internal layer. It is formed by a simple columnar epithelium and contains numerous tubular glands. In addition, a cell-dense connective tissue layer can be individuated at the level of this structure. Finally, a transition to squamous non-keratinized epithelium at the portio (squamocolumnar junction) can be appreciated. Functionally, the endometrium can be divided into two sublayers: the so-called stratum basale, which represents the basal layer, and the so-called stratum functionale, which is the real functional layer. The endometrium resembles a typical mucosa layer. Finally, the myometrium constitutes the intermediate layer between the perimetrium and the endometrium and represents the muscularis structure of the uterus. The uterine musculature shows properly the typical characteristics of the smooth muscle tissue. More precisely, the myometrium is composed of three smooth muscle layers: the subvascular layer, which is quite thin; the vascular layer, which is rather strong and well-perfused; and the supravascular layer, which is composed of a complex of crossing muscle fibers. The subvascular layer is

mainly involved in the separation of the endometrium during the menstrual cycle. The vascular layer runs around the uterus and, in doing this, it forms a kind of net for the perfusion of the tissue. It plays a major role during labor within the complex mechanism that regulates the uterine contractions during the partum [1]. The supravascular, with its muscle fibers, stabilizes the uterine wall [2,3]. The cells of the myometrium can transform themselves into uterine leiomyoma cells. So, the uterine leiomyoma is a pathology that involves, in detail, the myometrium. The uterine fibroids incidence in reproductive age women is approximately 60%, and if we consider black women, this percentage reaches 80% [4]. The symptomatology of uterine fibroids is very heavy. One of the most relevant clinical symptoms is prolonged or heavy menstrual bleeding. In addition, the irregular and excessive bleeding often experienced by the women affected by uterine leiomyomas, a lot of times, leads to anemia. Other symptoms of the uterine fibroids are represented by pelvic pain or pressure, pain at the level of the back of the legs, a pressure sensation at the level of the lower part of abdomen, bowel and bladder dysfunctions and pain during sexual intercourse.

In addition to all these physical ailments, uterine leiomyomas may also impact the pregnancy outcome. Depending on their position, size and number, uterine leiomyomas can be a cause of infertility and recurrent miscarriage [5–9]. Although uterine leiomyomas are not malignant tumors, they can cause significant morbidity. Thus, this pathology represents one of the most important public health problems worldwide [10]. This fact becomes also more relevant if we bear in mind that, at the moment, no long-term medical treatments are available for fibroids resolution [11].

Considering the role played by estrogens and progesterone in the leiomyoma growth [11,12], for the treatment of uterine fibroids, the U.S. Food and Drug Administration (FDA) approved leuprolide acetate, which is a gonadotropin-releasing hormone analog. However, these kinds of molecules, in particular in young women, can provoke several side effects, above all, a hypogonadal state; this is the reason why the duration of therapy is currently limited. Uterine leiomyomas usually start to grow again after breaking off the treatment [13,14]. Nevertheless, it was demonstrated that leuprolide acetate can be effectively used in order to decrease the volume of the uterine fibroids with improved fibroid-related symptoms. [15–17]. Of the treatments that have been studied up to now, the focus has above all been on those belonging to two categories: antiprogestin and selective progesterone receptor modulators (SPRMs). Thus, clinical trial results suggested mifepristone, which is an antiprogestinic molecule [18], and asoprisnil [19] and telapristone acetate (CDB-4124) [20], both belonging to the SPRMs category, as candidate therapeutic drugs against uterine fibroids (https://clinicaltrials.gov accessed on 7 April 2021). In particular, 17a-acetoxy-11b-(4-*N*,*N*-dimethylaminophenyl)-19-norpregna-4,9-diene-3,20-dione, also referred to as CDB-2914 and ulipristal acetate (UPA) [21–24], is an SPRM molecule, and it is very interesting to study because of the high affinity that it has shown in binding progesterone receptor isoforms A and B [25,26].

Currently, in the international literature, there is a debate about the usefulness and safety of the use of UPA [27].

A few years ago, we demonstrated that UPA can exert a downregulation effect at the level of the mRNA of activin A, a pro-fibrotic factor for leiomyoma. The UPA causes a similar impairing effect also on follistatin (FST), activin receptor type II (ActRIIB) and activin receptor-like kinase 4 (ALK4) mRNAs [28]. All these molecules together represent the activin pathway and these results consider activin A and its receptors as UPA targets and at the same time reinforce the validity of UPA as a treatment for uterine fibroids.

In 2012, the European Medicines Agency (EMA) approved the clinical use of UPA 5 mg, sold under the trade name Esmya (or generic medicines), but limited it to a three-month period and pre-surgery. However, in 2018, other limitations occurred since cases of severe liver toxicity had been reported. Following cases of liver damage that even required transplantation, in November 2020, the EMA recommended limiting the prescription of UPA 5 mg (Esmya or generic medicines) as much as possible. So, currently, Esmya and

generic medicines containing UPA 5 mg are only allowed to treat uterine fibroids in pre-menopausal women for whom surgical procedures (including uterine fibroid embolisation) are not appropriate or have not worked. On the other hand, these medicines must not be used for controlling the symptoms of uterine fibroids in the pre-surgical phase. Besides, it had already been demonstrated that in the women that had been pre-surgically treated with UPA, the myomas appeared softer and showed less clear cleavage planes. So, the result was that it was less easy to enucleate if compared to the enucleation modalities of the myomas belonging to women not pre-surgically treated with UPA [29]. In addition to all this, after the patients stop taking the UPA, leiomyomas revert [30,31]. Nowadays, hysterectomy remains the definitive treatment against uterine fibroids. In fact, at the moment, uterine leiomyomas represent the most common indication for hysterectomy in the world. However, it represents itself an additional problem concerning uterine fibroids and also the less invasive myomectomy leads to a serious postoperative morbidity [32,33]. Hysterectomy exerts also a very significant economic impact on the healthcare system all over the world, reaching an amount almost equal to $2.2 billion/year for the United States of America alone [34].

According to their anatomical location, uterine fibroids can be classified into three different types: submucosal fibroids, intramural fibroids and subserosal fibroids [35].

Uterine fibroids present themselves as solid, rounded masses, with an inhomogeneous eco-structure [36].

From a histological point of view, uterine fibroids can be classified into different types: usual leiomyoma, cellular leiomyoma that shows increased cellularity [37], lipoleiomyoma that exhibits adipocytes [38], apoplectic leiomyoma that shows stellate zones of recent hemorrhage [39] and rare, bizarre leiomyoma [40,41]. Among them, the usual leiomyoma is the most common histological variant with an incidence equal to approximately 94% and it is what is commonly referred to as a leiomyoma unless otherwise specified.

Usual leiomyomas are the ones considered a fibrotic disorder [42,43].

The leiomyomas were described as typical fibrotic tissues because they exhibit the up-regulation of the extracellular matrix (ECM) proteins—above all, collagen 1A1, fibronectin and versican [44]. In particular, numerous authors showed that the uterine fibroids contain approximately 50% more ECM than the corresponding myometrium [45–49]. In addition, the ECM was suggested to represent a reservoir for growth factors, cytokines, chemokines, angiogenic and inflammatory response mediators, and proteases [43,50–53], which are all molecules thought to be involved in the initiation and development of the uterine fibroids [11].

In this regard, a very important matter about uterine fibroids is that their etiopathogenesis has not yet been clarified [53].

Nowadays, some major risk factors associated with the uterine leiomyomas are known and, among them, the following are the most important ones: early menarche, nulliparity, age (meaning late reproductive years), polycystic ovary syndrome, diabetes, hypertension, obesity, and heredity [10,50,54]. In addition to this, since black women have a high incidence rate of uterine leiomyomas [4], ethnicity may also be considered as a potential risk factor for this pathology.

Of the most important factors involved in the pathogenesis of uterine leiomyoma, in the literature, it has been reported that chromosomal abnormalities, both at the level of alterations of karyotypic character and at the level of alterations of cytogenetic character, are present in about 50% of leiomyomas [55–57]. In addition, in the leiomyomas, the chromosomes 2, 3, 6, 7, 8, 10, 11, 12, 13, 14 and 22 were demonstrated to present genetic alterations with the genes MED12, HMGA2, HMGA1, FH, BHD, TSC2, PCOLCE, ORC5L, and LHFPL3 supposed to be mutated in some way [50,58–66]. Mutations at the level of these genes and, in particular, MED12 mutation, FH inactivation and HMGA2 overexpression, as well as COL4A6-COL4A5 deletion were confirmed also by studies based on the modern high-throughput sequencing techniques [67].

Furthermore, as well as genetic factors, molecules and cellular events belonging to typical epigenetic pathways, such as several microRNAs, DNA methylation and histone modification, have also been described to be involved in leiomyomas [68–71]. In particular, uterine leiomyomas have been shown to present a dysregulation about a lot of different microRNAs and, among them also let7, miR-21, miR-93, miR-106b, and miR-200 and their predicted target genes. In addition, the same type of dysregulation has not been found in the healthy myometrium [68,72–78]. In addition, other potential gene-markers for the uterine leiomyoma can be provided through the use of gene set enrichment analysis [79].

Moreover, it has been clearly highlighted that estrogens and progesterone, the most important female hormones, as well as their correspondent receptors, exert a very relevant effect on uterine leiomyoma growth, and it was shown that, in doing this, the action of these molecules undergoes the mediation of other molecules such as growth factors, cytokines, and chemokines [11,80]. Sometimes, in the postmenopausal period, women need hormone replacement treatment (HRT) based on estrogens and progesterone in order to cope with some of the typical menopausal symptoms. So, also in postmenopausal women affected by uterine leiomyoma, estrogens and progesterone due to HRT can exert an important effect on uterine leiomyoma growth. For this reason, the use of these hormones should be limited [81].

Epidermal growth factor (EGF), heparin-binding epidermal growth factor (HB-EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), transforming growth factor-alfa (TGF-α), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (acidic-FGF), basic fibroblast growth factor (basic-FGF), activin and myostatin are the most important growth factors that mediate the estrogens and progesterone action within the uterine leiomyoma physiology [54,80,82–84]. In addition to this, interleukin (IL)-1, IL-6, IL-11, IL-13, IL-15, tumor necrosis factor-alfa (TNF-α), granulocyte macrophage-colony-stimulating factor (GM-CSF) and erythropoietin (EPO) are all cytokines that interact with estrogens and progesterone, playing an important role in uterine leiomyoma growth [85–88]. Additionally, chemokines, with their receptors and in particular, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T cell expression and presumably secreted (RANTES), Eotaxin, Eotaxin-2, IL-8, chemokine CC-motif receptor (CCR) 1, CCR3, CCR5, C-X-C chemokine receptor (CXCR) 1, CXCR2 and monocyte chemoattractant protein-1 (MCP-1) stimulate the uterine leiomyoma growth after the interaction with estrogens and progesterone [88,89].

So, not only were growth factors [54,80,82], cytokines [11], chemokines [89], inflammatory response mediators [90], proteases [43,91–93] and the ECM, in particular as a reservoir of these molecules [43,50–52], shown to represent important actors in the establishment and in the growth of uterine fibroids [11], but also genetic alterations [50,55,64,94,95] and epigenetic mechanisms [69,70] as well as estrogens [96,97] and progesterone [97–104] can be considered as promoters of fibroid growth (Figure 1).

So far, we have discussed the anatomical environment and the histological features of the uterine fibroids as well as their incidence, their heavy symptoms, the available treatments and those still under study, the risk factors and also what is known about their pathogenesis. In this review, we will continue the discussion, thoroughly summarizing the role of the inflammatory process in uterine fibroid development and growth with particular regard towards the importance of the macrophages and the immune response in the uterine fibroids, trying to contribute to shed light on their etiopathogenesis.

The inflammatory process seems to have a noteworthy role in the establishment of the uterine fibroids [105]. In fact, we have just mentioned that the leiomyomas were described as typical fibrotic tissues [44] with a great deal of ECM [45–48].

In general, the fibrotic response arises from the recruitment of inflammatory cells such as monocytes and macrophages by means of inflammatory signals into the site of injury in every tissue and the consequent activation of fibroblasts that start producing collagen [106].

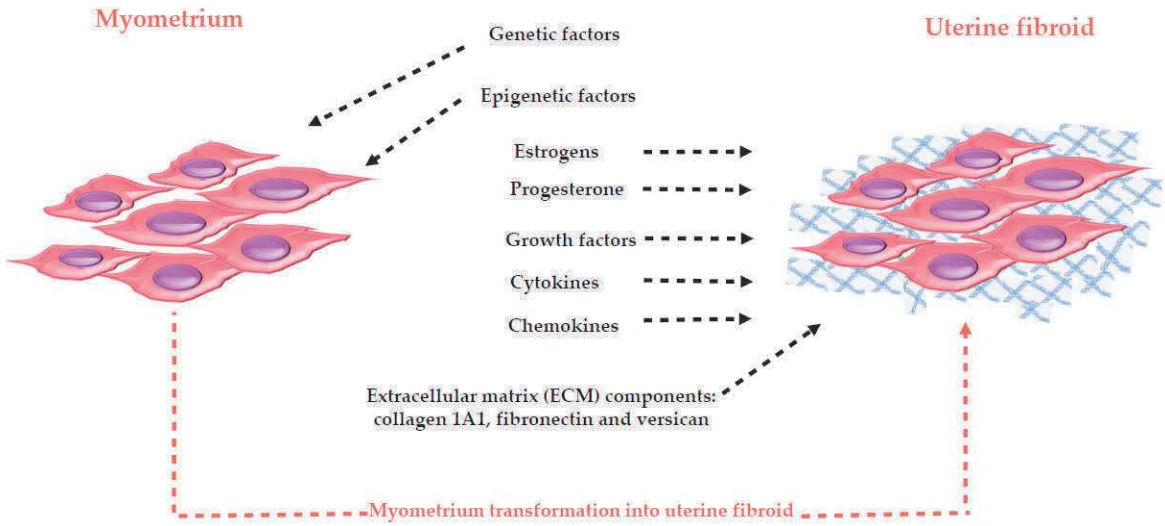


Figure 1. Illustration of the promoters of fibroid growth. The blue net represents the typical extracellular matrix (ECM) proteins: collagen 1A1, fibronectin and versican. The abundant ECM in uterine fibroids (approximately 50% more than the corresponding myometrium) was suggested to represent a reservoir for the other promoters of fibroid growth.

These fibroblasts are usually activated by inflammatory signals and they differentiate into myofibroblasts. They head the ECM turnover [107], leading to tissue homeostasis restoration [108,109]. A dysregulation in the myofibroblasts action can generate pathological fibrosis [106]. In fertile women, transient inflammation is a physiological and important process for the correct achievement of menstruation, ovulation, and parturition. An altered response can produce chronic inflammation in the uterus, ultimately leading to dysregulated tissue repair [90]. In particular, about leiomyoma development and growth, Leppert and her group suggested a model according to which, after a tissue injury, an abnormal response to tissue repair could occur, leading to disordered healing [42]. In a leiomyoma, smooth muscle cells, as well as fibroblasts or stem cells, can gain a myofibroblastic phenotype. In a dysregulated process, after myofibroblast transformation, the myofibroblasts cannot undergo apoptosis with the consequent formation of an altered ECM [30], which is a distinctive trait of the leiomyomas [44–48]. About this, it was noticed that fibroids exhibit a remarkable similarity to keloids, especially because of the disordered appearance of ECM and dysregulation of many genes in the ECM. In fact, microarray experiments have shown that fibroids possess gene features that resemble keloids [42]. So, fibroids could represent a disorder of wound healing and could arise in response to dysregulated extracellular signals as well as keloids [42]. Additionally, myomectomy and caesarean section, which have already been demonstrated to be causes of uterine rupture, may themselves represent a kind of damage followed by a wound healing response. In women showing disordered extracellular signals because of these alterations, a fibroid may develop [110].

2. The Role of Macrophages in Tissue Repair and Fibrosis in Several Organs

Although a lot of different cellular types such as fibroblasts, epithelial cells, endothelial cells, stem cells, neutrophils, innate lymphoid cells (ILCs), NK cells, B cells and T cells join to the complex inflammatory response that leads to tissue repair [109], macrophages develop a key regulatory role in every stage that characterizes the tissue repair and fibrosis [111]. This capability could be due to the macrophages' highly flexible programming [112]. In fact, within the injured tissue, the macrophages can be found in several different phenotypic states, and this flexibility allows them to perform many functions beginning from the promotion and resolution of inflammation, including the removal of apoptotic cells, up to the support of cell proliferation following injury [113]. After tissue injury, through chemokine gradients and some different adhesion molecules, a lot of inflammatory monocytes and macrophage precursors are recalled from the bone marrow to the injured site.

These recruited cells outnumber the resident tissue macrophages [114,115]. At this point, the release in the local tissue microenvironment of cytokines and growth factors represents the signal for the proliferation of both the recruited and resident macrophages [116,117]. In addition, in response to these signals, the macrophages also change their aspect in order to develop their functions [116,117]. In this way, macrophages assume the phenotype that could be called "pro-inflammatory macrophages" and so they can lead the initial phase of the response to injury since they represent an important source of chemokines, matrix metalloproteinases and other inflammatory mediators such as TNF-α [111]. The inflammatory process in response to injury goes on because of the macrophages' high flexibility [112]. In fact, they assume the phenotype that could be called "wound healing macrophages", which are specialized in the production and consequent secretion of several growth factors such as PDGF, transforming growth factor-beta 1 (TGF-β1), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor-alfa (VEGF-α) [118–122]. These molecules stimulate cell proliferation and angiogenesis [118–122]. In addition, under the effect of the soluble mediators produced by the wound healing macrophages, local and recruited tissue fibroblasts are induced to differentiate into myofibroblasts that drive the wound contraction and closure especially through the synthesis of extracellular matrix components [123] such as collagen 1A1, fibronectin and versican. Wound healing macrophages develop their regulatory role [111] also towards neighboring parenchymal and stromal cells' proliferation and expansion, and they can recruit additional stem cell and local progenitor cell populations in order to make them join to tissue repair in case of severe injury. At this point, the macrophages again change their aspect, gaining another phenotype, which can be called "anti-inflammatory macrophages" [124]. Anti-inflammatory macrophages act in response to several inhibitory mediators such as IL-10 and in turn they release a wide range of anti-inflammatory mediators such as IL-10 and TGF-β1 and show as cell surface receptors the proteins programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2), which represent the principal molecules involved in the immune system suppression and in the resolution of the inflammation [125–128] (Figure 2). Therefore, wound healing is a process that must be tightly regulated, otherwise it may lead to the formation of chronic wounds that in turn may facilitate the development of pathological fibrosis [129]. The macrophages, with their great flexibility that allows them to adopt different phenotypes [112,113], could play a unique, important and critical role at each stage of the wound healing, from the initiation and maintenance up to the resolution of the tissue repair process. Different studies have highlighted the macrophages' great flexibility. In the literature, this plasticity is often reported as the M1/M2 dichotomy of macrophages. It describes the different macrophage subtypes that are involved in the tissue repair process. The M1/M2 dichotomy describes the macrophage subsets showing the M1 subtypes expressing higher levels of several pro-inflammatory cytokines, such as TNF-α and interleukin-1 beta (IL-1β) and the M2 subtypes expressing increased levels of anti-inflammatory cytokines, such as IL-10 and TGF-β [130–132]. Even if this is a widespread nomenclature, it is now thought that the M1/M2 dichotomy is not sufficient at all to describe the several different phenotypes and functions of macrophages in vivo [133], also because both M1 and M2 markers can often be expressed at the same time [134]. In addition to this, studies about tissue repair in skeletal muscle showed that in vivo macrophage activation signaling pathways do not correspond to in vitro M1/M2 ones. Among them, for example, we can mention the signal transducer and activator of transcription 1 (STAT1)/interferon gamma (IFN-γ) receptor [135], canonical M2 markers induced by IL-4 [135], the transducer and activator of transcription 6 (STAT6) in IL-4 signaling [136], the IL-4/IL-13 signaling [137], and last but not least, hypoxia-inducible factors (HIFs) in M1/M2 gene expression [138] and in macrophage accumulation [139] pathways. Therefore, it can be affirmed the M1/M2 macrophage dichotomy was conceived by studying macrophages in culture and it is not suitable in order to describe macrophages in vivo straightforward [140]. The most noteworthy concept we have to focus on is that both definitions of M1/M2 macrophages and pro-inflammatory/wound healing/anti-inflammatory macrophages agree with the fact

that macrophages have a great flexibility so that they can assume several different phenotypes [112,113], and this capability may enable them to lead the tissue repair process. Indeed, something dysregulated such as macrophage proliferation, accumulation and infiltration, within the reported macrophage action could lead to uncontrolled repair tissue and to the consequent pathological fibrosis. Several studies have been carried out in order to characterize the macrophages' behavior within the initiation, maintenance and resolution of the tightly regulated wound healing response in different organs.

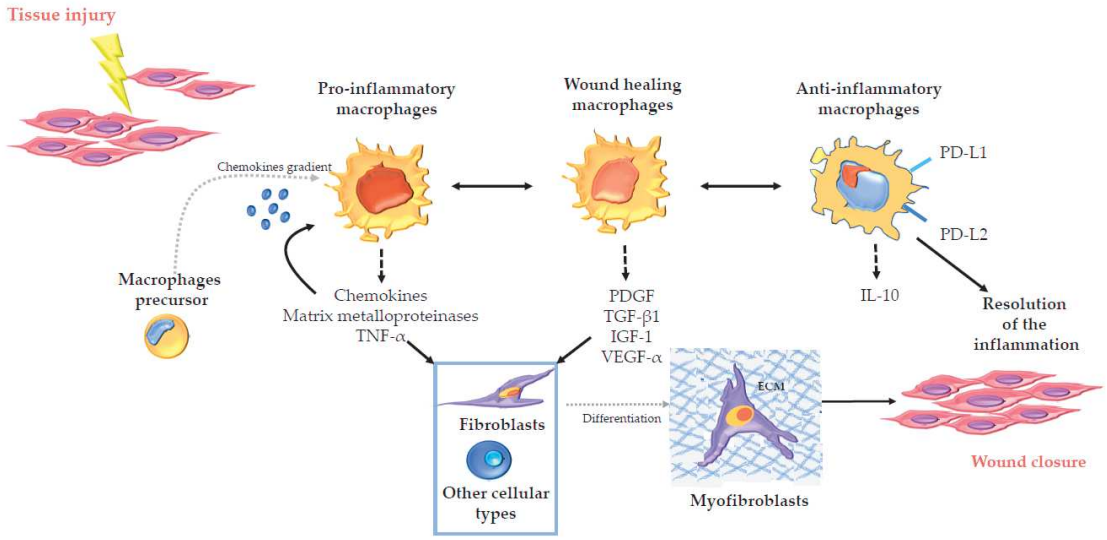


Figure 2. Illustration of the role of the macrophages and their highly flexible programming in tissue repair and fibrosis in several organs. Macrophages, because of their high flexibility, can play a key regulatory role in every stage that characterizes the tissue repair and fibrosis from the promotion to the resolution of the inflammation leading to the wound closure. The figure shows the principal events and principal molecules: chemokines, Matrix metalloproteinases, tumor necrosis factor-alfa (TNF-α), platelet-derived growth factor (PDGF), transforming growth factor-beta 1 (TGF-β1), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor-alfa (VEGF-α), programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2), interleukin-10 (IL-10) involved in the process, highlighting the different phenotypic states that the macrophages can assume in the process. The blue net represents the extracellular matrix (ECM) that is produced by myofibroblasts after that fibroblasts or other cellular types differentiated into them.

3. Macrophages in Uterine Fibroids

As it has been mentioned before, inflammation plays an important role in the pathophysiology of the uterine leiomyoma [105], which was defined as a typical fibrotic tissue [42,43].

Several studies have highlighted the involvement and importance of the macrophages in the inflammation and consequent fibrosis that are typical features of leiomyoma tissue [42–48,89,90,105,106].

Through the use of the glycosylated transmembrane glycoprotein antigen (CD68) that belongs to a family of lysosomal granules [141] as a marker of mature and activated macrophages, Miura et al. studied the macrophages' infiltration in different types of uterine leiomyomas. They demonstrated the myoma nodules and the autologous endometrium of the submucosal myomas (SMM) and intramural myomas (IMM) show a higher level of macrophage infiltration compared to the corresponding tissues of the subserosal myomas (SSM) or to the eutopic endometrium belonging to women without uterine myomas used as a control [142]. In addition to this, the authors showed a similar pattern also for the MCP-1 concentration. Moreover, MCP-1 concentration was shown to be positively correlated with the macrophage infiltration in SMM and IMM myoma nodules and endometrium [142]. So, the overproduction of MCP-1, which is one of the most important chemokines involved in the monocytes'/macrophages' migration and infiltration [143], may represent the cause

of the macrophages' infiltration in women with SMM and IMM, and this accumulation of inflammatory macrophages could lead to a negative effect on reproductive outcomes in women with SMM or IMM [142]. Anyway, the increased infiltration and accumulation of macrophages within some subtypes of fibroid tissue may represent proof of the macrophages' importance within leiomyoma pathology.

In support of all this, Khan and colleagues demonstrated that endometria belonging to women with uterine fibroids undergoing gonadotrophin-releasing hormone agonist (GnRHa) therapy exhibited decreased values of macrophage infiltration and MCP-1 levels when compared to corresponding values of macrophage infiltration and MCP-1 levels in endometria belonging to women with uterine fibroids that had not undergone GnRHa therapy [144].

On the other hand, a previous study conducted by Sozen highlighted that the myometrium of the women with uterine fibroids taking GnRHa and in particular the endothelial cells of blood vessels in myometrial tissues surrounding the leiomyoma show higher MCP-1 levels than the myometrium of the women with uterine fibroids not taking GnRHa [145]. This difference was not accompanied by a significant difference in the number of tissue macrophages between women who had undergone GnRHa therapy and women who had not undergone GnRHa therapy [145]. In this study, Sozen and colleagues expected to detect a macrophage infiltration increase following the MCP-1 increase because of the GnRHa use, but these results were disproved [145]. It is known that the uterus after GnRHa exposition shows a reduced arterial blood flow [146,147] and this may impair the macrophage accumulation, representing the explanation for why a macrophage infiltration increase does not accompany the MCP-1 increase in the myometrium of the women with uterine fibroids taking GnRHa [145].

In addition, estrogens and progesterone, which are recognized to be important promoters of the leiomyoma growth [96–104], impair MCP-1 expression [89].

Therefore, the discrepancy between the results obtained by Khan [144] and the results obtained by Sozen [145] may be due to the use of different tissue types or to the difference in tissue specificity and number of analyzed samples. The most important point to focus on is that within the complex network of molecules that are involved in the leiomyomas' development and growth, MCP-1 can also carry out an important role, taking part in the regulation of the macrophage infiltration. The MCP-1 regulatory action on macrophage infiltration may in turn be important for the development of the uterine fibroids. In addition to this, the cited studies about GnRHa, which is known to be commonly used for the treatment of uterine myomas, testify in any case that macrophages represent an aspect to be taken into consideration for the treatment and further clarification of the etiopathogenesis of uterine fibroids.

In addition, Kitaya and Yasuo have provided further evidence of the involvement and importance of the macrophages in the pathology of uterine fibroids. They analyzed the leukocyte density and composition in the human cycling endometrium in women affected by uterine fibroids. By immunohistochemical analysis, the authors compared endometrium with neighboring nodules with autologous endometrium without neighboring nodules and with allogeneic endometrium belonging to women without uterine fibroids. In particular, the macrophage (CD68 positive cells) density is significantly higher in the endometrium close to the leiomyoma nodules compared to the autologous endometrium far from the leiomyoma, as well as compared to the allogenic endometrium of women without uterine fibroids in the mid-to-late secretory phase [148]. The authors reported also that the endometrium far from the leiomyoma nodules had more macrophages than the endometrium of women without uterine fibroids in the proliferative and late secretory phase [148].

In addition, according to the results obtained by Miura et al. [142], the authors highlighted that the macrophage density is significantly higher in SMM than in IMM and SSM [148].

In addition, Kitaya and Yasuo showed that the whole stromal pan-leukocyte density is altered in the endometrium containing neighboring nodules of the women affected by uterine fibroids. Above all, they highlighted that the increased stromal pan-leukocyte density in endometrium with neighboring nodules during the proliferative phase is largely due to the increased macrophage density [148]. These findings testify once again that macrophages represent a very important aspect within the pathology of uterine fibroids.

Another aspect that is important to highlight about the involvement of the macrophages in uterine fibroids is the GM-CSF expression in leiomyoma and in myometrium. In fact, this cytokine represents the most important growth factor for macrophage proliferation, differentiation and functional activation [149].

In addition to this, GM-CSF has been demonstrated to determine the fibrotic reaction in several tissues [150–154]. In particular, thinking about the association between the overexpression of TGF-β and the establishment of tissue fibrosis through the stimulation of the conversion of fibroblasts into myofibroblasts in various sites throughout the body [155–157], GM-CSF has been shown to be involved in a fibrotic process that includes the accumulation of α smooth muscle actin-rich myofibroblasts through a mechanism involving TGF-β expression [150–154,157]. In addition, bearing in mind that it was demonstrated that TGF-β synthesis and release are increased in uterine fibroids [158], all of this makes GM-CSF one of the most important cytokines that may be able to play a key role in the initiation and maintenance of uterine leiomyoma, which is a typical fibrotic disorder [44].

In addition, since GM-CSF is considered to be the most important growth factor for macrophage proliferation, differentiation and functional activation [149], we could think that GM-CSF action and macrophage infiltration as they have been previously described could be interconnected within the development of the uterine fibroid pathology.

Considering the relationship between macrophages and uterine fibroids development, it is relevant that TGF-β is involved in tissue fibrosis in several sites throughout the body [155,156], is overexpressed in leiomyomas [158], and at the same time is the most important growth factor produced by macrophages [159]. In addition, TGF-β contributes to myofibroblast transformation [159], which represents another important aspect leading to the development of uterine fibroids [42,53].

It is very important to highlight that macrophages secrete not only TGF-β, which plays a key role in the progression of the fibrosis [159], but also produce activin A, an immunoregulator belonging to the TGF-β family [160]. Our group showed that, in primary leiomyoma cells, activin A acts as a pro-fibrotic factor leading to the expression of ECM proteins [161] that are upregulated in leiomyoma [44]. In addition to this, we later demonstrated in leiomyoma that activin A mRNA expression is upregulated by TNF-α [53] according to the literature, where the same effect is reported also in human bone marrow stromal cells and monocytes, human bone marrow stromal cell lines, cultured fibroblasts and keratinocytes [162–165]. The most remarkable aspect about activin A upregulation by TNF-α is that TNF-α is also mainly produced by macrophages [166] (Figure 3).

Studying leiomyomas, we found, according to the other results reported in this paper, that macrophage infiltration inside the leiomyoma is significantly higher compared with autologous myometrium more than 1.5 cm from the leiomyoma [53]. More precisely, by CD68 staining, our group found that macrophages predominantly localize inside leiomyoma and in the myometrium tissue next to leiomyoma. On the contrary, autologous distant myometrium showed low levels of CD68-positive macrophages [53] (Figure 4a,b). So, these findings highlight unequivocally the importance of inflammation, and above all, the key role of the macrophages in the development and growth of uterine fibroids.

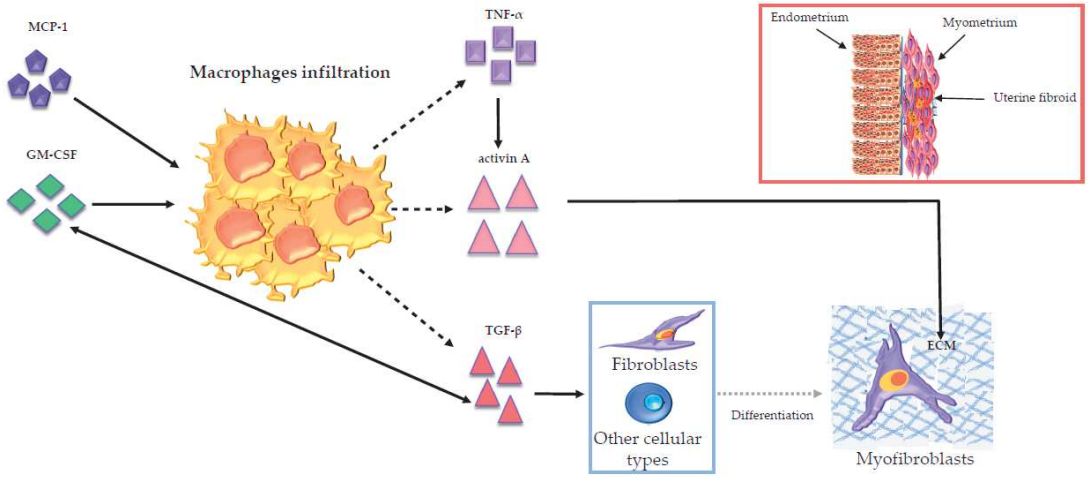


Figure 3. Illustration of the macrophages' (yellow in the figure) role in uterine fibroids. Monocyte chemoattractant protein-1 (MCP-1) takes part in the regulation of the macrophages' infiltration. The granulocyte macrophage-colony-stimulating factor (GM-CSF) is considered the most important growth factor for macrophage proliferation. GM-CSF can establish regulatory interactions with the transforming growth factor-beta (TGF-β), which was shown to be the most important growth factor secreted by macrophages. In uterine fibroids, TGF-β is overexpressed and it contributes to myofibroblast differentiation. Macrophages also secrete activin A, an immuno-regulator belonging to the TGF-β family. Activin A develops a pro-fibrotic action leading to the expression of the extracellular matrix (ECM) proteins (represented by the blue net in the figure), which are overexpressed in uterine fibroids. Activin A mRNA expression in uterine fibroids is upregulated by the tumor necrosis factor-alfa (TNF-α), an inflammatory mediator mainly produced by macrophages. On the right, above the image that portrays the myofibroblasts, the uterine fibroids (red in the figure) with the myometrium (pink in the figure), the endometrium (brown in the figure), the macrophages (yellow in the figure) and the overexpressed ECM proteins (blue net in the figure) are represented. The blood vessels within endometrium are also represented (red lines in the figure).

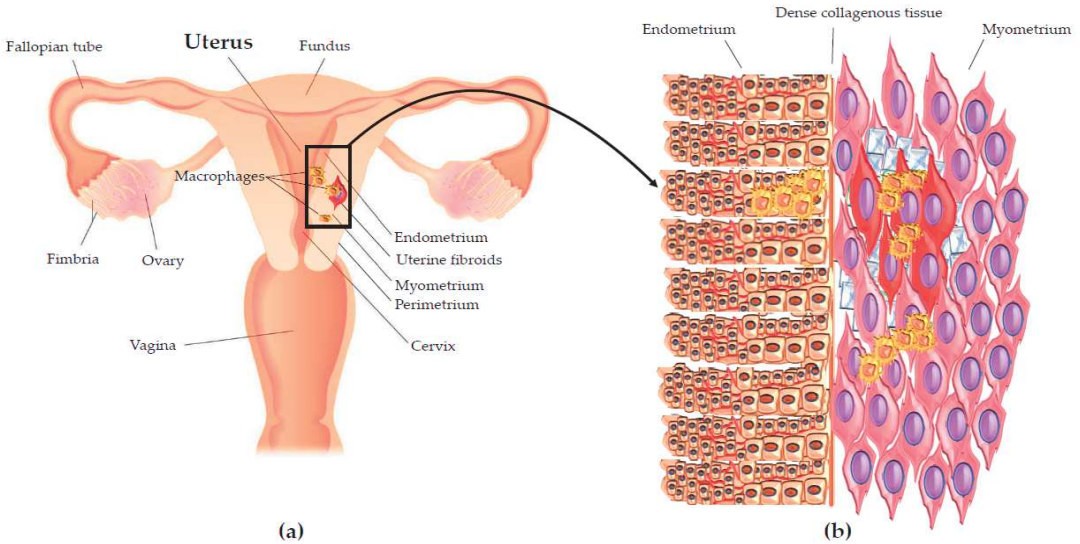


Figure 4. Macrophages in uterine fibroids. (**a**) Illustration of uterus showing the macrophage density in uterine fibroids pathology. (**b**) Enlargement of the detail showing the macrophage density in uterine fibroids pathology. Macrophages (yellow in the figure) predominantly localize inside uterine fibroids (red in the figure) and in the myometrium tissue (pink in the figure) next to them. Autologous distant myometrium shows low levels of macrophage infiltration. The macrophage density is higher also in the endometrium (brown in the figure) next to uterine fibroids than in the autologous endometrium far from uterine fibroid nodules. The extracellular matrix (ECM) around and within the uterine fibroids is also represented (blue net in the figure). The blood vessels within endometrium are also represented (red lines in the figure).

Since these results were obtained by studying different histotypes of leiomyomas, we could add that the reported macrophage localization is valid for both cellular and

usual leiomyomas with the cellular leiomyoma showing higher levels of CD68-positive macrophages compared with usual leiomyoma [53].

So, our group proposed a possible phase mechanism for the leiomyoma development. According to this mechanism, cellular leiomyoma histotype, as also suggested by Dixon et al. [54], could be considered as the first step in the tumoral transformation. In fact, cellular leiomyomas show low levels of the typical ECM proteins. On the other hand, our group noticed that cellular leiomyomas, in addition to higher levels of CD68 positive macrophages, also have an increased number of leukocytes and mast cells that are other types of inflammatory cells [53]. This aspect could represent a response to an inflammatory stimulus. As a result, these first step cells of the cellular leiomyoma undergo myofibroblast differentiation with the consequent upregulation of the typical ECM proteins [53]. In fact, usual leiomyomas that could be considered as the late-phase tumor show a larger amount of ECM proteins than we observed [53] (Figure 5).

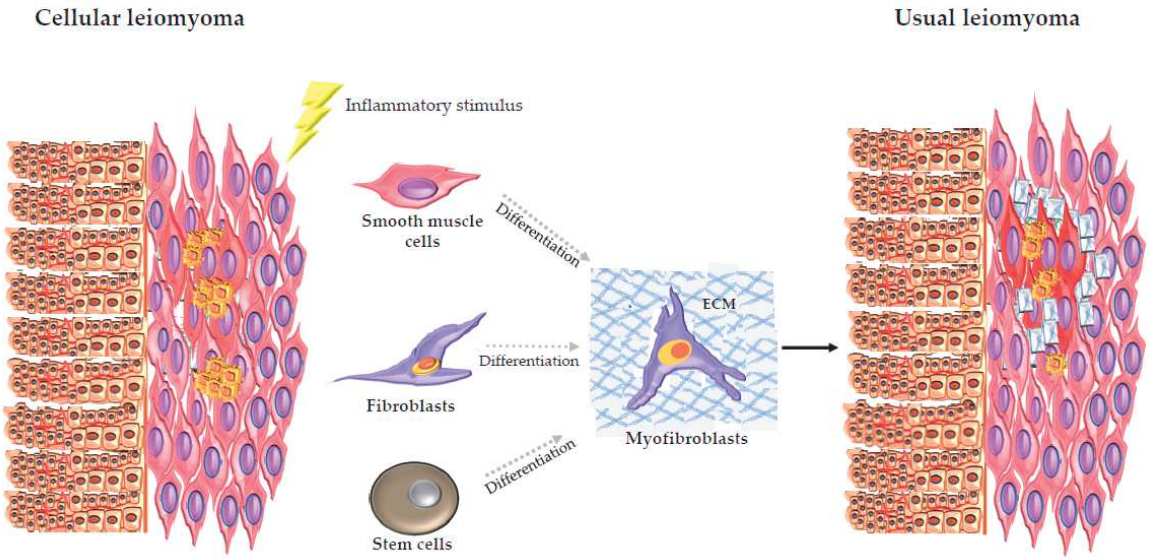


Figure 5. Illustration of the possible phase mechanism of leiomyoma development proposed by our group. Cellular leiomyoma is considered as the first step in the tumoral transformation. In fact, cellular leiomyoma shows higher levels of macrophage (yellow in the figure) infiltration and an increased number of inflammatory cells. This aspect could represent a response to an inflammatory stimulus that leads some cellular leiomyoma cells to myofibroblast differentiation with the consequent upregulation of the typical extracellular matrix (ECM) proteins. In fact, usual leiomyoma shows a larger amount of ECM proteins and low levels of macrophage infiltration. So, usual leiomyoma could be considered as the late-phase tumor. The blue net represents the typical ECM proteins: collagen 1A1, fibronectin and versican. The red color represents the uterine fibroids (light red for cellular leiomyoma histotype and dark red for usual leiomyoma histotype). The pink color represents the myometrium; the brown color represents the endometrium. The blood vessels within endometrium are also represented (red lines in the figure).

So, the data published by our group provide additional proof of the involvement of inflammation and the importance of the macrophages' action in the pathophysiology of uterine fibroids.

4. Conclusions

About uterine fibroids, whose etiopathogenesis has not yet understood at all, a deregulated inflammatory process leading to an exaggerated tissue repair may explain the abundant ECM, a typical feature of the uterine fibroids that, just because of this characteristic, is considered a typical fibrotic tissue. In particular, a key role in this complex network can be played by the macrophages when a deregulation in their action happens. In fact, the macrophages are important tissue repair actors by means of their highly flexible programming and their consequent plasticity. So, during the inflammation process and

the consequent wound healing that the inflammatory mechanisms lead to, macrophages have to proliferate and infiltrate within the damaged tissue; then, they assume different phenotypes and produce molecules that start, drive and finally stop the tissue repair up to the wound closure. So, there are a lot of critical checkpoints that need to be tightly regulated. In the uterine fibroids, sufficient proof of deregulated macrophage action was provided. In fact, increased infiltration and accumulation of macrophages within some subtypes of fibroid tissue were demonstrated. In addition to this, the importance of cytokines and chemokines such as GM-CSF and MCP-1 for the proliferation and infiltration of the macrophages in the uterine fibroids was shown. Furthermore, their expression in leiomyomas has been altered. All this, in turn, has an impact on the molecules that are secreted by macrophages. Among these molecules, the inflammation mediator TNF-α and the growth factors activin A and TGF-β can be considered the most important ones because they are known to be involved in the fibrosis that characterizes the uterine fibroids. In addition, these molecules, secreted by macrophages, were demonstrated to be interconnected with each other and with the GM-CSF. In this way, they establish in the uterine fibroids a complex network that, because of a dysregulation, at one or more levels, may explain the mechanisms that occur from an excessive wound healing driven by the inflammatory process to the fibrosis.

Better understanding the process leading to the increased infiltration and accumulation of macrophages in leiomyomas and the molecules involved within the consequent exaggerated tissue repair that arises from it, may represent proof of the macrophages' importance for the leiomyoma pathology. All this can also contribute to shed light on uterine fibroids etiology.

In turn, better understanding the uterine fibroids' etiology may represent the starting point to identify possible new therapy targets. This could improve the quality of life of the women affected by uterine fibroids.

Last but not least, a therapy against this pathology could also bring about better outcomes for pregnant women affected by this pathology.

Author Contributions: All authors participated in the writing, read and accepted the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. McEvoy, A.; Sabir, S. *Physiology, Pregnancy Contractions*; StatPearls: Treasure Island, FL, USA, 2021.
2. Valente, R.; Malesani, M.G. *Dizionario Medico*; Larousse: Paris, France, 1984; p. 928.
3. Gentile, F. *Enciclopedia Italiana*; Grolier: New Delhi, India, 1987; Volume 16, p. 271.
4. Day Baird, D.; Dunson, D.B.; Hill, M.C.; Cousins, D.; Schectman, J.M. High cumulative incidence of uterine leiomyoma in black and white women: Ultrasound evidence. *Am. J. Obstet. Gynecol.* **2003**, *188*, 100–107. [CrossRef] [PubMed]
5. Stewart, E.A. Uterine fibroids. *Lancet* **2001**, *357*, 293–298. [CrossRef]
6. Buttram, V.C., Jr.; Reiter, R.C. Uterine leiomyomata: Etiology, symptomatology, and management. *Fertil. Steril.* **1981**, *36*, 433–445. [PubMed]
7. Eldar-Geva, T.; Meagher, S.; Healy, D.L.; MacLachlan, V.; Breheny, S.; Wood, C. Effect of intramural, subserosal, and submucosal uterine fibroids on the outcome of assisted reproductive technology treatment. *Fertil. Steril.* **1998**, *70*, 687–691. [CrossRef]
8. Evans, P.; Brunsell, S. Uterine fibroid tumors: Diagnosis and treatment. *Am. Fam. Physician* **2007**, *75*, 1503–1508.
9. Marsh, E.E.; Bulun, S.E. Steroid hormones and leiomyomas. *Obstet. Gynecol. Clin. N. Am.* **2006**, *33*, 59–67. [CrossRef]
10. Okolo, S. Incidence, aetiology and epidemiology of uterine fibroids. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2008**, *22*, 571–588. [CrossRef] [PubMed]
11. Islam, M.S.; Protic, O.; Stortoni, P.; Grechi, G.; Lamanna, P.; Petraglia, F.; Castellucci, M.; Ciarmela, P. Complex networks of multiple factors in the pathogenesis of uterine leiomyoma. *Fertil. Steril.* **2013**, *100*, 178–193. [CrossRef]

12. Ciarmela, P.; Islam, M.S.; Reis, F.M.; Gray, P.C.; Bloise, E.; Petraglia, F.; Vale, W.; Castellucci, M. Growth factors and myometrium: Biological effects in uterine fibroid and possible clinical implications. *Hum. Reprod. Update* **2011**, *17*, 772–790. [CrossRef]
13. Lethaby, A.; Puscasiu, L.; Vollenhoven, B. Preoperative medical therapy before surgery for uterine fibroids. *Cochrane Database Syst. Rev.* **2017**, *11*, CD000547. [CrossRef]
14. Angioni, S.; D'Alterio, M.N.; Daniilidis, A. Highlights on Medical Treatment of Uterine Fibroids. *Curr. Pharm. Des.* **2021**. [CrossRef] [PubMed]
15. Friedman, A.J.; Hoffman, D.I.; Comite, F.; Browneller, R.W.; Miller, J.D. Treatment of leiomyomata uteri with leuprolide acetate depot: A double-blind, placebo-controlled, multicenter study. The Leuprolide Study Group. *Obstet. Gynecol.* **1991**, *77*, 720–725.
16. Schlaff, W.D.; Zerhouni, E.A.; Huth, J.A.; Chen, J.; Damewood, M.D.; Rock, J.A. A placebo-controlled trial of a depot gonadotropin-releasing hormone analogue (leuprolide) in the treatment of uterine leiomyomata. *Obstet. Gynecol* **1989**, *74*, 856–862. [PubMed]
17. Stovall, T.G.; Muneyyirci-Delale, O.; Summitt, R.L., Jr.; Scialli, A.R. GnRH agonist and iron versus placebo and iron in the anemic patient before surgery for leiomyomas: A randomized controlled trial. Leuprolide Acetate Study Group. *Obstet. Gynecol.* **1995**, *86*, 65–71. [CrossRef]
18. Carbonell, J.L.; Acosta, R.; Perez, Y.; Garces, R.; Sanchez, C.; Tomasi, G. Treatment of Uterine Myoma with 2.5 or 5 mg Mifepristone Daily during 3 Months with 9 Months Posttreatment Followup: Randomized Clinical Trial. *ISRN Obstret. Gynecol.* **2013**, *2013*, 649030. [CrossRef]
19. Chwalisz, K.; Larsen, L.; Mattia-Goldberg, C.; Edmonds, A.; Elger, W.; Winkel, C.A. A randomized, controlled trial of asoprisnil, a novel selective progesterone receptor modulator, in women with uterine leiomyomata. *Fertil. Steril.* **2007**, *87*, 1399–1412. [CrossRef] [PubMed]
20. Wiehle, R.D.; Goldberg, J.; Brodniewicz, T.; Jarus-Dziedzic, K.; Jabiry-Zieniewicz, Z. Effects of a new progesterone receptor modulator, CDB-4124, on fibroid size and uterine bleeding. *Obstet. Gynecol.* **2008**, *3*, 17–20.
21. Levens, E.D.; Potlog-Nahari, C.; Armstrong, A.Y.; Wesley, R.; Premkumar, A.; Blithe, D.L.; Blocker, W.; Nieman, L.K. CDB-2914 for uterine leiomyomata treatment: A randomized controlled trial. *Obstret. Gynecol.* **2008**, *111*, 1129–1136. [CrossRef]
22. Nieman, L.K.; Blocker, W.; Nansel, T.; Mahoney, S.; Reynolds, J.; Blithe, D.; Wesley, R.; Armstrong, A. Efficacy and tolerability of CDB-2914 treatment for symptomatic uterine fibroids: A randomized, double-blind, placebo-controlled, phase IIb study. *Fertil. Steril.* **2011**, *95*, 767–772.e2. [CrossRef]
23. Donnez, J.; Tatarchuk, T.F.; Bouchard, P.; Puscasiu, L.; Zakharenko, N.F.; Ivanova, T.; Ugocsai, G.; Mara, M.; Jilla, M.P.; Bestel, E. Ulipristal acetate versus placebo for fibroid treatment before surgery. *N. Engl. J. Med.* **2012**, *366*, 409–420. [CrossRef]
24. Donnez, J.; Tomaszewski, J.; Vázquez, F.; Bouchard, P.; Lemieszczuk, B.; Baró, F.; Nouri, K.; Selvaggi, L.; Sodowski, K.; Bestel, E. Ulipristal acetate versus leuprolide acetate for uterine fibroids. *N. Engl. J. Med.* **2012**, *366*, 421–432. [CrossRef] [PubMed]
25. Blithe, D.L.; Nieman, L.K.; Blye, R.P.; Stratton, P.; Passaro, M. Development of the selective progesterone receptor modulator CDB-2914 for clinical indications. *Steroids* **2003**, *68*, 1013–1017. [CrossRef]
26. Attardi, B.J.; Burgenson, J.; Hild, S.A.; Reel, J.R.; Blye, R.P. CDB-4124 and its putative monodemethylated metabolite, CDB-4453, are potent antiprogestins with reduced antiglucocorticoid activity: In vitro comparison to mifepristone and CDB-2914. *Mol. Cell. Endocrinol.* **2002**, *188*, 111–123. [CrossRef]
27. Del Forno, S.; Degli Esposti, E.; Salucci, P.; Leonardi, D.; Iodice, R.; Arena, A.; Raimondo, D.; Paradisi, R.; Seracchioli, R. Liver function, tolerability and satisfaction during treatment with ulipristal acetate in women with fibroids: A single center experience. *Gynecol. Endocrinol.* **2020**, *36*, 445–447. [CrossRef] [PubMed]
28. Ciarmela, P.; Carrarelli, P.; Islam, M.S.; Janjusevic, M.; Zupi, E.; Tosti, C.; Castellucci, M.; Petraglia, F. Ulipristal acetate modulates the expression and functions of activin a in leiomyoma cells. *Reprod. Sci.* **2014**, *21*, 1120–1125. [CrossRef]
29. Frasca, C.; Arena, A.; Degli Esposti, E.; Raimondo, D.; Del Forno, S.; Moro, E.; Zanello, M.; Mabrouk, M.; Seracchioli, R. First Impressions Can Be Deceiving: Surgical Outcomes of Laparoscopic Myomectomy in Patients Pretreated with Ulipristal Acetate. *J. Minim. Invasive Gynecol.* **2020**, *27*, 633–638. [CrossRef]
30. Friedman, A.J.; Rein, M.S.; Harrison-Atlas, D.; Garfield, J.M.; Doubilet, P.M. A randomized, placebo-controlled, double-blind study evaluating leuprolide acetate depot treatment before myomectomy. *Fertil. Steril.* **1989**, *52*, 728–733. [CrossRef]
31. Islam, M.S.; Protic, O.; Giannubilo, S.R.; Toti, P.; Tranquilli, A.L.; Petraglia, F.; Castellucci, M.; Ciarmela, P. Uterine leiomyoma: Available medical treatments and new possible therapeutic options. *J. Clin. Endocrinol. Metab.* **2013**, *98*, 921–934. [CrossRef]
32. Wallach, E.E.; Vlahos, N.F. Uterine myomas: An overview of development, clinical features, and management. *Obstret. Gynecol.* **2004**, *104*, 393–406. [CrossRef]
33. Kim, T.; Purdy, M.P.; Kendall-Rauchfuss, L.; Habermann, E.B.; Bews, K.A.; Glasgow, A.E.; Khan, Z. Myomectomy associated blood transfusion risk and morbidity after surgery. *Fertil. Steril.* **2020**, *114*, 175–184. [CrossRef]
34. Flynn, M.; Jamison, M.; Datta, S.; Myers, E. Health care resource use for uterine fibroid tumors in the United States. *Am. J. Obstet. Gynecol.* **2006**, *195*, 955–964. [CrossRef]
35. Zepiridis, L.I.; Grimbizis, G.F.; Tarlatzis, B.C. Infertility and uterine fibroids. *Best Pract. Res. Clin. Obstret. Gynaecol.* **2016**, *34*, 66–73. [CrossRef] [PubMed]
36. Kurman, R.; Ellenson, L.; Ronnett, B. Blaustein's Pathology of the Female Genital Tract. *Int. J. Gynecol. Pathol.* **2016**. [CrossRef]
37. Rosai, J.; Ackerman, L. *Rosai and Ackerman's Surgical Pathology*, 10th ed.; Elsevier: Amsterdam, The Netherlands, 2012; pp. 1508–1513.

38. Avritscher, R.; Iyer, R.B.; Ro, J.; Whitman, G. Lipoleiomyoma of the uterus. *AJR Am. J. Roentgenol.* **2001**, *177*, 856. [CrossRef] [PubMed]
39. Myles, J.L.; Hart, W.R. Apoplectic leiomyomas of the uterus. A clinicopathologic study of five distinctive hemorrhagic leiomyomas associated with oral contraceptive usage. *Am. J. Surg. Pathol.* **1985**, *9*, 798–805. [CrossRef] [PubMed]
40. Ciarmela, P.; Islam, M.S.; Lamanna, P.; Tranquilli, A.L.; Castellucci, M. Healthy and pathological changes of myometrium: Pregnant myometrium, uterine fibroids and leiomyosarcoma. *Rev. Argent. Anatomía Clínica* **2012**, *4*, 7–13. [CrossRef]
41. Toledo, G.; Oliva, E. Smooth muscle tumors of the uterus: A practical approach. *Arch. Pathol. Lab. Med.* **2008**, *132*, 595–605. [CrossRef] [PubMed]
42. Leppert, P.C.; Catherino, W.H.; Segars, J.H. A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays. *Am. J. Obstret. Gynecol.* **2006**, *195*, 415–420. [CrossRef] [PubMed]
43. Malik, M.; Norian, J.; McCarthy-Keith, D.; Britten, J.; Catherino, W.H. Why leiomyomas are called fibroids: The central role of extracellular matrix in symptomatic women. *Semin. Reprod. Med.* **2010**, *28*, 169–179. [CrossRef]
44. Gelse, K.; Pöschl, E.; Aigner, T. Collagens-structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* **2003**, *55*, 1531–1546. [CrossRef]
45. Fujita, M. Histological and biochemical studies of collagen in human uterine leiomyomas. *Hokkaido J. Med. Sci.* **1985**, *60*, 602–615. [PubMed]
46. Arici, A.; Sozen, I. Transforming growth factor-beta3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation. *Fertil. Steril.* **2000**, *73*, 1006–1011. [CrossRef]
47. Norian, J.M.; Malik, M.; Parker, C.Y.; Joseph, D.; Leppert, P.C.; Segars, J.H.; Catherino, W.H. Transforming growth factor beta3 regulates the versican variants in the extracellular matrix-rich uterine leiomyomas. *Reprod. Sci.* **2009**, *16*, 1153–1164. [CrossRef] [PubMed]
48. Stewart, E.A.; Friedman, A.J.; Peck, K.; Nowak, R.A. Relative overexpression of collagen type I and collagen type III messenger ribonucleic acids by uterine leiomyomas during the proliferative phase of the menstrual cycle. *J. Clin. Endocrinol. Metab.* **1994**, *79*, 900–906. [PubMed]
49. Giuliani, A.; Greco, S.; Pacile, S.; Zannotti, A.; Delli Carpini, G.; Tromba, G.; Giannubilo, S.R.; Ciavattini, A.; Ciarmela, P. Advanced 3D Imaging of Uterine Leiomyoma's Morphology by Propagation-based Phase-Contrast Microtomography. *Sci. Rep.* **2019**, *9*, 10580. [CrossRef]
50. Walker, C.L.; Stewart, E.A. Uterine fibroids: The elephant in the room. *Science* **2005**, *308*, 1589–1592. [CrossRef]
51. Hulboy, D.L.; Rudolph, L.A.; Matrisian, L.M. Matrix metalloproteinases as mediators of reproductive function. *Mol. Hum. Reprod.* **1997**, *3*, 27–45. [CrossRef]
52. Ohara, N. Sex steroidal modulation of collagen metabolism in uterine leiomyomas. *Clin. Exp. Obstet. Gynecol.* **2009**, *36*, 10.
53. Protic, O.; Toti, P.; Islam, M.S.; Occhini, R.; Giannubilo, S.R.; Catherino, W.H.; Cinti, S.; Petraglia, F.; Ciavattini, A.; Castellucci, M.; et al. Possible involvement of inflammatory/reparative processes in the development of uterine fibroids. *Cell Tissue Res.* **2016**, *364*, 415–427. [CrossRef]
54. Flake, G.P.; Andersen, J.; Dixon, D. Etiology and pathogenesis of uterine leiomyomas: A review. *Environ. Health Perspect.* **2003**, *111*, 1037–1054. [CrossRef]
55. Kiechle-Schwarz, M.; Sreekantaiah, C.; Berger, C.S.; Pedron, S.; Medchill, M.T.; Surti, U.; Sandberg, A.A. Nonrandom cytogenetic changes in leiomyomas of the female genitourinary tract. A report of 35 cases. *Cancer Genet. Cytogenet.* **1991**, *53*, 125–136. [CrossRef]
56. Rein, M.S.; Friedman, A.J.; Barbieri, R.L.; Pavelka, K.; Fletcher, J.A.; Morton, C.C. Cytogenetic abnormalities in uterine leiomyomata. *Obstet. Gynecol.* **1991**, *77*, 923–926.
57. Meloni, A.M.; Surti, U.; Contento, A.M.; Davare, J.; Sandberg, A.A. Uterine leiomyomas: Cytogenetic and histologic profile. *Obstet. Gynecol.* **1992**, *80*, 209–217. [PubMed]
58. Cha, P.C.; Takahashi, A.; Hosono, N.; Low, S.K.; Kamatani, N.; Kubo, M.; Nakamura, Y. A genome-wide association study identifies three loci associated with susceptibility to uterine fibroids. *Nat. Genet.* **2011**, *43*, 447–450. [CrossRef]
59. Ligon, A.H.; Scott, I.C.; Takahara, K.; Greenspan, D.S.; Morton, C.C. PCOLCE deletion and expression analyses in uterine leiomyomata. *Cancer Genet. Cytogenet.* **2002**, *137*, 133–137. [CrossRef]
60. Ptacek, T.; Song, C.; Walker, C.L.; Sell, S.M. Physical mapping of distinct 7q22 deletions in uterine leiomyoma and analysis of a recently annotated 7q22 candidate gene. *Cancer Genet. Cytogenet.* **2007**, *174*, 116–120. [CrossRef] [PubMed]
61. Velagaleti, G.V.; Tonk, V.S.; Hakim, N.M.; Wang, X.; Zhang, H.; Erickson-Johnson, M.R.; Medeiros, F.; Oliveira, A.M. Fusion of HMGA2 to COG5 in uterine leiomyoma. *Cancer Genet. Cytogenet.* **2011**, *202*, 11–16. [CrossRef]
62. Mäkinen, N.; Mehine, M.; Tolvanen, J.; Kaasinen, E.; Li, Y.; Lehtonen, H.J.; Gentile, M.; Yan, J.; Enge, M.; Taipale, M. MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. *Science* **2011**, *334*, 252–255. [CrossRef]
63. Nezhad, M.H.; Drieschner, N.; Helms, S.; Meyer, A.; Tadayyon, M.; Klemke, M.; Belge, G.; Bartnitzke, S.; Burchardt, K.; Frantzen, C. 6p21 rearrangements in uterine leiomyomas targeting HMGA1. *Cancer Genet. Cytogenet.* **2010**, *203*, 247–252. [CrossRef]
64. Sandberg, A.A. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: Leiomyoma. *Cancer Genet. Cytogenet.* **2005**, *158*, 1–26. [CrossRef]
65. El-Gharib, M.N.; Elsobky, E.S. Cytogenetic aberrations and the development of uterine leiomyomata. *J. Obstet. Gynaecol. Res.* **2010**, *36*, 101–107. [CrossRef]

66. Mehine, M.; Kaasinen, E.; Makinen, N.; Katainen, R.; Kampjarvi, K.; Pitkanen, E.; Heinonen, H.R.; Butzow, R.; Kilpivaara, O.; Kuosmanen, A.; et al. Characterization of uterine leiomyomas by whole-genome sequencing. *N. Engl. J. Med.* **2013**, *369*, 43–53. [CrossRef]
67. Mehine, M.; Makinen, N.; Heinonen, H.R.; Aaltonen, L.A.; Vahteristo, P. Genomics of uterine leiomyomas: Insights from high-throughput sequencing. *Fertil. Steril.* **2014**, *102*, 621–629. [CrossRef]
68. Marsh, E.E.; Lin, Z.; Yin, P.; Milad, M.; Chakravarti, D.; Bulun, S.E. Differential expression of microRNA species in human uterine leiomyoma versus normal myometrium. *Fertil. Steril.* **2008**, *89*, 1771–1776. [CrossRef]
69. Navarro, A.; Yin, P.; Monsivais, D.; Lin, S.M.; Du, P.; Wei, J.J.; Bulun, S.E. Genome-Wide DNA Methylation Indicates Silencing of Tumor Suppressor Genes in Uterine Leiomyoma. *PLoS ONE* **2012**, *7*, e33284. [CrossRef] [PubMed]
70. Wei, L.H.; Torng, P.L.; Hsiao, S.M.; Jeng, Y.M.; Chen, M.W.; Chen, C.A. Histone Deacetylase 6 Regulates Estrogen Receptor α in Uterine Leiomyoma. *Reprod. Sci.* **2011**, *18*, 755–762. [CrossRef] [PubMed]
71. Yang, Q.; Mas, A.; Diamond, M.P.; Al-Hendy, A. The Mechanism and Function of Epigenetics in Uterine Leiomyoma Development. *Reprod. Sci.* **2016**, *23*, 163–175. [CrossRef] [PubMed]
72. Wang, T.; Zhang, X.; Obijuru, L.; Laser, J.; Aris, V.; Lee, P.; Mittal, K.; Soteropoulos, P.; Wei, J.J. A micro-RNA signature associated with race, tumor size, and target gene activity in human uterine leiomyomas. *Genes Chromosomes Cancer* **2007**, *46*, 336–347. [CrossRef] [PubMed]
73. Liu, J.; Matsuo, H.; Xu, Q.; Chen, W.; Wang, J.; Maruo, T. Concentration-dependent effects of a selective estrogen receptor modulator raloxifene on proliferation and apoptosis in human uterine leiomyoma cells cultured in vitro. *Hum. Reprod.* **2007**, *22*, 1253–1259. [CrossRef] [PubMed]
74. Georgieva, B.; Milev, I.; Minkov, I.; Dimitrova, I.; Bradford, A.P.; Baev, V. Characterization of the uterine leiomyoma microRNAome by deep sequencing. *Genomics* **2012**, *93*, 275–281. [CrossRef] [PubMed]
75. Ciebiera, M.; Wlodarczyk, M.; Zgliczynski, S.; Lozinski, T.; Walczak, K.; Czekierdowski, A. The Role of miRNA and Related Pathways in Pathophysiology of Uterine Fibroids-From Bench to Bedside. *Int. J. Mol. Sci.* **2020**, *21*, 3016. [CrossRef]
76. Nothnick, W.B. Non-coding RNAs in Uterine Development, Function and Disease. *Adv. Exp. Med. Biol.* **2016**, *886*, 171–189. [CrossRef]
77. Baranov, V.S.; Osinovskaya, N.S.; Yarmolinskaya, M.I. Pathogenomics of Uterine Fibroids Development. *Int. J. Mol. Sci.* **2019**, *20*, 6151. [CrossRef]
78. McWilliams, M.M.; Chennathukuzhi, V.M. Recent Advances in Uterine Fibroid Etiology. *Semin. Reprod. Med.* **2017**, *35*, 181–189. [CrossRef] [PubMed]
79. Mallik, S.; Maulik, U. MiRNA-TF-gene network analysis through ranking of biomolecules for multi-informative uterine leiomyoma dataset. *J. Biomed. Inf.* **2015**, *57*, 308–319. [CrossRef]
80. Ciarmela, P.; Bloise, E.; Gray, P.C.; Carrarelli, P.; Islam, M.S.; De Pascalis, F.; Severi, F.M.; Vale, W.; Castellucci, M.; Petraglia, F. Activin-A and myostatin response and steroid regulation in human myometrium: Disruption of their signalling in uterine fibroid. *J. Clin. Endocrinol. Metab.* **2011**, *96*, 755–765. [CrossRef] [PubMed]
81. Moro, E.; Degli Esposti, E.; Borghese, G.; Manzara, F.; Zanello, M.; Raimondo, D.; Gava, G.; Arena, A.; Casadio, P.; Meriggiola, M.C.; et al. The Impact of Hormonal Replacement Treatment in Postmenopausal Women with Uterine Fibroids: A State-of-the-Art Review of the Literature. *Medicina (Kaunas)* **2019**, *55*, 549. [CrossRef] [PubMed]
82. Sozen, I.; Arici, A. Interactions of cytokines, growth factors, and the extracellular matrix in the cellular biology of uterine leiomyomata. *Fertil. Steril.* **2002**, *78*, 1–12. [CrossRef]
83. Ciarmela, P.; Wiater, E.; Vale, W. Activin-A in myometrium: Characterization of the actions on myometrial cells. *Endocrinology* **2008**, *149*, 2506–2516. [CrossRef] [PubMed]
84. Ciarmela, P.; Wiater, E.; Smith, S.M.; Vale, W. Presence, actions, and regulation of myostatin in rat uterus and myometrial cells. *Endocrinology* **2009**, *150*, 906–914. [CrossRef]
85. Hatthachote, P.; Gillespie, J.I. Complex interactions between sex steroids and cytokines in the human pregnant myometrium: Evidence for an autocrine signaling system at term. *Endocrinology* **1999**, *140*, 2533–2540. [CrossRef]
86. Litovkin, K.V.; Domenyuk, V.P.; Bubnov, V.V.; Zaporozhan, V.N. Interleukin-6-174G/C polymorphism in breast cancer and uterine leiomyoma patients: A population-based case control study. *Exp. Oncol.* **2007**, *29*, 295–298.
87. Kurachi, O.; Matsuo, H.; Samoto, T.; Maruo, T. Tumor necrosis factor-α expression in human uterine leiomyoma and its down-regulation by progesterone. *J. Clin. Endocrinol. Metab.* **2001**, *86*, 2275–2280. [CrossRef] [PubMed]
88. Syssoev, K.A.; Kulagina, N.V.; Chukhlovin, A.B.; Morozova, E.B.; Totolian, A.A. Expression of mRNA for chemokines and chemokine receptors in tissues of the myometrium and uterine leiomyoma. *Bull. Exp. Biol. Med.* **2008**, *145*, 84–89. [CrossRef]
89. Sozen, I.; Olive, D.L.; Arici, A. Expression and hormonal regulation of monocyte chemotactic protein-1 in myometrium and leiomyomata. *Fertil. Steril.* **1998**, *69*, 1095–1102. [CrossRef]
90. Nair, S.; Al-Hendy, A. Adipocytes enhance the proliferation of human leiomyoma cells via TNF-α proinflammatory cytokine. *Reprod. Sci.* **2011**, *18*, 1186–1192. [CrossRef] [PubMed]
91. Bodner-Adler, B.; Bodner, K.; Kimberger, O.; Czerwenka, K.; Leodolter, S.; Mayerhofer, K. Expression of matrix metalloproteinases in patients with uterine smooth muscle tumors: An immunohistochemical analysis of MMP-1 and MMP-2 protein expression in leiomyoma, uterine smooth muscle tumor of uncertain malignant potential, and leiomyosarcoma. *J. Soc. Gynecol. Investig.* **2004**, *11*, 182–186. [CrossRef]

92. Wolanska, M.; Sobolewski, K.; Bańkowski, E.; Jaworski, S. Matrix metalloproteinases of human leiomyoma in various stages of tumor growth. *Gynecol. Obstet. Investig.* **2004**, *58*, 14–18. [CrossRef]
93. Bogusiewicz, M.; Stryjecka-Zimmer, M.; Postawski, K.; Jakimiuk, A.J.; Rechberger, T. Activity of matrix metalloproteinase-2 and-9 and contents of their tissue inhibitors in uterine leiomyoma and corresponding myometrium. *Gynecol. Endocrinol.* **2007**, *23*, 541–546. [CrossRef]
94. Walker, C.L.; Hunter, D.; Everitt, J.I. Uterine leiomyoma in the Eker rat: A unique model for important diseases of women. *Genes Chromosomes Cancer* **2003**, *38*, 349–356. [CrossRef] [PubMed]
95. Cook, J.D.; Walker, C.L. The Eker rat: Establishing a genetic paradigm linking renal cell carcinoma and uterine leiomyoma. *Curr. Mol. Med.* **2004**, *4*, 813–824. [CrossRef]
96. Andersen, J.; Barbieri, R.L. Abnormal gene expression in uterine leiomyomas. *J. Soc. Gynecol. Investig.* **1995**, *2*, 663–672. [CrossRef] [PubMed]
97. Maruo, T.; Ohara, N.; Wang, J.; Matsuo, H. Sex steroidal regulation of uterine leiomyoma growth and apoptosis. *Hum. Reprod. Update* **2004**, *10*, 207–220. [CrossRef]
98. Sadan, O.; Van Iddekinge, B.; Van Gelderen, C.J.; Savage, N.; Becker, P.J.; Van Der Walt, L.A.; Robinson, M. Oestrogen and progesterone receptor concentrations in leiomyoma and normal myometrium. *Ann. Clin. Biochem.* **1987**, *24*, 263–267. [CrossRef] [PubMed]
99. Kim, J.J.; Sefton, E.C. The role of progesterone signaling in the pathogenesis of uterine leiomyoma. *Mol. Cell. Endocrinol.* **2011**, *358*, 223–231. [CrossRef] [PubMed]
100. Brandon, D.D.; Bethea, C.L.; Strawn, E.Y.; Novy, M.J.; Burry, K.A.; Harrington, M.S.; Erickson, T.E.; Warner, C.; Keenan, E.J.; Clinton, G.M. Progesterone receptor messenger ribonucleic acid and protein are overexpressed in human uterine leiomyomas. *Am. J. Obstet. Gynecol.* **1993**, *169*, 78–85. [CrossRef]
101. Marelli, G.; Codegoni, A.M.; Bizzi, A. Estrogen and progesterone receptors in leiomyomas and normal uterine tissues during reproductive life. *Acta Eur. Fertil.* **1989**, *20*, 19–22.
102. Viville, B.; Charnock-Jones, D.S.; Sharkey, A.M.; Wetzka, B.; Smith, S.K. Distribution of the A and B forms of the progesterone receptor messenger ribonucleic acid and protein in uterine leiomyomata and adjacent myometrium. *Hum. Reprod.* **1997**, *12*, 815–822. [CrossRef]
103. Ying, Z.; Weiyuan, Z. Dual actions of progesterone on uterine leiomyoma correlate with the ratio of progesterone receptor A:B. *Gynecol. Endocrinol.* **2009**, *25*, 520–523. [CrossRef]
104. Fujimoto, J.; Hirose, R.; Ichigo, S.; Sakaguchi, H.; Li, Y.; Tamaya, T. Expression of progesterone receptor form A and B mRNAs in uterine leiomyoma. *Tumor Biol.* **1998**, *19*, 126–131. [CrossRef]
105. Protic, O.; Islam, M.S.; Greco, S.; Giannubilo, S.R.; 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**, *35*, 499–509. [CrossRef]
106. Kisseleva, T.; Brenner, D.A. Mechanisms of fibrogenesis. *Exp. Biol. Med.* **2008**, *233*, 109–122. [CrossRef] [PubMed]
107. Hinz, B.; Phan, S.H.; Thannickal, V.J.; Prunotto, M.; Desmouliere, A.; Varga, J.; De Wever, O.; Mareel, M.; Gabbiani, G. Recent developments in myofibroblast biology: Paradigms for connective tissue remodeling. *Am. J. Pathol.* **2012**, *180*, 1340–1355. [CrossRef] [PubMed]
108. Hinz, B. Formation and function of the myofibroblast during tissue repair. *J. Investig. Derm.* **2007**, *127*, 526–537. [CrossRef] [PubMed]
109. Wynn, T.A. Cellular and molecular mechanisms of fibrosis. *J. Pathol.* **2008**, *214*, 199–210. [CrossRef] [PubMed]
110. Kacperczyk, J.; Bartnik, P.; Romejko-Wolniewicz, E.; Dobrowolska-Redo, A. Postmyomectomic Uterine Rupture Despite Cesarean Section. *Anticancer Res.* **2016**, *36*, 1011–1013.
111. Wynn, T.A.; Barron, L. Macrophages: Master regulators of inflammation and fibrosis. *Semin. Liver Dis.* **2010**, *30*, 245–257. [CrossRef]
112. Mosser, D.M.; Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **2008**, *8*, 958–969. [CrossRef]
113. Lech, M.; Anders, H.J. Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair. *Biochim. Biophys. Acta* **2013**, *1832*, 989–997. [CrossRef]
114. Davies, L.C.; Jenkins, S.J.; Allen, J.E.; Taylor, P.R. Tissue-resident macrophages. *Nat. Immunol.* **2013**, *14*, 986–995. [CrossRef]
115. Galli, S.J.; Borregaard, N.; Wynn, T.A. Phenotypic and functional plasticity of cells of innate immunity: Macrophages, mast cells and neutrophils. *Nat. Immunol.* **2011**, *12*, 1035–1044. [CrossRef] [PubMed]
116. Jenkins, S.J.; Ruckerl, D.; Cook, P.C.; Jones, L.H.; Finkelman, F.D.; van Rooijen, N.; MacDonald, A.S.; Allen, J.E. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* **2011**, *332*, 1284–1288. [CrossRef] [PubMed]
117. Jenkins, S.J.; Ruckerl, D.; Thomas, G.D.; Hewitson, J.P.; Duncan, S.; Brombacher, F.; Maizels, R.M.; Hume, D.A.; Allen, J.E. IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. *J. Exp. Med.* **2013**, *210*, 2477–2491. [CrossRef]
118. Berse, B.; Brown, L.F.; Van de Water, L.; Dvorak, H.F.; Senger, D.R. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol. Biol. Cell* **1992**, *3*, 211–220. [CrossRef] [PubMed]

119. Chujo, S.; Shirasaki, F.; Kondo-Miyazaki, M.; Ikawa, Y.; Takehara, K. Role of connective tissue growth factor and its interaction with basic fibroblast growth factor and macrophage chemoattractant protein-1 in skin fibrosis. *J. Cell Physiol.* **2009**, *220*, 189–195. [CrossRef]
120. Rappolee, D.A.; Mark, D.; Banda, M.J.; Werb, Z. Wound macrophages express TGF-alpha and other growth factors in vivo: Analysis by mRNA phenotyping. *Science* **1988**, *241*, 708–712. [CrossRef]
121. Shimokado, K.; Raines, E.W.; Madtes, D.K.; Barrett, T.B.; Benditt, E.P.; Ross, R. A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. *Cell* **1985**, *43*, 277–286. [CrossRef]
122. Willenborg, S.; Lucas, T.; van Loo, G.; Knipper, J.A.; Krieg, T.; Haase, I.; Brachvogel, B.; Hammerschmidt, M.; Nagy, A.; Ferrara, N.; et al. CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. *Blood* **2012**, *120*, 613–625. [CrossRef]
123. Murray, P.J.; Wynn, T.A. Protective and pathogenic functions of macrophage subsets. *Nat. Rev. Immunol.* **2011**, *11*, 723–737. [CrossRef] [PubMed]
124. Ramachandran, P.; Iredale, J.P.; Fallowfield, J.A. Resolution of liver fibrosis: Basic mechanisms and clinical relevance. *Semin. Liver Dis.* **2015**, *35*, 119–131. [CrossRef]
125. Khalil, N.; Bereznay, O.; Sporn, M.; Greenberg, A.H. Macrophage production of transforming growth factor beta and fibroblast collagen synthesis in chronic pulmonary inflammation. *J. Exp. Med.* **1989**, *170*, 727–737. [CrossRef]
126. Said, E.A.; Dupuy, F.P.; Trautmann, L.; Zhang, Y.; Shi, Y.; El-Far, M.; Hill, B.J.; Noto, A.; Ancuta, P.; Peretz, Y.; et al. Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. *Nat. Med.* **2010**, *16*, 452–459. [CrossRef] [PubMed]
127. Shouval, D.S.; Biswas, A.; Goettel, J.A.; McCann, K.; Conaway, E.; Redhu, N.S.; Mascanfroni, I.D.; Al Adham, Z.; Lavoie, S.; Ibourk, M.; et al. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity* **2014**, *40*, 706–719. [CrossRef]
128. Zigmond, E.; Bernshtein, B.; Friedlander, G.; Walker, C.R.; Yona, S.; Kim, K.W.; Brenner, O.; Krauthgamer, R.; Varol, C.; Muller, W.; et al. Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. *Immunity* **2014**, *40*, 720–733. [CrossRef] [PubMed]
129. Wynn, T.A.; Ramalingam, T.R. Mechanisms of fibrosis: Therapeutic translation for fibrotic disease. *Nat. Med.* **2012**, *18*, 1028–1040. [CrossRef] [PubMed]
130. Tidball, J.G. Regulation of muscle growth and regeneration by the immune system. *Nat. Rev. Immunol.* **2017**, *17*, 165–178. [CrossRef]
131. Arnold, L.; Henry, A.; Poron, F.; Baba-Amer, Y.; van Rooijen, N.; Plonquet, A.; Gherardi, R.K.; Chazaud, B. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* **2007**, *204*, 1057–1069. [CrossRef]
132. Saini, J.; McPhee, J.S.; Al-Dabbagh, S.; Stewart, C.E.; Al-Shanti, N. Regenerative function of immune system: Modulation of muscle stem cells. *Ageing Res. Rev.* **2016**, *27*, 67–76. [CrossRef]
133. Martinez, F.O.; Gordon, S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Rep.* **2014**, *6*, 13. [CrossRef]
134. Stables, M.J.; Shah, S.; Camon, E.B.; Lovering, R.C.; Newson, J.; Bystrom, J.; Farrow, S.; Gilroy, D.W. Transcriptomic analyses of murine resolution-phase macrophages. *Blood* **2011**, *118*, e192–e208. [CrossRef]
135. Varga, T.; Mounier, R.; Horvath, A.; Cuvellier, S.; Dumont, F.; Poliska, S.; Ardjoune, H.; Juban, G.; Nagy, L.; Chazaud, B. Highly Dynamic Transcriptional Signature of Distinct Macrophage Subsets during Sterile Inflammation, Resolution, and Tissue Repair. *J. Immunol.* **2016**, *196*, 4771–4782. [CrossRef]
136. Varga, T.; Mounier, R.; Patsalos, A.; Gogolak, P.; Peloquin, M.; Horvath, A.; Pap, A.; Daniel, B.; Nagy, G.; Pintye, E.; et al. Macrophage PPARgamma, a Lipid Activated Transcription Factor Controls the Growth Factor GDF3 and Skeletal Muscle Regeneration. *Immunity* **2016**, *45*, 1038–1051. [CrossRef]
137. Heredia, J.E.; Mukundan, L.; Chen, F.M.; Mueller, A.A.; Deo, R.C.; Locksley, R.M.; Rando, T.A.; Chawla, A. Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. *Cell* **2013**, *153*, 376–388. [CrossRef] [PubMed]
138. Takeda, N.; O'Dea, E.L.; Doedens, A.; Kim, J.W.; Weidemann, A.; Stockmann, C.; Asagiri, M.; Simon, M.C.; Hoffmann, A.; Johnson, R.S. Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis. *Genes Dev.* **2010**, *24*, 491–501. [CrossRef]
139. Gondin, J.; Theret, M.; Duhamel, G.; Pegan, K.; Mathieu, J.R.; Peyssonnaux, C.; Cuvellier, S.; Latroche, C.; Chazaud, B.; Bendahan, D.; et al. Myeloid HIFs are dispensable for resolution of inflammation during skeletal muscle regeneration. *J. Immunol.* **2015**, *194*, 3389–3399. [CrossRef] [PubMed]
140. Oishi, Y.; Manabe, I. Macrophages in inflammation, repair and regeneration. *Int. Immunol.* **2018**, *30*, 511–528. [CrossRef]
141. Holness, C.L.; Simmons, D.L. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood* **1993**, *81*, 1607–1613. [CrossRef] [PubMed]
142. Miura, S.; Khan, K.N.; Kitajima, M.; Hiraki, K.; Moriyama, S.; Masuzaki, H.; Samejima, T.; Fujishita, A.; Ishimaru, T. Differential infiltration of macrophages and prostaglandin production by different uterine leiomyomas. *Hum. Reprod.* **2006**, *21*, 2545–2554. [CrossRef] [PubMed]

143. Deshmane, S.L.; Kremlev, S.; Amini, S.; Sawaya, B.E. Monocyte chemoattractant protein-1 (MCP-1): An overview. *J. Interferon Cytokine Res.* **2009**, *29*, 313–326. [CrossRef]
144. Khan, K.N.; Kitajima, M.; Hiraki, K.; Fujishita, A.; Sekine, I.; Ishimaru, T.; Masuzaki, H. Changes in tissue inflammation, angiogenesis and apoptosis in endometriosis, adenomyosis and uterine myoma after GnRH agonist therapy. *Hum. Reprod.* **2010**, *25*, 642–653. [CrossRef]
145. Sozen, I.; Senturk, L.M.; Arici, A. Effect of gonadotropin-releasing hormone agonists on monocyte chemotactic protein-1 production and macrophage infiltration in leiomyomatous uterus. *Fertil. Steril.* **2001**, *76*, 792–796. [CrossRef]
146. Aleem, F.A.; Predanic, M. The hemodynamic effect of GnRH agonist therapy on uterine leiomyoma vascularity: A prospective study using transvaginal color Doppler sonography. *Gynecol. Endocrinol.* **1995**, *9*, 253–258. [CrossRef]
147. Matta, W.H.; Stabile, I.; Shaw, R.W.; Campbell, S. Doppler assessment of uterine blood flow changes in patients with fibroids receiving the gonadotropin-releasing hormone agonist Buserelin. *Fertil. Steril.* **1988**, *49*, 1083–1085. [CrossRef]
148. Kitaya, K.; Yasuo, T. Leukocyte density and composition in human cycling endometrium with uterine fibroids. *Hum. Immunol.* **2010**, *71*, 158–163. [CrossRef]
149. Rasko, J.E.; Ben, H. Granulocyte-macrophage colony stimulating factor. In *The Cytokine Handbook*; Thomson, A., Ed.; Academic Press: London, UK, 1994; pp. 343–369.
150. Andreutti, D.; Gabbiani, G.; Neuville, P. Early granulocyte-macrophage colony-stimulating factor expression by alveolar inflammatory cells during bleomycin-induced rat lung fibrosis. *Lab. Investig.* **1998**, *78*, 1493–1502. [PubMed]
151. Rubbia-Brandt, L.; Sappino, A.P.; Gabbiani, G. Locally applied GM-CSF induces the accumulation of alpha-smooth muscle actin containing myofibroblasts. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* **1991**, *60*, 73–82. [CrossRef]
152. Xing, Z.; Gauldie, J.; Tremblay, G.M.; Hewlett, B.R.; Addison, C. Intradermal transgenic expression of granulocyte-macrophage colony-stimulating factor induces neutrophilia, epidermal hyperplasia, Langerhans' cell/macrophage accumulation, and dermal fibrosis. *Lab. Investig. A J. Tech. Methods Pathol.* **1997**, *77*, 615.
153. Xing, Z.; Tremblay, G.M.; Sime, P.J.; Gauldie, J. Overexpression of granulocyte-macrophage colony-stimulating factor induces pulmonary granulation tissue formation and fibrosis by induction of transforming growth factor-beta 1 and myofibroblast accumulation. *Am. J. Pathol.* **1997**, *150*, 59–66. [PubMed]
154. Xing, Z.; Ohkawara, Y.; Jordana, M.; Graham, F.; Gauldie, J. Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J. Clin. Investig.* **1996**, *97*, 1102–1110. [CrossRef]
155. Border, W.A.; Noble, N.A. Transforming growth factor beta in tissue fibrosis. *N. Engl. J. Med.* **1994**, *331*, 1286–1292. [CrossRef] [PubMed]
156. Roberts, A.B. Transforming growth factor-beta: Activity and efficacy in animal models of wound healing. *Wound Repair Regen* **1995**, *3*, 408–418. [CrossRef]
157. Vyalov, S.; Desmouliere, A.; Gabbiani, G. GM-CSF-induced granulation tissue formation: Relationships between macrophage and myofibroblast accumulation. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* **1993**, *63*, 231–239. [CrossRef]
158. Ciebiera, M.; Wlodarczyk, M.; Wrzosek, M.; Meczekalski, B.; Nowicka, G.; Lukaszuk, K.; Ciebiera, M.; Slabuszewska-Jozwiak, A.; Jakiel, G. Role of Transforming Growth Factor beta in Uterine Fibroid Biology. *Int. J. Mol. Sci.* **2017**, *18*, 2435. [CrossRef]
159. Fallowfield, J.A.; Mizuno, M.; Kendall, T.J.; Constandinou, C.M.; Benyon, R.C.; Duffield, J.S.; Iredale, J.P. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J. Immunol.* **2007**, *178*, 5288–5295. [CrossRef] [PubMed]
160. Sierra-Filardi, E.; Puig-Kroger, A.; Blanco, F.J.; Nieto, C.; Bragado, R.; Palomero, M.I.; Bernabeu, C.; Vega, M.A.; Corbi, A.L. Activin A skews macrophage polarization by promoting a proinflammatory phenotype and inhibiting the acquisition of anti-inflammatory macrophage markers. *Blood* **2011**, *117*, 5092–5101. [CrossRef] [PubMed]
161. Islam, M.S.; Catherino, W.H.; Protic, O.; Janjusevic, M.; Gray, P.C.; Giannubilo, S.R.; Ciavattini, A.; Lamanna, P.; Tranquilli, A.L.; Petraglia, F.; et al. Role of activin-A and myostatin and their signaling pathway in human myometrial and leiomyoma cell function. *J. Clin. Endocrinol. Metab.* **2014**, *99*, E775–E785. [CrossRef]
162. Hubner, G.; Werner, S. Serum growth factors and proinflammatory cytokines are potent inducers of activin expression in cultured fibroblasts and keratinocytes. *Exp. Cell Res.* **1996**, *228*, 106–113. [CrossRef]
163. Shao, L.; Frigon, N.L., Jr.; Sehy, D.W.; Yu, A.L.; Lofgren, J.; Schwall, R.; Yu, J. Regulation of production of activin A in human marrow stromal cells and monocytes. *Exp. Hematol.* **1992**, *20*, 1235–1242. [PubMed]
164. Shao, L.E.; Frigon, N.L., Jr.; Yu, A.; Palyash, J.; Yu, J. Contrasting effects of inflammatory cytokines and glucocorticoids on the production of activin A in human marrow stromal cells and their implications. *Cytokine* **1998**, *10*, 227–235. [CrossRef]
165. Takahashi, S.; Uchimaru, K.; Harigaya, K.; Asano, S.; Yamashita, T. Tumor necrosis factor and interleukin-1 induce activin A gene expression in a human bone marrow stromal cell line. *Biochem. Biophys. Res. Commun* **1992**, *188*, 310–317. [CrossRef]
166. Mantovani, A.; Sica, A.; Sozzani, S.; Allavena, P.; Vecchi, A.; Locati, M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* **2004**, *25*, 677–686. [CrossRef] [PubMed]

RBMO

ELSEVIER

ARTICLE

Quercetin and indole-3-carbinol inhibit extracellular matrix expression in human primary uterine leiomyoma cells

BIOGRAPHY

Dr Ciarmela is an Associate Professor of Human Anatomy at Università Politecnica delle Marche, Italy. Her research is focused on the investigation of uterine physiology and pathophysiology and exploration of the role of growth factors. The studies conducted are aimed at understanding the pathogenesis of uterine fibroids and at developing potential therapeutic agents.

Stefania Greco[1,†], Md Soriful Islam[1,*,‡], Alessandro Zannotti[1,2], Giovanni Delli Carpini[2], Stefano Raffaele Giannubilo[2], Andrea Ciavattini[2], Felice Petraglia[3], Pasquapina Ciarmela[1,*]

KEY MESSAGE

This study demonstrates the in-vitro anti-fibrotic (reduction of collagen 1A1 and fibronectin) and anti-migratory effects of quercetin and indole-3-carbinol in myometrial and leiomyoma cells, as well as the anti-proliferative effect in myometrial cells. This demonstrates the scientific basis for the development of new therapeutic, preventive agents, or both, for uterine leiomyomas.

ABSTRACT

Research question: What is the effect of quercetin and indole-3-carbinol (I3C) on extracellular matrix expression, cell migration and proliferation in human myometrial and uterine leiomyoma cells.

Design: Myometrial and leiomyoma cells were treated with quercetin or I3C at different concentrations (10 μg/ml; 50 μg/ml; 100 μg/ml; and 250 μg/ml) for 48 h to measure mRNA and protein expressions of extracellular matrix (collagen 1A1, fibronectin and versican), as well as cell migration and the proliferation rate.

Results: Quercetin decreased mRNA levels of collagen 1A1 in myometrial ($P < 0.0001$) and leiomyoma cells ($P < 0.0001$). Quercetin reduced mRNA and protein levels of fibronectin in myometrial cells ($P < 0.05$) and fibronectin protein in leiomyoma cells ($P < 0.05$). I3C reduced collagen 1A1 mRNA levels in myometrial ($P < 0.05$) and leiomyoma cells at higher dose ($P < 0.05$). The protein levels of fibronectin were also reduced in both myometrial and leiomyoma cells with highest dose of I3C ($P < 0.05$), although mRNA levels were not affected in leiomyoma cells. Neither quercetin nor I3C treatment altered versican mRNA levels in both cell types. A significant reduction of the migration of both myometrial and leiomyoma cells in response to quercetin was observed ($P < 0.05$) and I3C ($P < 0.05$ for myometrial and $P < 0.01$ for leiomyoma cells) treatment. Both quercetin and I3C significantly reduced myometrial cell proliferation ($P < 0.05$).

Conclusions: The in-vitro anti-fibrotic, anti-migratory and anti-proliferative effects of quercetin and I3C form the scientific basis for developing new therapeutic, preventive agents, or both, for uterine leiomyomas.

[1] Department of Experimental and Clinical Medicine, Faculty of Medicine, Università Politecnica delle Marche, 60020 via Tronto 10/a Ancona, Italy
[2] Department of Medical Biotechnology and Department of Molecular and Developmental, Medicine, Obstetrics, and Gynaecology, Università Politecnica delle Marche, Ancona 60020, Italy
[3] Department of Experimental and Clinical Biomedical Sciences "Mario Serio", Obstetrics and Gynaecology, University of Florence, Italy
†Equally contributed.
‡Present address: Department of Gynecology and Obstetrics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

KEYWORDS
Antifibrotic
Dietary phytochemicals
Extracellular matrix
Indole-3-carbinol
Quercetin
Uterine fibroid

© 2020 Published by Elsevier Ltd on behalf of Reproductive Healthcare Ltd.
*Corresponding author. E-mail address: p.ciarmela@univpm.it (P Ciarmela). https://doi.org/10.1016/j.rbmo.2020.01.006
1472-6483/© 2020 Published by Elsevier Ltd on behalf of Reproductive Healthcare Ltd.
Declaration: The authors report no financial or commercial conflicts of interest.

INTRODUCTION

Uterine leiomyoma, or uterine fibroid, is the most common benign tumour in gynaecology, and affects many women, especially during their fertile years. This tumour develops in the myometrium or muscular layer of the uterus (*Walker and Stewart, 2005*). Fibroids cause pelvic pain, abnormal vaginal bleeding, pressure on the bladder, infertility and obstetric complications (*Ciavattini et al. 2015*; *Kashani et al. 2016*). Surgery, such as hysterectomy and myomectomy, has been the definitive treatment for symptomatic fibroids. Gonadotrophin-releasing hormone agonist (*Friedman et al. 1989*; *Stovall et al., 1995*) and ulipristal acetate (*Donnez et al., 2015*) plays an important role as presurgical treatments in reducing fibroid size as well as its associated symptoms (such as abnormal bleeding). The long-term use of gonadotrophin-releasing hormone agonists, however, is associated with menopausal symptoms and bone loss (*Friedman et al., 1991*; *Leather et al., 1993*). Recently, concern about the risk of rare but serious liver injury with ulipristal acetate treatment has been raised (*Donnez, 2018*).

Uterine fibroids consist of an excessive amount of extracellular matrix (ECM) proteins, including collagen, fibronectin and versican (*Islam et al., 2017a*). The growth of fibroids is primarily influenced by cell–ECM interaction, and the rigid structure of ECM is believed to be the cause of abnormal bleeding in the uterus (*Islam et al., 2017a*). Therefore, compounds that can regulate ECM production could be an ideal way to control fibroid growth.

Dietary phytochemicals are plant origin chemical compounds that have disease-controlling properties (*Islam et al., 2014a*; *2017c*). Quercetin is a flavonoid, found in most edible fruits and vegetables, such as tea, lemon, tomato, onion leaves and strawberry. This flavonoid is known to exert antifibrotic effects in hepatic fibrosis (*Lee et al., 2003*), pulmonary fibrosis (*Baowen et al., 2010*) and kidney fibroblasts (*Hu et al., 2009*). Quercetin suppresses collagen production in lung fibroblasts induced by transforming growth factor beta (*Nakamura et al., 2011*), as well as collagen and fibronectin production, and transforming growth factor beta/Smad-signalling pathway in keloid fibroblasts (*Phan et al., 2003a*; *2003b*).

Epidemiological studies have reported that intake of quercetin-rich foods reduces the risk of gastric cancer by 43% and colon cancer by 32%. Consumption of quercetin was also reported to reduce lung cancer risk by 51% and even in heavy smokers by 65% (*Dunnick and Hailey, 1992*; *Pereira et al., 1996*).

Indole-3-carbinol (I3C) is produced from naturally occurring glucosinolates contained in a wide variety of plants, including members of the Cruciferae family and particularly members of the genus Brassica. I3C has been reported to inhibit cell proliferation as well as expression levels of alpha-smooth muscle actin, type I collagen in hepatic stellate cells (*Ping et al., 2011*). The combination of epidemiological and experimental data provides suggestive evidence that a high intake of cruciferous vegetables protects against some cancers at various sites. In a nationwide study of postmenopausal women in Sweden, consumption of cruciferous vegetables was inversely associated with breast cancer risk (*Terry et al., 2001*). Cruciferous vegetables, however, have a number of cancer-preventing compounds; I3C alone showed efficacy for the prevention of breast (*Bradlow et al., 1991*), endometrial (*Kojima et al. 1994*) and cervical cancers (*Jin et al. 1999*) in animal models. Importantly, I3C showed efficacy for treatment of precancerous lesions of the cervix in translational human studies (*Auborn et al., 2003*). The aim of the present study was to investigate the effects of quercetin and I3C on extracellular matrix expression as well as the cell migration and proliferation of myometrial and leiomyoma cells.

MATERIALS AND METHODS

Primary cell cultures

The present study included samples of myometrial and usual type of leiomyoma tissue excised from 21 women undergoing hysterectomy for symptomatic fibroids. The most homogeneous sample possible was included considering the high variability; for example, differing ages (range 41–49 years), race, hormonal milieu, tumour size and location of tumours. In fact, all patients were white and in the proliferative phase of the menstrual cycle. The location of leiomyomas was intramural and measured 7–10 cm in diameter. All patients who had not received exogenous hormones in the previous 3 months were considered. All patients provided informed consent and the permission of the Human Investigation Committee was granted on 10 February 2016 (reference number: 2015 0486 OR).

After surgery, the myometrial and leiomyoma samples were collected in Hank's Balanced Salt Solution (Euroclone, Milan, Italy), and immediately transported to the laboratory. The samples were washed several times with Dulbecco's phosphate buffered saline (PBS) (Invitrogen, ThermoFischer, Carlsbad, CA, USA) to remove excess blood. After cutting tissue into small pieces, the samples 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 a water bath with manual shaking. After digesting the cell suspension, it was centrifuged at 250 g for 10 min, and the collagenase was inactivated with fetal bovine serum (FBS) (Gibco, ThermoFischer Scientific).

Finally, the cell pellet was dispersed in DMEM containing 10% FBS (Gibco) (ThermoFischer, Waltham, MA, USA), 1% antibiotic (penicillin-streptomycin) (Euroclone SpA, Milan, Italy), 1% fungizone (Amphotericin B) (Lonza Group Ag, Basel, Switzerland), and 1% glutamine (Gibco) (ThermoFischer, Waltham, MA, USA) in plastic dishes, and incubated at 37°C in 95% air, 5% CO_2. The growth medium was changed after 24 or 48 h 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-alpha-smooth muscle actin) (Sigma-Aldrich, Milan, Italy). Almost all cells were strongly positive for alpha-smooth muscle actin.

The lower passage number (P0–P4) of cells was used for experiments to avoid changes in phenotype and gene expression. Myometrial and leiomyoma cells were treated with quercetin and I3C (10 µg/ml; 50 µg/ml; 100 µg/ml; and 250 µg/ml) (Sigma-Aldrich, Milan, Italy) for 48 h. After treatment, the cells were detached from the petri dish by the TRIzol® reagent (Invitrogen), ThermoFisher, Waltham, MA, USA).

This reagent produces three phases: the upper white transparent phase containing the RNA, a white disk with the DNA and a lower pink phase containing the proteins.

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted using the white transparent phase from TRIzol® reagent (Invitrogen, ThermoFisher, Waltham, MA, USA), according to the manufacturer's instructions. Samples were digested with a ribonuclease-free deoxyribonuclease (Promega Corporation, Madison, WI, USA), and the RNA was cleaned up and concentrated using ReliaPrep™ RNA Cell Miniprep System (Promega Corporation, Madison, WI, USA). Reverse transcriptase was carried out using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, ThermoFischer, Foster, CA, USA) with 1 µg RNA, and the TaqMan real-time polymerase chain reaction (PCR) was carried out for all the genes analysed. TaqMan gene expression assays were used (Applied Biosystems), (ThermoFischer, Waltham, MA, USA): collagen 1A1 (Hs00164004_m1), fibronectin (Hs00365052_m1), versican (Hs00171642_m1) and the housekeeping genes, hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), (Hs99999909_m1) and β-actin (*ACTB*) (Hs99999903_m1). The following thermal cycle protocol was used (initial denaturation at 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s) using 100 ng cDNA in a final reaction volume of 10 µl. The blank for each reaction, consisting of amplifications carried out in the absences of reverse transcriptase enzyme, was measured.

Western blotting

Proteins were extracted using the pink phase from TRIzol® reagent (Invitrogen) (ThermoFisher, Waltham, MA, USA), 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, Waltham, MA, USA), 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, Waltham, MA, USA) according to the manufacturer's instructions. Ponceau S solution (Euroclone) was used to detect proteins on nitrocellulose membranes. After blocking the membrane with 5% (weight by volume) non-fat 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 mouse monoclonal anti-fibronectin (Sigma-Aldrich, Milan, Italy), and 1: 3000 dilutions for mouse monoclonal anti-β-tubulin (Sigma-Aldrich, Milan, Italy). The next day, the membrane was washed four times in TBST and incubated with 1:10000 dilution horseradish peroxidase conjugated anti-mouse immunoglobulin antibody from sheep (Amersham) for 2 h. The membrane was washed four times in TBST, and immunoreactive proteins were visualized using Clarity™ western enhanced chemiluminescence substrate (Bio-Rad Laboratories, Hercules, CA, USA). Protein levels were measured using Image J 1.49n software (National Institutes of Health, http://imagej.nih.gov/ij).

Immunocytochemistry

A total of 1000 myometrial and leiomyoma cells were seeded in each chamber of tissue culture slides with eight wells and allowed to divide. Myometrial and leiomyoma cells were treated with quercetin and I3C (10 µg/ml; 50 µg/ml; 100 µg/ml; and 250 µg/ml) (Sigma-Aldrich, Milan, Italy) for 48 h; at the end of treatment, cells were washed three times with PBS, treated with 0.2% Triton X-100 in PBS for 5 min and washed three times with PBS. To inhibit endogenous peroxidase activity, cells were incubated for 10 min with 3% hydrogen peroxide in deionized water. Cells were washed three times with PBS and, to block non-specific background, cells were incubated for 20 min at room temperature with normal horse serum diluted 1:75 in 1% bovine serum albumin in PBS. Cells were then incubated with Fibronectin mouse monoclonal antibody (Sigma-Aldrich, Milan, Italy) at 1:600 and alpha-smooth muscle actin mouse monoclonal antibody (Dako) (Agilent Technologies, Santa Clara, California, USA) at 1:1000 dilution for 1 h at room temperature. After washing with PBS, cells were incubated with biotinylated anti-mouse immunoglobulin made in horse diluted 1:200. The peroxidase ABC method was carried out for 1 h at room temperature using 3,3′-diaminobenzidine as chromogen. Sections were counterstained in Mayer's haematoxylin, dehydrated and mounted with Eukitt solution.

Wound closure assay

Myometrial and leiomyomas 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 serum-free media in the absence or presence of quercetin or I3C (250 µg/ml) for 48 h. Digital images were taken of the initial wound and at 12 h, 24 h and 48 h (*Rodriguez et al. 2005*; *Liang et al., 2007*; *Wright et al., 2012*). 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:

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

Cell proliferation assay

Cellular growth curves were measured using the CyQuant cell proliferation assay kit according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA). Myometrial and leiomyoma cells were seeded in 96-well plates at various initial density of cells: 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 quercetin or I3C at 250 µg/ml, and left untreated (Invitrogen, Life Technologies, Carlsbad, CA, USA), and allowed to divide for the number of days indicated (days 1, 2 and 4). At the indicated times, media were discarded, and plates were frozen at –80°C. On the day of the assay, plates were thawed, cells were lysed and total cellular nucleic acid was measured using fluorescence at 520 nm emission after excitation at 480 nm (*Ciarmela et al., 2008*; *Ciarmela et al., 2009*; *Islam et al. 2014b*).

Data analysis

GraphPad Prism version 6.01 for Windows (GraphPad, San Diego, CA) was used for statistical analyses. The data were analysed using non-parametric Kruskal–Wallis analysis of variance, followed by post-hoc Dunn test for multiple comparisons. Differences were considered significant when $P < 0.05$. All experiments were carried out in triplicate, except for the real-time PCR and the cell proliferation assay ($n = 6$).

RESULTS

Effects of quercetin and indole-3-carbinol on extracellular matrix mRNA expression in primary myometrial and leiomyoma cells

To determine the antifibrotic effect of quercetin and I3C, primary myometrial and leiomyoma cells were treated with either compounds at different concentrations: 10 μg/ml; 50 μg/ml; 100 μg/ml; and 250 μg/ml for 48 h. The mRNA expression was measured by real-time PCR. Quercetin reduced collagen 1A1 mRNA in primary myometrial and leiomyoma cells at all concentrations. In myometrial cells, fibronectin mRNA was significantly reduced at all doses of quercetin ($P < 0.05$ to $P < 0.01$), whereas, in leiomyoma cells, a significant reduction only took place at the lowest dose ($P < 0.05$).

I3C reduced collagen 1A1 mRNA levels in both myometrial and leiomyoma cells at higher dose. Fibronectin mRNA was reduced only in myometrial cells with highest dose of I3C. The versican mRNA expression levels were affected by neither of these compounds in both cell types (FIGURE 1).

Effects of quercetin and indole-3-carbinol on protein expression of fibronectin in primary myometrial and leiomyoma cells

Data from western blotting showed that protein expression levels of fibronectin were reduced after treatments with quercetin or I3C in primary leiomyoma and myometrial cells compared with untreated controls (FIGURE 2).

Immunocytochemistry micrographs also showed decreased expression levels of fibronectin in primary leiomyoma and myometrial cells in response to quercetin or I3C treatment (FIGURE 3).

Effect of quercetin and indole-3-carbinol on migration of primary myometrial and leiomyoma cells

To evaluate the effect of quercetin and I3C (250 μg/ml) on cell migration, myometrial and leiomyoma cells were treated with these compounds at 250 μg/ml or left untreated for 48 h, and digital images were captured from the initial wound and after 12 h, 24 h (data not shown) and 48 h. Results showed that quercetin and I3C significantly reduced the migration of both myometrial (both quercetin and I3C; $P < 0.05$) and leiomyoma cells ($P < 0.05$ and $P < 0.01$, respectively, for quercetin and I3C) (FIGURE 4A and FIGURE 4B).

Effect of quercetin and indole-3-carbinol on proliferation of primary myometrial and leiomyoma cells

To demonstrate the antiproliferative effect of quercetin and I3C, myometrial and leiomyoma cells were treated with these two compounds at 250 μg/ml or left untreated for different days of interval (days 1, 2 and 4). Results showed that leiomyoma cell proliferation was not affected by quercetin and I3C, but they reduced myometrial cell proliferation (FIGURE 5).

DISCUSSION

Uterine leiomyoma is not a malignant disease; however, it is associated with several reproductive and gynaecological problems, including menorrhagia, dysmenorrhoea, chronic pelvic pain, infertility, recurrent miscarriage, premature birth and postpartum haemorrhage (*Buttram and Reiter, 1981*). Compared with the burden of disease to women's health, medical treatments are limited, so the present study focuses on seeking preventive therapies.

The role of cell proliferation, and of ECM proteins, in producing the leiomyoma bulk structure and growth provides an important target to control this tumour by developing anti-fibrotic and anti-proliferative agents.

Recent studies have reported the importance of the role of phytochemical agents on uterine leiomyomas. The present results suggest that quercetin, the main active compound of the onion, may be a potential adjuvant for the treatment of uterine disorders, in line with a previous study conducted *in vivo* on rats (*Wu et al., 2015*). In addition, the following effects have been shown: isoliquiritigenin, a licorice flavonoid, induced antiproliferative effects on Eker rat uterine leiomyoma ELT3 cells (*Lin et al. 2019a*); resveratrol, a polyphenol was found to be a potent anti-proliferative agent against human leiomyoma cells *in vitro* and in murine models (*Chen et al., 2019*); and an anti-proliferative effect of adlay seeds was demonstrated on rat uterine leiomyoma ELT3 cells (*Lin et al. 2019b*).

In the present study, the in-vitro anti-fibrotic, anti-migratory and anti-proliferative effects of two phytochemicals (quercetin and I3C) in myometrial and leiomyoma cells were evaluated. We demonstrated anti-fibrotic effects of quercetin and I3C by assessing the expression levels of three major ECM components (collagen 1A1, fibronectin and versican). Collagen acts as a central structural component of the ECM that maintains cellular morphology. It plays important roles in regulating proliferation, migration, differentiation, survival, and wound healing and the fibrotic processes (*Pickering, 2001*). Leiomyoma cells demonstrate an overexpression of collagen types I mRNA in leiomyomas compared with the adjacent myometrium (*Leppert et al., 2006*). Fibronectin, a glycoprotein of the ECM protein, plays an important role in cell adhesion, migration, growth and differentiation as well as in fibrosis. Leiomyoma cells expresses elevated levels of fibronectin compared with myometrial cells (*Arici and Sozen, 2000*). Versican, a large chondroitin sulphate proteoglycan, plays an important role in cell migration, cell adhesion, cell proliferation, tissue stabilization, tissue homeostasis and inflammation (*Islam et al., 2016*). The up-regulation of versican expression has been reported in leiomyoma cells compared with healthy counterparts (*Norian et al. 2009*).

We found that quercetin and I3C reduced collagen 1A1 mRNA expression in myometrial and leiomyoma cells, as well as fibronectin mRNA expression in myometrial cells. Quercetin was effective at all doses used (10 μg/ml; 50 μg/ml; 100 μg/ml; 250 μg/ml), whereas I3C was effective only at 250 μg/ml. Although fibronectin mRNA reduction was not statistically significant in leiomyoma cells after quercetin (apart from at the lowest dose) or I3C treatment, Western blotting analysis showed a significant reduction of fibronectin protein expression in myometrial and leiomyoma cells. The visible reduction of fibronectin protein expression was also observed in myometrial and leiomyoma cells, compared with untreated controls, as measured by immunocytochemistry. In addition to antifibrotic effects of quercetin and I3C, they also reduced cell migration of myometrial and leiomyoma cells. Both compounds were unable to inhibit growth of leiomyoma cells whereas myometrial cells were affected.

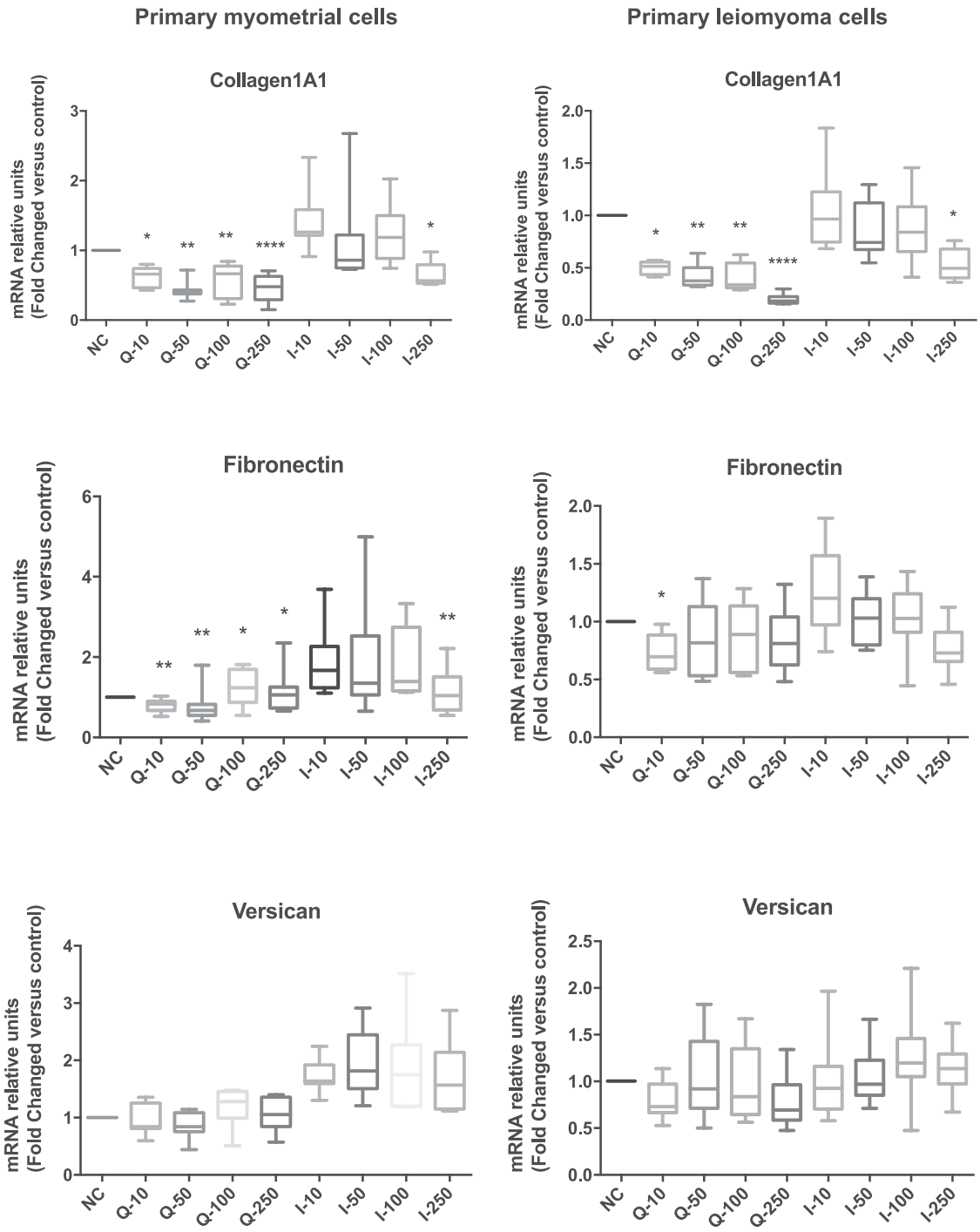


FIGURE 1 Effect of quercetin and indole-3-carbinol on extracellular matrix expression in myometrial and leiomyoma cells. Results of real-time polymerase chain reaction for relative amounts (versus HPRT 1) of collagen 1A1, fibronectin and versican in myometrial and leiomyoma cells treated for 48 h with different concentrations of quercetin at 10 μg/ml (Q-10), 50 μg/ml (Q-50), 100 μg/ml (Q-100), 250 μg/ml (Q-250), or indole-3-carbinol at 10 μg/ml (I-10), 50 μg/ml (I-50), 100 μg/ml (I-100), 250 μg/ml (I-250) compared with negative control (untreated cells). Data are expressed as median, first quartiles, third quartiles and minimum and maximum value ($n = 6$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). NC, negative control.

The anti-fibrotic effects of quercetin and I3C have been demonstrated in different cell types (*Phan et al. 2003a*; *2003b*; *Hu et al. 2009*; *Nakamura et al. 2011*; *Ping et al. 2011*). The present study further confirms their anti-fibrotic potential. In our previous study, we demonstrated that strawberry extracts were able to inhibit expression of ECM protein components, including collagen 1A1, fibronectin and versican in leiomyoma cells (*Islam et al., 2017b*; *Giampieri et al. 2019*). Strawberries contain several bioactive compounds, such as ellagic acid, kaempferol, catechins, anthocyanins, including quercetin. As expected, similar to strawberry extract, quercetin also inhibited expression of ECM

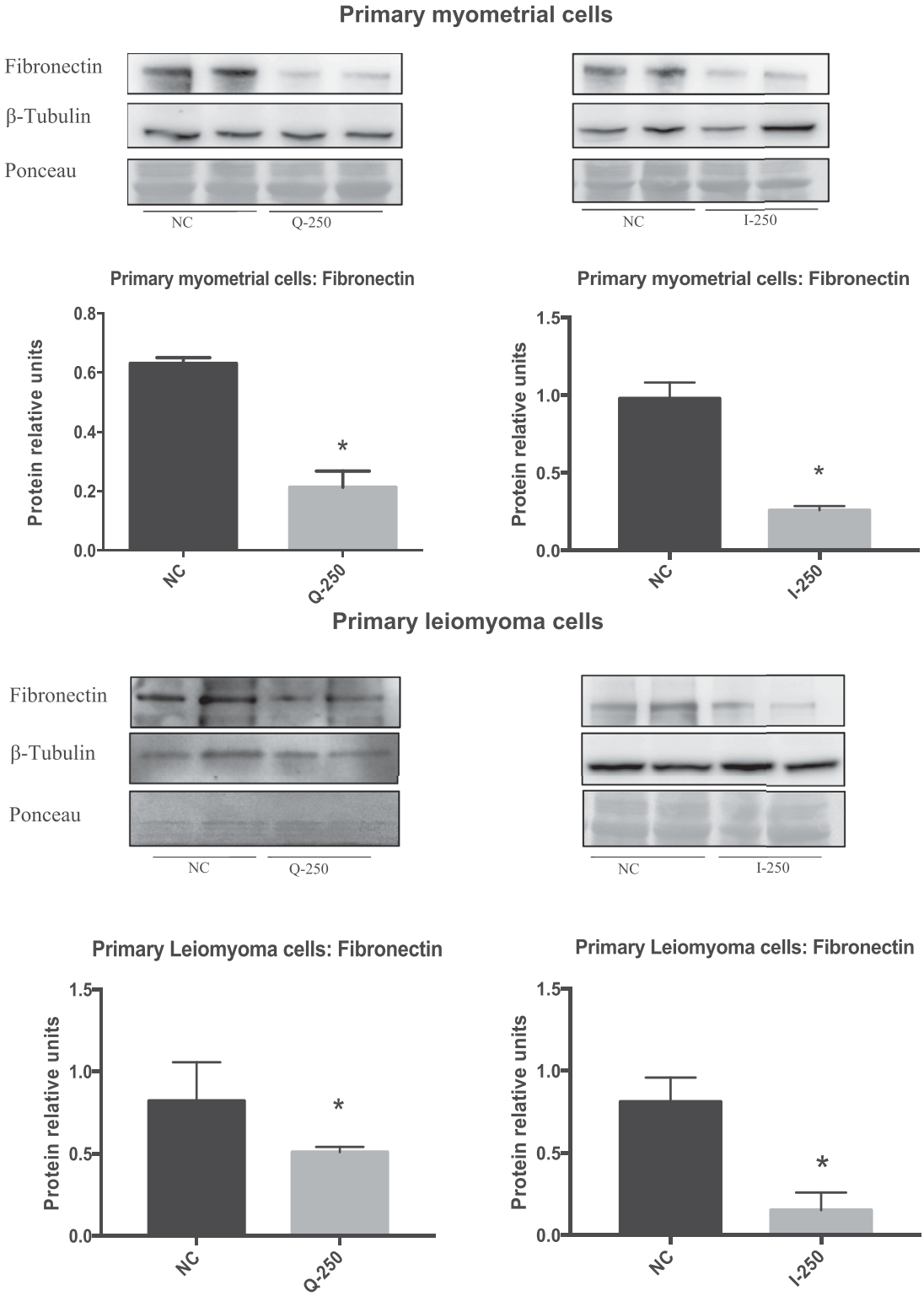


FIGURE 2 Effect of quercetin and indole-3-carbinol on fibronectin protein expression in myometrial and leiomyoma cells. Western blotting showing the protein expression of fibronectin in myometrial and leiomyoma cells treated for 48 h with quercetin at 250 μg/ml (Q-250) and indole-3-carbinol at 250 μg/ml (I-250) or left untreated. Representative gels and data analysis showing the decrease expression of fibronectin after treatment with quercetin or indole-3-carbinol compared with negative control (untreated cells). Data are expressed as mean ± SD (n = 3, *P < 0.05). NC, negative control.

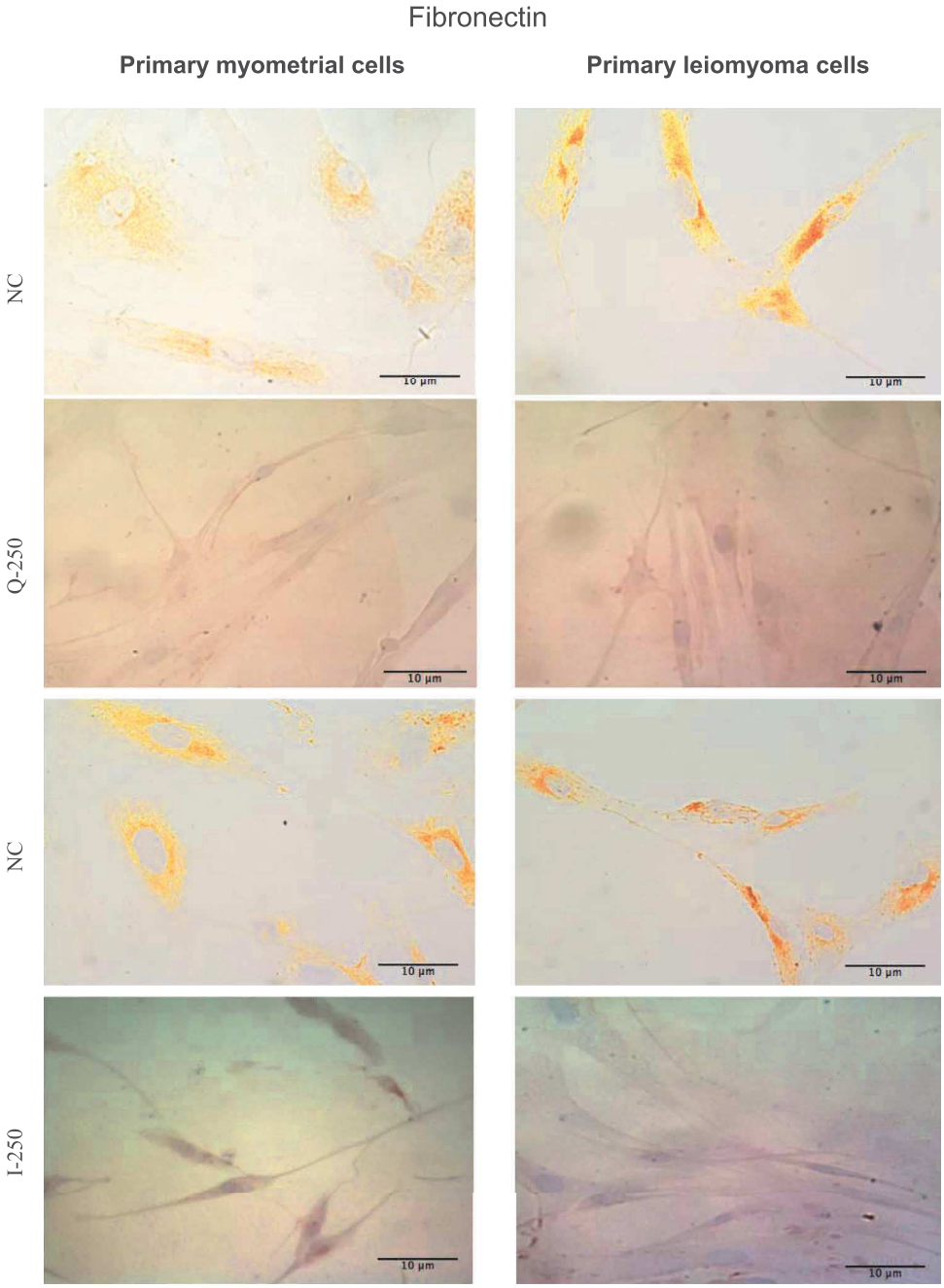
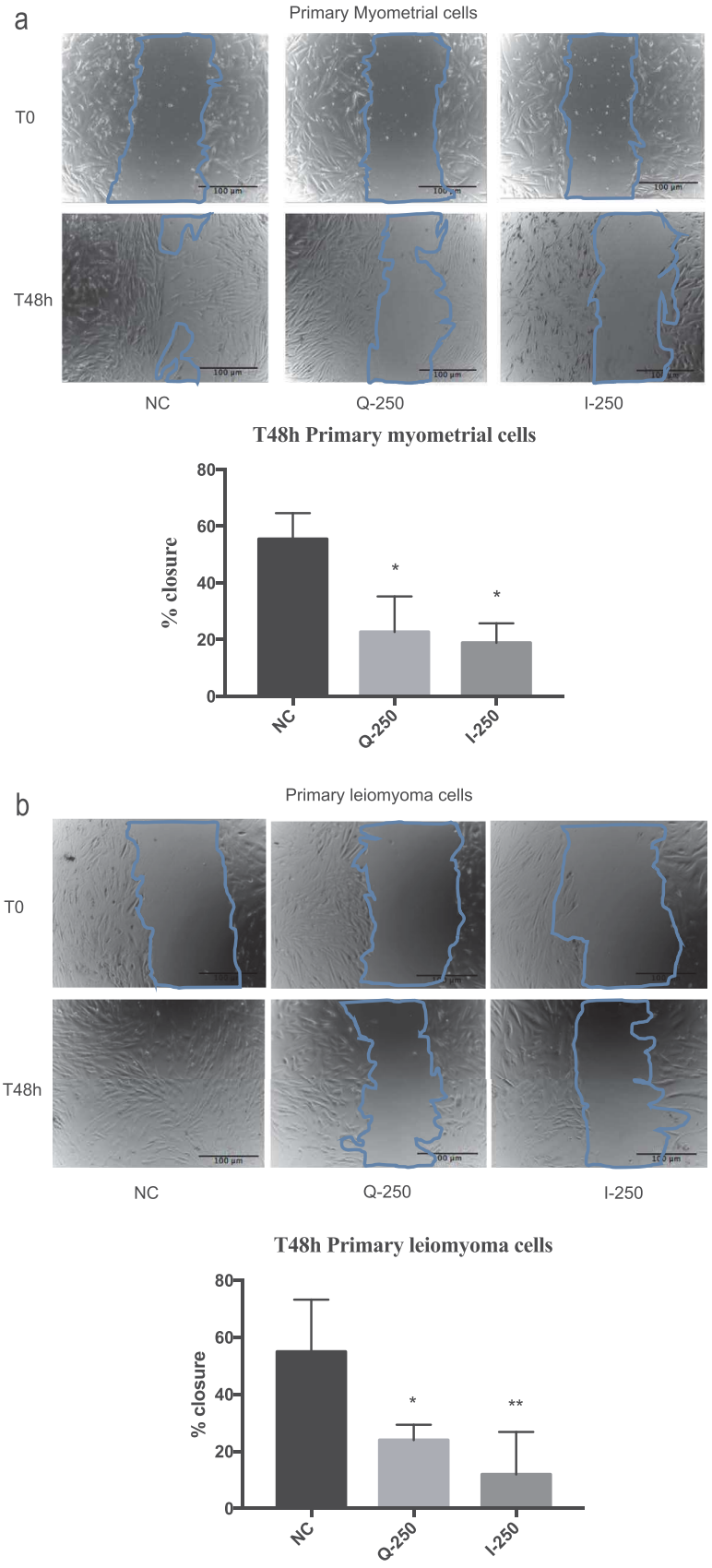


FIGURE 3 Effect of quercetin and indole-3-carbinol on fibronectin expression in myometrial and leiomyoma cells. Immunocytochemical staining showing the effects of quercetin and indole 3-carbinol on fibronectin expression in myometrial and leiomyoma cells in response to treatments of quercetin at 250 µg/ml (Q-250) and indole-3-carbinol at 250 µg/ml (I-250) after 48 h compared with untreated cells (negative control). Microscope observation showing the decrease expression levels of fibronectin after treatments with quercetin and indole 3-carbinol compared with negative control (untreated cells) (n = 3). NC, negative control.

components, collagen 1A1 and fibronectin in leiomyoma cells, demonstrating that quercetin is one of the active antifibrotic compounds of strawberry fruit.

Because of their therapeutic potential, quercetin and I3C are now under clinical trial for different pathologies. Phase I and phase II clinical trials of quercetin have been completed or are now recruiting for multiple conditions, including squamous cell carcinoma (NCT03476330). Quercetin was administered twice daily at an adjusted dose based on weight for a maximum total daily dose of 4000 mg/day. If the patient is 70 kg or more, the dose is automatically assigned at the maximum dose of 4000 mg/day. For patients with type 2 diabetes (NCT01839344), the maximum dose of quercetin is 250 mg capsules, whereas, for patients with chemotherapy-induced oral mucositis, the single oral dose is 2000 mg (NCT01732393). Patients in the intervention group were administered two, 250-mg quercetin capsules daily for 3 weeks. Patients in the placebo group received two placebo capsules containing lactose. Patients were examined every other day for evaluation



FIGURES 4 a and b: Effect of quercetin and indole-3-carbinol on migration of myometrial and leiomyoma cells. Representative photographs, and graphs showing the effects of quercetin at 250 µg/ml (Q-250) or indole-3-carbinol at 250 µg/ml (I-250) treatments on wound closure of scratch-wounded confluent myometrial and leiomyoma cells; both treatments significantly inhibit wound closure in myometrial and leiomyoma cells. Image J software was used analyse images. Data are expressed as mean ± SD (n = 3). *P < 0.05; **P < 0.01. NC, negative control.

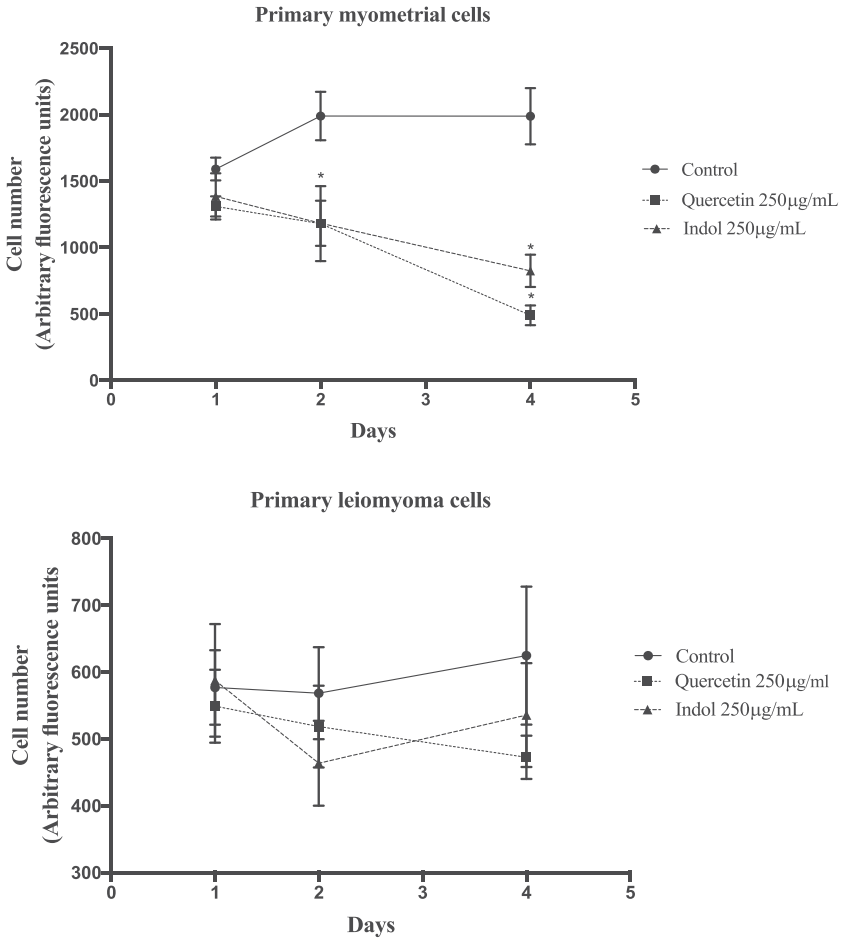


FIGURE 5 The effect of quercetin and indole-3-carbinol on proliferation of myometrial and leiomyoma cells. Cell proliferation assay. Cell proliferation was measured using the CyQuant cell proliferation assay kit. Representative graphs showing the effects of quercetin (250 µg/ml) or indole-3-carbinol (250 µg/ml) treatments. Both compounds significantly inhibit proliferation of myometrial cells while leiomyoma cells were not affected. Data are expressed as mean ± SD (n = 6). *P < 0.05. NC, negative control.

of initiation and severity of oral mucositis. In addition, a phase III, randomized, double blind clinical trial of quercetin for coronary artery disease progression has recently been listed (not yet recruiting) (NCT03943459). In a 60-day trial, 60 postmenopausal women with coronary artery disease were divided into three groups with 20 women assigned to each group. Group 1 were given quercetin (500 mg/day). Furthermore, a phase IV clinical trial of quercetin containing onion peel extract has been completed on endothelial function (NCT02180022). A phase I clinical trial of I3C has been completed for preventing cancers in healthy participants (NCT00100958 and NCT00033345). In addition, clinical trial phases II and III of I3C on prostate-specific antigen recurrence in patients who have undergone prostatectomies have also been completed (NCT00579332). Advanced clinical trials of quercetin and I3C support the possible use of these two compounds or their dietary sources, and also as preventive, therapeutic agents, or both, for uterine fibroids. In particular, patients could benefit most from these therapeutic agents in the following circumstances: while seeking pregnancy or during the pregnancy itself; when a significant growth of fibroids has been identified; with the possible onset of adverse obstetric outcomes (*Ciavattini et al. 2016*); and when use of other pharmacological agents is considered impossible. Moreover, as the severity of fibroid-related symptoms is reduced after the menopause, these compounds may be used in perimenopausal women, potentially avoiding the need for surgery.

In conclusion, the present data support the hypothesis that the two natural compounds quercetin and I3C can be developed as therapeutic interventions and preventive dietary supplements, or as locally administrated phytochemicals for uterine fibroids.

REFERENCES

Arici, A., Sozen, I. **Transforming growth factor-beta3 is expressed at high levels in leiomyoma where it stimulates fibronectin expression and cell proliferation.** Fertility and Sterility. 2000; 73: 1006–1011

Auborn, K.J., Fan, S., Rosen, E.M., Goodwin, L., Chandraskaren, A., Williams, D.E., Chen, D., Carter, T.H. **Indole-3-carbinol is a negative regulator of estrogen.** J. Nutr. 2003; 133: 2470S–2475S

Baowen, Q., Yulin, Z., Xin, W., Wenjing, X., Hao, Z., Zhizhi, C., Xingmei, D., Xia, Z., Yuquan, W., Lijuan, C. **A further investigation concerning correlation between anti-fibrotic effect of liposomal quercetin and inflammatory cytokines in pulmonary fibrosis.** European journal of pharmacology. 2010; 642: 134–139

Bradlow, H.L., Michnovicz, J., Telang, N.T., Osborne, M.P. **Effects of dietary indole-3-carbinol on estradiol metabolism and spontaneous mammary tumours in mice.** Carcinogenesis. 1991; 12: 1571–1574

Buttram, V.C.Jr., Reiter, R.C. **Uterine leiomyomata: Etiology, symptomatology, and management.** Fertil. Steril. 1981; 36: 433–445

Chen, H.Y., Lin, P.H., Shih, Y.H., Wang, K.L., Hong, Y.H., Shieh, T.M., Huang, T.C., Hsia, S.M.

Natural antioxidant resveratrol suppresses uterine fibroid cell growth and extracellular matrix formation in vitro and in vivo. Antioxidants (Basel) 2019; 8

Ciarmela, P., Wiater, E., Smith, S.M., Vale, W. **Presence, actions, and regulation of myostatin in rat uterus and myometrial cells.** Endocrinology. 2009; 150: 906–914

Ciarmela, P., Wiater, E., Vale, W. **Activin-a in myometrium: Characterization of the actions on myometrial cells.** Endocrinology. 2008; 149: 2506–2516

Ciavattini, A., Clemente, N., Delli Carpini, G., Di Giuseppe, J., Giannubilo, S.R., Tranquilli, A.L. **Number and size of uterine fibroids and obstetric outcomes.** J. Matern. Fetal. Neonatal. Med. 2015; 28: 484–488

Ciavattini, A., Delli Carpini, G., Clemente, N., Moriconi, L., Gentili, C., Di Giuseppe, J. **Growth trend of small uterine fibroids and human chorionic gonadotropin serum levels in early pregnancy: An observational study.** Fertil. Steril. 2016; 105: 1255–1260

Donnez, J. **Liver injury and ulipristal acetate: An overstated tragedy?** Fertility and sterility 2018; 110: 593–595

Donnez, J., Hudecek, R., Donnez, O., Matule, D., Arhendt, H.J., Zatik, J., Kasilovskiene, Z., Dumitrascu, M.C., Fernandez, H., Barlow, D.H., Bouchard, P., Fauser, B.C., Bestel, E., Terrill, P., Osterloh, I., Loumaye, E. **Efficacy and safety of repeated use of ulipristal acetate in uterine fibroids.** Fertil. Steril. 2015; 103

Dunnick, J.K., Hailey, J.R. **Toxicity and carcinogenicity studies of quercetin, a natural component of foods.** Fundam. Appl. Toxicol. 1992; 19: 423–431

Friedman, A.J., Hoffman, D.I., Comite, F., Browneller, R.W., Miller, J.D. **Treatment of leiomyomata uteri with leuprolide acetate depot: A double-blind, placebo-controlled, multicenter study. The leuprolide study group.** Obstetrics and Gynaecology 1991; 77: 720–725

Friedman, A.J., Rein, M.S., Harrison-Atlas, D., Garfield, J.M., Doubilet, P.M. **A randomized, placebo-controlled, double-blind study evaluating leuprolide acetate depot treatment before myomectomy.** Fertility and Sterility 1989; 52: 728–733

Giampieri, F., Islam, M.S., Greco, S., Gasparrini, M., Forbes Hernandez, T.Y., Delli Carpini, G., Giannubilo, S.R., 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. 2019; 234: 7622–7633

Hu, Q., Noor, M., Wong, Y.F., Hylands, P.J., Simmonds, M.S.J., Xu, Q., Jiang, D., Hendry, B.M., Xu, Q. **In vitro anti-fibrotic activities of herbal compounds and herbs.** Nephrology Dialysis Transplantation. 2009; 24: 3033–3041

Islam, M.S., Akhtar, M.M., Ciavattini, A., Giannubilo, S.R., Protic, O., Janjusevic, M., Procopio, A.D., Segars, J.H., Castellucci, M., Ciarmela, P. **Use of dietary phytochemicals to target inflammation, fibrosis, proliferation, and angiogenesis in uterine tissues: Promising options for prevention and treatment of uterine fibroids?** Mol. Nutr. Food Res. 2014; 58: 1667–1684

Islam, M.S., Catherino, W.H., Protic, O., Janjusevic, M., Gray, P.C., Giannubilo, S.R., Ciavattini, A., Lamanna, P., Tranquilli, A.L., Petraglia, F., Castellucci, M., Ciarmela, P. **Role of activin-a and myostatin and their signaling pathway in human myometrial and leiomyoma cell function.** J. Clin. Endocrinol. Metab. 2014; 99: E775–E785

Islam, M.S., Ciavattini, A., Petraglia, F., Castellucci, M., Ciarmela, P. **Extracellular matrix in uterine leiomyoma pathogenesis: A potential target for future therapeutics.** Human reproduction update. 2017; 24: 59–85

Islam, M.S., Giampieri, F., Janjusevic, M., Gasparrini, M., Forbes-Hernandez, T.Y., Mazzoni, L., Greco, S., Giannubilo, S.R., 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; 8: 23575–23587

Islam, M.S., Greco, S., Janjusevic, M., Ciavattini, A., Giannubilo, S.R., D'adderio, A., Biagini, A., Fiorini, R., Castellucci, M., Ciarmela, P. **Growth factors and pathogenesis.** Best Pract. Res. Clin. Obstet. Gynaecol. 2016; 34: 25–36

Islam, M.S., Segars, J.H., Castellucci, M., Ciarmela, P. **Dietary phytochemicals for possible preventive and therapeutic option of uterine fibroids: Signaling pathways as target.** Pharmacological Reports. 2017; 69: 57–70

Jin, L., Qi, M., Chen, D.Z., Anderson, A., Yang, G.Y., Arbeit, J.M., Auborn, K.J. **Indole-3-carbinol prevents cervical cancer in human papilloma virus type 16 (hpv16) transgenic mice.** Cancer Res. 1999; 59: 3991–3997

Kashani, B.N., Centini, G., Morelli, S.S., Weiss, G., Petraglia, F. **Role of medical management for uterine leiomyomas.** Best Pract. Res. Clin. Obstet. Gynaecol. 2016; 34: 85–103

Kojima, T., Tanaka, T., Mori, H. **Chemoprevention of spontaneous endometrial cancer in female donryu rats by dietary indole-3-carbinol.** Cancer Res. 1994; 54: 1446–1449

Leather, A.T., Studd, J.W.W., Watson, N.R., Holland, E.F.N. **The prevention of bone loss in young women treated with gnrh analogues with" add-back" estrogen therapy.** Obstetrics & Gynaecology 1993; 81: 104–107

Lee, E.S., Lee, H.E., Shin, J.Y., Yoon, S., Moon, J.O. **The flavonoid quercetin inhibits dimethylnitrosamine-induced liver damage in rats.** Journal of pharmacy and pharmacology. 2003; 55: 1169–1174

Leppert, P.C., Catherino, W.H., Segars, J.H. **A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays.** Am. J. Obstet. Gynecol. 2006; 195: 415–420

Liang, C.C., Park, A.Y., Guan, J.L. **In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro.** Nat. Protoc. 2007; 2: 329–333

Lin, P.H., Kung, H.L., Chen, H.Y., Huang, K.C., Hsia, S.M. **Isoliquiritigenin suppresses e2-induced uterine leiomyoma growth through the modulation of cell death program and the repression of ecm accumulation.** Cancers (Basel) 2019; 11

Lin, P.H., Shih, C.K., Yen, Y.T., Chiang, W., Hsia, S.M. **Adlay (coix lachryma-jobi l. Var. Ma-yuen stapf.) hull extract and active compounds inhibit proliferation of primary human leiomyoma cells and protect against sexual hormone-induced mice smooth muscle hyperproliferation.** Molecules 2019; 24

Nakamura, T., Matsushima, M., Hayashi, Y., Shibasaki, M., Imaizumi, K., Hashimoto, N., Shimokata, K., Hasegawa, Y., Kawabe, T. **Attenuation of transforming growth factor-beta-stimulated collagen production in fibroblasts by quercetin-induced heme oxygenase-1.** American journal of respiratory cell and molecular biology. 2011; 44: 614–620

Norian, J.M., Malik, M., Parker, C.Y., Joseph, D., Leppert, P.C., Segars, J.H., Catherino, W.H. **Transforming growth factor beta3 regulates the versican variants in the extracellular matrix-rich uterine leiomyomas.** Reproductive Sciences. 2009; 16: 1153–1164

Pereira, M.A., Grubbs, C.J., Barnes, L.H., Li, H., Olson, G.R., Eto, I., Juliana, M., Whitaker, L.M., Kelloff, G.J., Steele, V.E., Lubet, R.A. **Effects of the phytochemicals, curcumin and quercetin, upon azoxymethane-induced colon cancer and 7,12-dimethylbenz[a]anthracene-induced mammary cancer in rats.** Carcinogenesis. 1996; 17: 1305–1311

Phan, T.T., Lim, I.J., Sun, L., Chan, S.Y., Bay, B.H., Tan, E.K., Lee, S.T. **Quercetin inhibits fibronectin production by keloid-derived fibroblasts. Implication for the treatment of excessive scars.** Journal of dermatological science 2003; 33: 192–194

Phan, T.T., See, P., Tran, E., Nguyen, T.T.T., Chan, S.Y., Lee, S.T., Huynh, H. **Suppression of insulin-like growth factor signalling pathway and collagen expression in keloid-derived fibroblasts by quercetin: Its therapeutic potential use in the treatment and/or prevention of keloids.** British Journal of Dermatology. 2003; 148: 544–552

Pickering, J.G. **Regulation of vascular cell behavior by collagen form is function.** Circulation research. 2001; 88: 458–459

Ping, J., Li, J.-T., Liao, Z.-X., Shang, L., Wang, H. **Indole-3-carbinol inhibits hepatic stellate cells proliferation by blocking nadph oxidase/reactive oxygen species/p38 mapk pathway.** European journal of pharmacology. 2011; 650: 656–662

Rodriguez, L.G., Wu, X., Guan, J.L. **Wound-healing assay.** Methods Mol. Biol. 2005; 294: 23–29

Stovall, T.G., Muneyyirci-Delale, O., Summitt, R.L.Jr, Scialli, A.R. **Gnrh agonist and iron versus placebo and iron in the anemic patient before surgery for leiomyomas: A randomized controlled trial. The leuprolide study group.** Obstetrics & Gynaecology 1995; 86: 65–71

Terry, P., Wolk, A., Persson, I., Magnusson, C. **Brassica vegetables and breast cancer risk.** JAMA. 2001; 285: 2975–2977

Walker, C.L., Stewart, E.A. **Uterine fibroids: The elephant in the room.** Science. 2005; 308: 1589–1592

Wright, C.S., Pollok, S., Flint, D.J., Brandner, J.M., Martin, P.E. **The connexin mimetic peptide gap27 increases human dermal fibroblast migration in hyperglycemic and hyperinsulinemic conditions in vitro.** J. Cell. Physiol. 2012; 227: 77–87

Wu, C.-H., Shieh, T.-M., Wang, K.-L., Huang, T.-C., Hsia, S.-M. **Quercetin, a main flavonoid in onion, inhibits the pgf2α-induced uterine contraction in vitro and in vivo.** Journal of Functional Foods 2015; 19: 495–504

Received 12 June 2019; received in revised form 19 December 2019; accepted 10 January 2020.

nutrients

MDPI

Review

Phytoprogestins: Unexplored Food Compounds with Potential Preventive and Therapeutic Effects in Female Diseases

Stefania Greco [1], Pamela Pellegrino [1], Alessandro Zannotti [1,2], Giovanni Delli Carpini [2], Andrea Ciavattini [2], Fernando M. Reis [3] and Pasquapina Ciarmela [1,*]

[1] Department of Experimental and Clinical Medicine, Università Politecnica delle Marche, 60126 Ancona, Italy; s.greco@staff.univpm.it (S.G.); p.pellegrino@pm.univpm.it (P.P.); a.zannotti@pm.univpm.it (A.Z.)
[2] Department of Specialist and Odontostomatological Clinical Sciences, Università Politecnica delle Marche, 60126 Ancona, Italy; g.dellicarpini@staff.univpm.it (G.D.C.); a.ciavattini@univpm.it (A.C.)
[3] Department of Obstetrics and Gynecology, Universidade Federal de Minas Gerais, Belo Horizonte 30130-100, Brazil; fmreis@ufmg.br
* Correspondence: p.ciarmela@univpm.it; Tel.: +39-0712206270

Abstract: In recent years, there has been an increasing interest in natural therapies to prevent or treat female diseases. In particular, many studies have focused on searching natural compounds with less side effects than standard hormonal therapies. While phytoestrogen-based therapies have been extensively studied, treatments with phytoprogestins reported in the literature are very rare. In this review, we focused on compounds of natural origin, which have progestin effects and that could be good candidates for preventing and treating female diseases. We identified the following phytoprogestins: kaempferol, apigenin, luteolin, and naringenin. In vitro studies showed promising results such as the antitumoral effects of kaempferol, apigenin and luteolin, and the anti-fibrotic effects of naringenin. Although limited data are available, it seems that phytoprogestins could be a promising tool for preventing and treating hormone-dependent diseases.

Keywords: female disease; progesterone; phytoprogestins; phytochemical compounds

check for updates

Citation: Greco, S.; Pellegrino, P.; Zannotti, A.; Delli Carpini, G.; Ciavattini, A.; Reis, F.M.; Ciarmela, P. Phytoprogestins: Unexplored Food Compounds with Potential Preventive and Therapeutic Effects in Female Diseases. *Nutrients* **2021**, *13*, 4326. https://doi.org/10.3390/nu13124326

Academic Editor: Elad Tako

Received: 4 November 2021
Accepted: 29 November 2021
Published: 30 November 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

In recent years, there has been an increasing interest in alternative and natural methods for the prevention or treatment of female diseases. In particular, many studies have focused on searching for adequate compounds with less side effects than standard hormonal therapies. Although the etiopathogenetic mechanisms of many gynecological diseases, such as endometriosis [1] and uterine fibroids [2] are still not clear, the role of steroid hormones is undoubted. Indeed, there is an important hormonal imbalance, for example, in endometriosis [3], uterine leiomyomas [4], ovarian cancer [5], and breast cancer [6].

The father of medicine, Hippocrates, proclaimed "Let food be the medicine and medicine be the food" around 25 centuries ago. In recent studies, there is a high interest in dietary phytochemicals. Phytochemicals are chemical compounds of natural origin that can be used as therapeutic or preventive agents.

Nutraceutical compounds can exert their effects on health in different ways, including through hormonal activity. Their mechanism of action is: 1. Competition with the hormone for binding to the corresponding receptor, thanks to a structural similarity; 2. Influence on the activity of key enzymes of the biosynthetic pathway, such as in the case of isoflavones, which are moderate aromatase inhibitors, thus reducing estrogen synthesis; 3. Influence on the epigenome by affecting DNA methylation activity, histone modification, and microRNA regulation [7].

Phytoestrogens and phytoprogestins are phytochemical compounds of natural origin, which have estrogenic and progestagenic effects, respectively [8,9]. While phytoestrogen-based therapies have been extensively studied in the clinical setting, treatments with

phytoprogestin are still in the preclinical stage, and their potential remains unexplored [8]. Therefore, we decided to review the current evidence supporting the preventive and therapeutic effects of phytoprogestins in female diseases.

2. Methods

In this narrative review, we performed a bibliographic search of studies evaluating the effects of dietary phytoprogestins on reproductive cells and tissues and the possible association of these nutritional compounds with gynecological diseases. The search was carried out on Pubmed using combinations of the following terms: phytochemicals [MeSH], flavonoids [MeSH], kaempferol, apigenin, naringenin, luteolin, women, uterine fibroids, endometriosis, ovarian cancer, and breast cancer. The search was narrowed to studies in humans or relevant animal models of human diseases and complemented by screening the reference lists of the selected articles. We also briefly review the pharmacological mechanisms of progesterone receptor activation and progesterone-based therapies in order to provide a background to the discussion of phytoprogestins.

3. Progesterone

Progesterone is a sex steroid hormone essential in female reproduction, including the menstrual cycle and the establishment and maintenance of pregnancy [10]. The etymology of the name derives from the Latin "pro gestationem" [11], as it allows the endometrium to pass from the proliferative to the secretory stage, facilitating the nesting of the blastocyst and is essential for maintaining pregnancy; in fact, it promotes the uterine growth and suppresses the contractility of the muscular tissue of the uterus (myometrium). In the mammary gland, it promotes the development of the gland for the secretion of milk. In addition, progesterone plays an essential role in the physiology of non-reproductive tissues, such as the cardiovascular system, the central nervous system, and bone tissue. In the brain, progesterone is neuroprotective, and its metabolite allopregnanolone is a GABAergic agonist [12,13] (Figure 1).

Steroids are ancestral molecules [11] characterized by a common base structure of cyclopentane–perhydro–phenanthrene, a polycyclic complex of 17 carbon atoms making a four-ring system. Based on the number of carbon atoms, sex steroids can be categorized into three groups: progesterone and progestins, with 21 carbon atoms, androgens, which have 19 carbon atoms, and finally estrogens, with 18 carbon atoms.

The biosynthesis of steroid hormones is the same in all organs where they are produced, such as the ovary, testis, adrenal cortex, brain, and placenta. The gonadal progesterone is mainly transported by blood to reach the target cells, while the progesterone produced by adrenal gland is mostly locally converted into glucocorticoids and androgens [14]. Progesterone circulates in the bloodstream bound to cortisol-binding globulin (approximately 10%) and serum albumin and has a relatively short half-life of only five minutes. The metabolites mainly produced in the liver are sulfates and glucuronides, which are excreted in the urine. Circulating progesterone is converted by the kidney into a mineralocorticoid, deoxycorticosterone (DOC). During the luteal phase, pregnancy, and administration of exogenous progesterone, most circulating DOC arises from this pathway and may bring unbearable side effects [14].

Progesterone exerts its physiological effect by binding to target cells via specific nuclear progesterone receptors (PR) or by binding to membrane receptors (progesterone receptor membrane component, PGRMC, or mPR). The binding with the nuclear receptors gives rise to a genomic pathway that requires a much longer response than the non-genomic one, which is triggered when progesterone binds to membrane receptors.

PRs are expressed in the human ovary [15], uterus [16], testis [17], brain [18], pancreas [19], bone tissue [20], mammary gland [21] and urinary tract [22]. PRs, together with the receptors for estradiol, mineralocorticoids, glucocorticoids, and androgens, belong to the superfamily of nuclear receptors. The nuclear progesterone receptor consists of a central binding domain for DNA (DBD) and a carboxylic terminal binding domain for the ligand

(LBD). In addition, the receptor has transcription activation function (TAF) domains that interact with coactivators and corepressors to regulate the downstream target genes [23] (Figure 2). The newly transcribed progesterone receptor is assembled into an inactive multiprotein chaperone complex in the cytoplasm [24]. The receptor at this level must be inactive [25] since its activation occurs only in the presence of a link with the hormone, which induces a conformational change of the receptor [26].

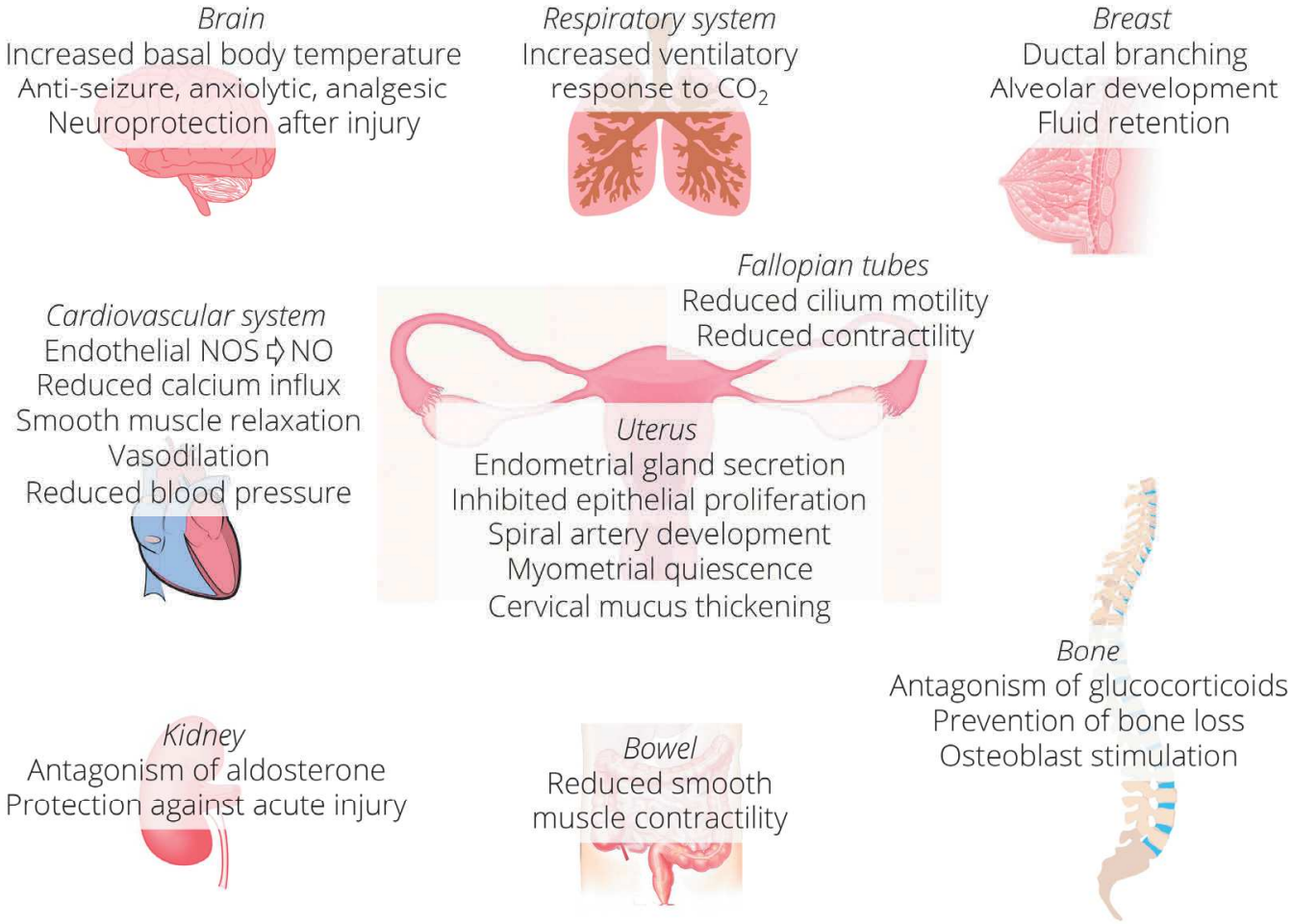


Figure 1. Schematic representation of the organs in which progesterone performs functions. Progesterone acts in reproductive as well as in non-reproductive tissues. NOS = nitric oxide synthase, NO = nitric oxide.

Two isoforms of PR are transcribed from a single gene by alternative splicing from two distinct promoters [27,28], giving rise to transcripts that encode, respectively, for the protein isoforms A (PRA) and B (PRB) (Figure 2). PRA and PRB are identical in sequence, except that PRA lacks 164 amino acids at the N-terminal, making it the shorter of the two proteins [28].

Progesterone may act through a genomic (slow process) or a non-genomic (fast process) pathway. The classical pathways of progesterone actions are mediated via nuclear receptors. Progesterone enters the cell and binds PRs, inducing their conformational change and dimerization. The complex of progesterone with PR translocates to the nucleus and interacts with DNA-binding elements in the genome, activating the transcription of progesterone-responsive genes (Figure 2). The non-genomic (also called non-classical or extranuclear) progesterone action initiates at the cell surface with the activation of the cytoplasmic PRs or membrane-bound PRs (mPRs) and determines an intracellular signaling that elicits a rapid response [29]. These proteins include the progesterone receptor membrane component 1

(PGRMC1), its counterpart PGRMC2, and the family of membrane progesterone receptors (mPR), also known as PAQR (progestin and adipoQ) receptors [30].

Studies in mice have shown that the elimination of the PRB isoform resulted in the unhealthy development of the mammary gland [31], while the elimination of PRA caused an abnormal development of the uterus and impaired its reproductive function [32]. Therefore, in animals, a dominant expression of one of the two isoforms seems to be necessary for the normal functioning and development of some organs. On the other hand, in humans, all healthy tissues, including those of the mammary gland and uterus, have epithelial cells that express PR with the co-expression of both the PRA and PRB isoforms [33,34]. This condition suggests that the colocalization and thus the cooperative activity of PRA and PRB mediate the action of PR in humans. Although the two isoforms are expressed in the same way in most human tissues, there is a different expression in the endometrium. In fact, during the secretory phase of the menstrual cycle, when there are high levels of circulating progesterone, the PRA isoform is poorly expressed, resulting in a clear predominance of PRB [33].

In breast and endometrial cancers, there are substantial differences in progesterone levels and its isoforms compared to normal tissues. In fact, in healthy tissues deriving from the mammary gland, epithelial cells equally express both PR isoforms [34], while in neoplastic biopsies, it is possible to see a significant increase in the expression, alternatively, of PRA or PRB [34,35]. Similarly, in endometrial cancer, it is common to find only one of the two isoforms expressed, either PRA or PRB, suggesting that the lack of co-expression of both isoforms is an early event of the onset of endometrial cancer [36].

A third isoform (PRC) has been identified in the human placenta [37]. PRC is an isoform with a truncated N-terminal domain, with a molecular mass of approximately 60 kDa, present in the cytoplasm. PRC lacks the first zinc finger of the DBD, but it can still bind progesterone. The actions of PRC are not clear, but it can form heterodimers with PRA and PRB and, in this way, regulate the transcriptional activity of the PR isoforms [37,38].

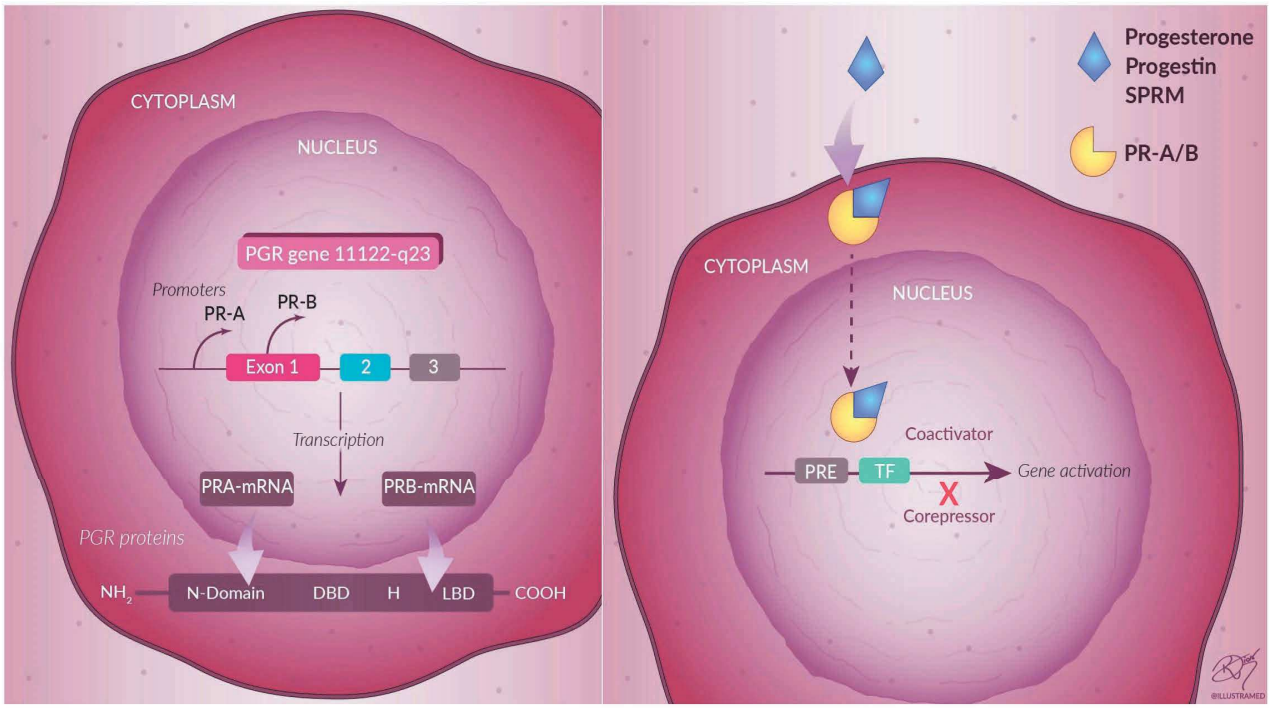


Figure 2. Progesterone receptors and their activation. The nuclear receptor is formed by two promoter regions on the PR gene, one for PRA and one for PRB, and these two promoters allow the synthesis of the two separate mRNA transcripts that code for the two different isoforms PRA and PRB. DBD = DNA-binding domain, H = hinge, LBD = ligand-binding domain, SPRM = selective progesterone receptor modulator, PRE = progesterone responsive element, TF = transcription factor.

4. Progesterone-Based Drug Therapy

Progestogens are the most common compounds used as drug therapy for the treatment of women's diseases. Many gynecological diseases are treated with synthetic progestin-based drugs. In the United States, endometrial cancer is one of the most common gynecological cancers, with 46,470 new cases and 8,120 deaths in 2011 [39]. Even if the molecular mechanisms involved in endometrial carcinogenesis are not clear, it seems that chronic exposure to estrogen and its metabolites without sufficient counterbalance of progesterone has proliferative effects [40,41] and is harmful to DNA [42,43]. Based on the antiestrogenic role of progesterone, many patients affected by endometrial cancer may have an indication to progesterone-based therapy, particularly in case of contraindications to surgery or desire for fertility maintenance. Indeed, women with endometrial hyperplasia and well-differentiated endometrial adenocarcinoma show a good response to progestogen therapy [44]. However, as the severity of the disease increases, the efficacy of progestogens decreases [45].

Other estrogen-dependent female pathologies with a high social impact, such as endometriosis, are often treated with progestin therapies [46,47], including synthetic progestins such as medroxyprogesterone acetate or dienogest [48,49]. Uterine fibroids may also be treated with progestins. Since the first reports of decades ago [50], studies have focused on the effects of different progestins on uterine fibroids, with different drug dosages and regimens. For example, medroxyprogesterone acetate [51] and dienogest [52,53] have shown a regressive effect on uterine fibroids.

Moreover, progestogens are widely used as a contraceptive method and in menopausal hormone therapy, in combination with estrogens. These therapies may also have an effect of prevention of ovarian cancer, but they increase the risk of venous thromboembolism and present side effects [54,55]. Therefore, the identification of alternative progestogens is clinically significant. Numerous studies in the literature indicate a great interest in developing phytoprogestogens, such as botanical extracts or food supplements, hoping to provide the beneficial effects of progestins while avoiding the side effects.

Selective progesterone receptor modulator (SPRM) is a class of synthetic ligands that act on the PR and tend to be more tissue-specific than progestins. The mechanism of action of SPRMs occurs through binding to PR, resulting in a conformational change of the receptor. The action can be agonistic, antagonistic, or mixed. The agonist action of SPRMs involves the recruitment of different coactivators to induce transcriptional activity and occurs in tissues where high levels of coactivators are present, while antagonist activity occurs where corepressors are in excess (Figure 2).

When the PR is inactive, SPRMs bind to the receptor and activate it. The binding involves nuclear import, which gives the receptor the property of dimerization. In the nucleus, the dimer interacts with the response element in the DNA, causing the up-regulation or down-regulation of the gene [56,57]. The action of SPRMs also depends on the ratio of PR-A and PR-B in the tissue and on the specific binding affinity of the SPRMs for each receptor isoform [58]. SPRMs have been developed for clinical applications, considering their tissue selectivity and low rate of side effects [59]. Their application is principally for the treatment of uterine fibroids [60], endometriosis [61], and breast cancer [62].

Despite having beneficial effects, for example, in the treatment of uterine fibroids, the prolonged use of SPRMs may lead to endometrial hyperplasia and other side effects. Indeed, it has been shown that long-term use of the SPRM asoprisnil results in long-term damage to the endometrium. Ulipristal acetate has been approved in Canada and Europe as a presurgical therapy for patients with uterine fibroids to control bleeding, and in the United States for emergency contraception. However, it has raised concerns due to liver toxicity [63], as well as telapristone acetate, which was stopped in 2009. Vilaprisan is still under study, and its possible collateral effects are not yet known [64].

5. Phytoprogestins

Phytoprogestins are chemical compounds of vegetal origin that have progesterone-like activity and can function as non-steroidal SPRMs. Unlike estrogenic counterparts, which have been extensively studied, the literature reports much fewer studies on phytoprogestins. The following phytoprogestins have been identified: kaempferol, apigenin, luteolin, and naringenin (Figure 3).

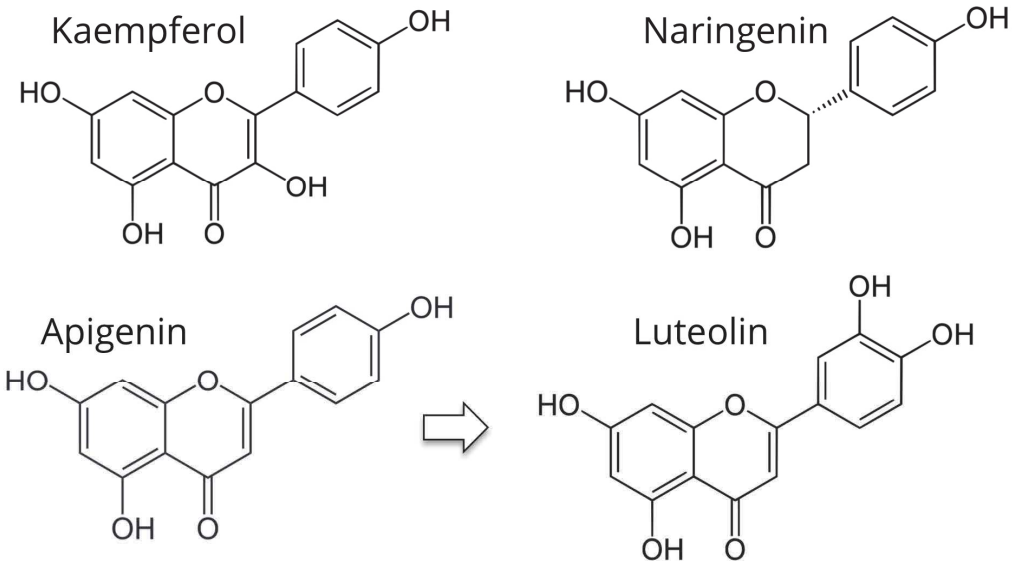


Figure 3. Chemical structure of phytoprogestins.

5.1. Kaempferol

Kaempferol (KP: 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavonoid found in several botanical families, including Pteridophyta, Pinophyta, and Magnoliophyta (Figure 3). Flavonoids are a group of secondary metabolites widespread in nature. These substances are known for the benefits of their consumption, which seems to reduce the risk of cancer and cardiovascular diseases [65]. A case–control study showed a 40% reduction (adjusted odds ratio 0.60) in breast cancer risk in Chinese women in the upper quartile of serum KP levels [66]. The risk of epithelial ovarian cancer was also decreased by 40% among women in the highest quintile of KP dietary intake of a large prospective cohort in the USA, the Nurses' Health Study [67]. Several studies have shown that KP has excellent antioxidant properties. In fact, it is able to decrease, even at low concentrations, the levels of the hydroxyl radical and peroxynitrite, highly reactive species capable of causing severe damage to DNA, proteins, and lipids [68]. In addition, KP has anti-inflammatory properties not only in vitro but also in vivo [69,70].

KP inhibits estrogen receptor-α, causing antiestrogenic effects, depending on the concentration of endogenous estrogens. The antiestrogenic activity of KP results in the inhibition of the growth of hormone-dependent tumors; this activity has been demonstrated in numerous in vitro studies, for example, in endometrial carcinoma cells [71] and two lines of breast cancer cells [72].

In uterine fibroids, despite being hormone-dependent tumors with severe symptoms, the effects of KP have not been extensively studied. KP treatment reduces the expression of the estrogen receptor, thus inhibiting the cell proliferation of human uterine leiomyoma cells in vitro [73], although its therapeutic effect in vivo remains unknown (Table 1).

Table 1. Effects of phytoprogestins that suggest their potential to treat women's diseases.

Substance	Study Design	Effects	Significance	References
Kaempferol	Experiments in mice and rats	Anti-inflammatory	Could be useful to treat chronic pelvic pain and its causes	[69,70]
	In vitro culture of human neutrophils	Antioxidant	Another potential therapeutic mechanism to treat endometriosis	[68]
	In vitro culture of endometrial cancer cells	Growth inhibition and apoptosis	Could be effective against endometrial hyperplasia and cancer	[71]
Apigenin	In vitro culture of human cancer cell lines	Growth inhibition and apoptosis VEGF inhibition	Could be effective against endometrial hyperplasia and cancer	[74,75]
Luteolin	Human breast tumor xenografts in nude mice	Inhibition of tumor growth and angiogenesis	Could be an adjuvant therapy of breast cancer	[76,77]
Naringenin	Mouse model in vivo	Analgesic, anti-inflammatory and antioxidant	Could be useful to treat chronic pelvic pain and its causes	[78]
	Rat model of hepatic injury in vivo	Antifibrotic	Could be effective to treat uterine fibroids	[79]

5.2. Apigenin and Luteolin

Apigenin (4',5,7-trihydroxyflavone) is found in a wide range of plants, including chamomile (*Matricaria recutita*). The traditional use of chamomile as a treatment for insomnia and anxiety has led to investigations of its active constituents, including apigenin. Apigenin is mainly present as a glycosylated compound in significant quantities in vegetables (parsley, celery, onions), fruit (oranges), herbs (chamomile, thyme, oregano, basil), and vegetable drinks (tea, beer, and wine) [80]. Apigenin is considered a phytoestrogen, although it has a much lower potency than other phytoestrogens such as genistein [81]. However, in recent studies, it has emerged that apigenin is also a phytoprogestin. A study found that apigenin reduces the risk of breast tumors in women exposed to prolonged treatment with medroxyprogesterone acetate [82,83]. A study by Horwitz and Sartorius showed that prolonged progestogen therapy could lead to the development of breast cancer through the activation of stem cells that differentiate into cancer cells [84]. In animals subjected to medroxyprogesterone therapy, apigenin administration decreased the incidence of tumors by 50% [82].

Apigenin has an antitumor effect by acting through a variety of mechanisms, including the induction of cell cycle arrest and apoptosis [74], attenuation of phosphorylation of MAP kinase [85] and inhibition of the proinflammatory cytokine interleukin-6 [86]. In vitro studies have shown that treating human breast cancer cell lines [75] with apigenin significantly reduced the expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 [87]. The significant reduction in VEGF disadvantages the tumor growth and development in breast tissue.

Apigenin taken orally is detectable in peripheral blood at concentrations sufficient to be biologically effective [88]. Immediately after ingestion, its concentration increases, and it remains in circulation for a long time, suggesting that it can accumulate within tissues to levels sufficient to exert chemo-preventive effects [89]. Furthermore, apigenin increased the endometrial expression of Hand2, which is a transcription factor stimulated by progesterone. The activation of Hand2 by progesterone allows an antiproliferative action in the endometrium, further suggesting that apigenin is a phytoprogestin. Apigenin appears to be non-toxic even at high doses, as suggested by a study in which it was

repeatedly administered to animals up to 50 mg/kg per 10–13 days, and no signs of toxicity were observed. Apigenin seems to reduce endometrial (Ishikawa) cell proliferation regardless of progesterone [90]. In vivo, apigenin is rapidly metabolized to luteolin.

Luteolin, a flavonoid found in more than 300 plant species, many of which are readily available in the human diet, has been demonstrated to be an excellent progesterone antagonist [91] (Figure 3). A study showed that luteolin effectively inhibits the growth of progestogen-dependent human xenograft tumors, inhibiting angiogenesis and limiting the conversion of breast cancer cells to stem cell-like cells [76,77]. Interestingly, preliminary results suggest that luteolin may inhibit the growth of endometriotic lesions in a mouse model [92].

5.3. Naringenin

Naringenin (4,5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen- 4-one) belongs to the subclass of flavanones (Figure 3). It is a colorless compound that gives the typical bitter taste in citrus, including grapefruit, orange, and lemon [93].

Naringenin has antioxidant, immunomodulatory, anti-inflammatory, nephroprotective, hepatoprotective, neuroprotective, antidiabetic, antitumor, and anti-atherosclerotic properties. In addition, naringenin has a high bioavailability [94,95].

Naringenin is able to inhibit the recruitment and generation of reactive oxygen species (ROS), thereby reducing oxidative stress [78,96]. Moreover, it acts directly on the NF-KB pathway in vitro and in vivo [97]. This signaling pathway is known to be activated by external agents such as pathogens. In the presence of external agents, pro-inflammatory cytokines such as IL-1 and TNF-α are recalled [98]. This stimulation and this recall involve the activation of the IKB kinase complex (IKK), which eventually phosphorylates IKK β. The phosphorylated IKB allows NF-KB to translocate into the nucleus, causing inflammatory responses [99]. Naringenin can prevent the degradation of IKB, inhibiting the transcription activity of NF-KB [98].

In numerous studies, it has emerged that naringenin is also an excellent anti-fibrotic agent [79]. In fact, naringenin was able to decrease the expression of collagen, fibronectin, and Smad3 induced by TGF-β and to inhibit Plasminogen Activator-1 (PAI-1) in hepatic cells [100]. Some of these mechanisms are similar to those fueled by progesterone in uterine fibroids [4].

In a study by Rosenberg et al. [101] it emerged that naringenin may also have progestin-like activity. More specifically, the study showed that the progestin activity of naringenin is weak and acts at concentrations around 10^{-5}–10^{-6} M. These concentration levels are similar to those deemed necessary for the action of resveratrol as a weak estrogen [102], but not for the activity of synthetic progestins such as norgestrel and norgestimate. In fact, the biological activity of naringenin compared to norgestimate is about 104-fold lower.

The effects that naringenin as a phytoprogestin could have on diseases such as endometriosis and uterine fibroids remain to be investigated. An in vitro study found that naringenin induced apoptosis and inhibited the proliferation of immortalized cell lines derived from the endocervical epithelium of a premenopausal woman undergoing hysterectomy for endometriosis [103].

6. Conclusions

There is large unexplored potential in using plant-derived substances to treat human diseases. Some of these phytochemicals have been characterized as phytoprogestins, based on their similarity with progesterone and their pharmacological interaction with PR, functioning as agonists, partial agonists, or antagonists. At least four phytoprogestins have been studied in vitro with promising results such as the antitumoral effects of KP, apigenin, and luteolin, and the anti-fibrotic effects of naringenin. Although there are limited data in the literature, it appears that phytoprogestins could be a good tool for preventing and treating hormone-dependent diseases such as endometriosis, uterine fibroids, ovarian cancer, and breast cancer, with potential reduction in the side effects of currently available

hormone treatments. The next step is to proceed with tests in well-characterized animal models to define the therapeutic mechanisms and safety of these substances, along with observational human studies correlating the dietary ingestion of phytoprogestins with the prevalence and incidence of gynecologic diseases.

Author Contributions: Writing, S.G., P.P., A.Z., G.D.C., A.C., F.M.R., P.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bulletti, C.; Coccia, M.E.; Battistoni, S.; Borini, A. Endometriosis and infertility. *J. Assist. Reprod. Genet.* **2010**, *27*, 441–447. [CrossRef]
2. Islam, M.S.; Greco, S.; Janjusevic, M.; Ciavattini, A.; Giannubilo, S.R.; D'Adderio, A.; Biagini, A.; Fiorini, R.; Castellucci, M.; Ciarmela, P. Growth factors and pathogenesis. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2016**, *34*, 25–36. [CrossRef]
3. Marquardt, R.M.; Kim, T.H.; Shin, J.H.; Jeong, J.W. Progesterone and Estrogen Signaling in the Endometrium: What Goes Wrong in Endometriosis? *Int. J. Mol. Sci.* **2019**, *20*, 3822. [CrossRef] [PubMed]
4. Reis, F.M.; Bloise, E.; Ortiga-Carvalho, T.M. Hormones and pathogenesis of uterine fibroids. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2016**, *34*, 13–24. [CrossRef] [PubMed]
5. Momenimovahed, Z.; Tiznobaik, A.; Taheri, S.; Salehiniya, H. Ovarian cancer in the world: Epidemiology and risk factors. *Int. J. Womens Health* **2019**, *11*, 287–299. [CrossRef]
6. Group, E.C.W. Hormones and breast cancer. *Hum. Reprod. Update* **2004**, *10*, 281–293. [CrossRef] [PubMed]
7. Dietz, B.M.; Hajirahimkhan, A.; Dunlap, T.L.; Bolton, J.L. Botanicals and Their Bioactive Phytochemicals for Women's Health. *Pharmacol. Rev.* **2016**, *68*, 1026–1073. [CrossRef]
8. Hajirahimkhan, A.; Dietz, B.M.; Bolton, J.L. Botanical modulation of menopausal symptoms: Mechanisms of action? *Planta Med.* **2013**, *79*, 538–553. [CrossRef]
9. Zava, D.T.; Dollbaum, C.M.; Blen, M. Estrogen and progestin bioactivity of foods, herbs, and spices. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 369–378. [CrossRef]
10. Scarpin, K.M.; Graham, J.D.; Mote, P.A.; Clarke, C.L. Progesterone action in human tissues: Regulation by progesterone receptor (PR) isoform expression, nuclear positioning and coregulator expression. *Nucl. Recept. Signal* **2009**, *7*, e009. [CrossRef]
11. Taraborrelli, S. Physiology, production and action of progesterone. *Acta Obstet. Gynecol. Scand.* **2015**, *94*, 8–16. [CrossRef] [PubMed]
12. Guennoun, R. Progesterone in the Brain: Hormone, Neurosteroid and Neuroprotectant. *Int. J. Mol. Sci.* **2020**, *21*, 5271. [CrossRef] [PubMed]
13. Piette, P.C.M. The pharmacodynamics and safety of progesterone. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2020**, *69*, 13–29. [CrossRef]
14. Amadori, A.; Cavallari, C.; Giacomucci, E.; Macrelli, S.; Mastronuzzi, G.; Ucci, N. *Fisiologia Della Riproduzione*; CLUEB: Bologna, Italy, 1994; pp. 1–92.
15. Horie, K.; Takakura, K.; Fujiwara, H.; Suginami, H.; Liao, S.; Mori, T. Immunohistochemical localization of androgen receptor in the human ovary throughout the menstrual cycle in relation to oestrogen and progesterone receptor expression. *Hum. Reprod.* **1992**, *7*, 184–190. [CrossRef] [PubMed]
16. Thijssen, J.H. Progesterone receptors in the human uterus and their possible role in parturition. *J. Steroid Biochem. Mol. Biol.* **2005**, *97*, 397–400. [CrossRef] [PubMed]
17. Abid, S.; Gokral, J.; Maitra, A.; Meherji, P.; Kadam, S.; Pires, E.; Modi, D. Altered expression of progesterone receptors in testis of infertile men. *Reprod. Biomed. Online* **2008**, *17*, 175–184. [CrossRef]
18. Brinton, R.D.; Thompson, R.F.; Foy, M.R.; Baudry, M.; Wang, J.; Finch, C.E.; Morgan, T.E.; Pike, C.J.; Mack, W.J.; Stanczyk, F.Z.; et al. Progesterone receptors: Form and function in brain. *Front. Neuroendocrinol.* **2008**, *29*, 313–339. [CrossRef]
19. Doglioni, C.; Gambacorta, M.; Zamboni, G.; Coggi, G.; Viale, G. Immunocytochemical localization of progesterone receptors in endocrine cells of the human pancreas. *Am. J. Pathol.* **1990**, *137*, 999–1005.
20. Bland, R. Steroid hormone receptor expression and action in bone. *Clin. Sci.* **2000**, *98*, 217–240. [CrossRef]
21. Branchini, G.; Schneider, L.; Cericatto, R.; Capp, E.; Brum, I.S. Progesterone receptors A and B and estrogen receptor alpha expression in normal breast tissue and fibroadenomas. *Endocrine* **2009**, *35*, 459–466. [CrossRef]
22. Batra, S.C.; Iosif, C.S. Progesterone receptors in the female lower urinary tract. *J. Urol.* **1987**, *138*, 1301–1304. [CrossRef]

23. Lonard, D.M.; Lanz, R.B.; O'Malley, B.W. Nuclear receptor coregulators and human disease. *Endocr. Rev.* **2007**, *28*, 575–587. [CrossRef] [PubMed]
24. Smith, D.F.; Faber, L.E.; Toft, D.O. Purification of unactivated progesterone receptor and identification of novel receptor-associated proteins. *J. Biol. Chem.* **1990**, *265*, 3996–4003. [CrossRef]
25. Pratt, W.B.; Galigniana, M.D.; Morishima, Y.; Murphy, P.J. Role of molecular chaperones in steroid receptor action. *Essays Biochem.* **2004**, *40*, 41–58. [CrossRef] [PubMed]
26. Tata, J.R. Signalling through nuclear receptors. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 702–710. [CrossRef] [PubMed]
27. Gronemeyer, H.; Meyer, M.E.; Bocquel, M.T.; Kastner, P.; Turcotte, B.; Chambon, P. Progestin receptors: Isoforms and antihormone action. *J. Steroid. Biochem. Mol. Biol.* **1991**, *40*, 271–278. [CrossRef]
28. Kastner, P.; Krust, A.; Turcotte, B.; Stropp, U.; Tora, L.; Gronemeyer, H.; Chambon, P. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J.* **1990**, *9*, 1603–1614. [CrossRef]
29. Losel, R.M.; Besong, D.; Peluso, J.J.; Wehling, M. Progesterone receptor membrane component 1–many tasks for a versatile protein. *Steroids* **2008**, *73*, 929–934. [CrossRef]
30. Kowalik, M.K.; Rekawiecki, R.; Kotwica, J. The putative roles of nuclear and membrane-bound progesterone receptors in the female reproductive tract. *Reprod. Biol.* **2013**, *13*, 279–289. [CrossRef] [PubMed]
31. Mulac-Jericevic, B.; Lydon, J.P.; DeMayo, F.J.; Conneely, O.M. Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9744–9749. [CrossRef]
32. Mulac-Jericevic, B.; Mullinax, R.A.; De Mayo, F.J.; Lydon, J.P.; Conneely, O.M. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* **2000**, *289*, 1751–1754. [CrossRef] [PubMed]
33. Mote, P.A.; Balleine, R.L.; McGowan, E.M.; Clarke, C.L. Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle. *J. Clin. Endocrinol. Metab.* **1999**, *84*, 2963–2971. [CrossRef] [PubMed]
34. Mote, P.A.; Bartow, S.; Tran, N.; Clarke, C.L. Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis. *Breast Cancer Res. Treat.* **2002**, *72*, 163–172. [CrossRef]
35. Graham, J.D.; Yeates, C.; Balleine, R.L.; Harvey, S.S.; Milliken, J.S.; Bilous, A.M.; Clarke, C.L. Characterization of progesterone receptor A and B expression in human breast cancer. *Cancer Res.* **1995**, *55*, 5063–5068. [PubMed]
36. Arnett-Mansfield, R.L.; deFazio, A.; Wain, G.V.; Jaworski, R.C.; Byth, K.; Mote, P.A.; Clarke, C.L. Relative expression of progesterone receptors A and B in endometrioid cancers of the endometrium. *Cancer Res.* **2001**, *61*, 4576–4582.
37. Taylor, A.H.; McParland, P.C.; Taylor, D.J.; Bell, S.C. The cytoplasmic 60 kDa progesterone receptor isoform predominates in the human amniochorion and placenta at term. *Reprod. Biol. Endocrinol.* **2009**, *7*, 22. [CrossRef] [PubMed]
38. Wei, L.L.; Hawkins, P.; Baker, C.; Norris, B.; Sheridan, P.L.; Quinn, P.G. An amino-terminal truncated progesterone receptor isoform, PRc, enhances progestin-induced transcriptional activity. *Mol. Endocrinol.* **1996**, *10*, 1379–1387. [CrossRef]
39. Siegel, R.; Ward, E.; Brawley, O.; Jemal, A. Cancer statistics, 2011: The impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J. Clin.* **2011**, *61*, 212–236. [CrossRef]
40. Key, T.J.; Pike, M.C. The dose-effect relationship between 'unopposed' oestrogens and endometrial mitotic rate: Its central role in explaining and predicting endometrial cancer risk. *Br. J. Cancer* **1988**, *57*, 205–212. [CrossRef]
41. Siiteri, P.K. Steroid hormones and endometrial cancer. *Cancer Res.* **1978**, *38*, 4360–4366.
42. Doherty, J.A.; Weiss, N.S.; Fish, S.; Fan, W.; Loomis, M.M.; Sakoda, L.C.; Rossing, M.A.; Zhao, L.P.; Chen, C. Polymorphisms in nucleotide excision repair genes and endometrial cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **2011**, *20*, 1873–1882. [CrossRef] [PubMed]
43. Shibutani, S.; Ravindernath, A.; Suzuki, N.; Terashima, I.; Sugarman, S.M.; Grollman, A.P.; Pearl, M.L. Identification of tamoxifen-DNA adducts in the endometrium of women treated with tamoxifen. *Carcinogenesis* **2000**, *21*, 1461–1467. [CrossRef]
44. Gompel, A. Progesterone and endometrial cancer. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2020**, *69*, 95–107. [CrossRef] [PubMed]
45. Ethier, J.L.; Desautels, D.N.; Amir, E.; MacKay, H. Is hormonal therapy effective in advanced endometrial cancer? A systematic review and meta-analysis. *Gynecol. Oncol.* **2017**, *147*, 158–166. [CrossRef] [PubMed]
46. Vierikko, P.; Kauppila, A.; Ronnberg, L.; Vihko, R. Steroidal regulation of endometriosis tissue: Lack of induction of 17 beta-hydroxysteroid dehydrogenase activity by progesterone, medroxyprogesterone acetate, or danazol. *Fertil. Steril.* **1985**, *43*, 218–224. [CrossRef]
47. Brandon, D.D.; Erickson, T.E.; Keenan, E.J.; Strawn, E.Y.; Novy, M.J.; Burry, K.A.; Warner, C.; Clinton, G.M. Estrogen receptor gene expression in human uterine leiomyomata. *J. Clin. Endocrinol. Metab.* **1995**, *80*, 1876–1881. [CrossRef]
48. Soper, J.T.; McCarty, K.S., Jr.; Creasman, W.T.; Clarke-Pearson, D.L. Induction of cytoplasmic progesterone receptor in human endometrial carcinoma transplanted into nude mice. *Am. J. Obstet. Gynecol.* **1984**, *150*, 437–439. [CrossRef]
49. Murji, A.; Biberoglu, K.; Leng, J.; Mueller, M.D.; Romer, T.; Vignali, M.; Yarmolinskaya, M. Use of dienogest in endometriosis: A narrative literature review and expert commentary. *Curr. Med. Res. Opin.* **2020**, *36*, 895–907. [CrossRef]
50. Goodman, A.L. Progesterone therapy in uterine fibromyoma. *J. Clin. Endocrinol. Metab.* **1946**, *6*, 402–408. [CrossRef]
51. Lumbiganon, P.; Rugpao, S.; Phandhu-fung, S.; Laopaiboon, M.; Vudhikamraksa, N.; Werawatakul, Y. Protective effect of depot-medroxyprogesterone acetate on surgically treated uterine leiomyomas: A multicentre case-control study. *BJOG Int. J. Obstet. Gynaecol.* **1996**, *103*, 909–914. [CrossRef]

52. Schindler, A.E.; Campagnoli, C.; Druckmann, R.; Huber, J.; Pasqualini, J.R.; Schweppe, K.W.; Thijssen, J.H. Classification and pharmacology of progestins. *Maturitas* **2008**, *61*, 171–180. [CrossRef] [PubMed]
53. Ichigo, S.; Takagi, H.; Matsunami, K.; Suzuki, N.; Imai, A. Beneficial effects of dienogest on uterine myoma volume: A retrospective controlled study comparing with gonadotropin-releasing hormone agonist. *Arch. Gynecol. Obstet.* **2011**, *284*, 667–670. [CrossRef] [PubMed]
54. Rott, H. Thrombotic risks of oral contraceptives. *Curr. Opin. Obstet. Gynecol.* **2012**, *24*, 235–240. [CrossRef] [PubMed]
55. Practice Committee of the American Society for Reproductive Medicine. Combined hormonal contraception and the risk of venous thromboembolism: A guideline. *Fertil. Steril.* **2017**, *107*, 43–51. [CrossRef]
56. DeMarzo, A.M.; Beck, C.A.; Onate, S.A.; Edwards, D.P. Dimerization of mammalian progesterone receptors occurs in the absence of DNA and is related to the release of the 90-kDa heat shock protein. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 72–76. [CrossRef]
57. Smith, C.L.; O'Malley, B.W. Coregulator function: A key to understanding tissue specificity of selective receptor modulators. *Endocr. Rev.* **2004**, *25*, 45–71. [CrossRef]
58. Bouchard, P.; Chabbert-Buffet, N.; Fauser, B.C. Selective progesterone receptor modulators in reproductive medicine: Pharmacology, clinical efficacy and safety. *Fertil. Steril.* **2011**, *96*, 1175–1189. [CrossRef]
59. Wilkens, J.; Male, V.; Ghazal, P.; Forster, T.; Gibson, D.A.; Williams, A.R.; Brito-Mutunayagam, S.L.; Craigon, M.; Lourenco, P.; Cameron, I.T.; et al. Uterine NK cells regulate endometrial bleeding in women and are suppressed by the progesterone receptor modulator asoprisnil. *J. Immunol.* **2013**, *191*, 2226–2235. [CrossRef]
60. Donnez, J. Uterine Fibroids and Progestogen Treatment: Lack of Evidence of Its Efficacy: A Review. *J. Clin. Med.* **2020**, *9*, 3948. [CrossRef]
61. Bressler, L.H.; Bernardi, L.A.; Snyder, M.A.; Wei, J.J.; Bulun, S. Treatment of endometriosis-related chronic pelvic pain with Ulipristal Acetate and associated endometrial changes. *HSOA J. Reprod. Med. Gynaecol. Obstet.* **2017**, *2*. [CrossRef]
62. Lee, O.; Sullivan, M.E.; Xu, Y.; Rogers, C.; Muzzio, M.; Helenowski, I.; Shidfar, A.; Zeng, Z.; Singhal, H.; Jovanovic, B.; et al. Selective Progesterone Receptor Modulators in Early-Stage Breast Cancer: A Randomized, Placebo-Controlled Phase II Window-of-Opportunity Trial Using Telapristone Acetate. *Clin. Cancer Res.* **2020**, *26*, 25–34. [CrossRef]
63. Dinis-Oliveira, R.J. Pharmacokinetics, toxicological and clinical aspects of ulipristal acetate: Insights into the mechanisms implicated in the hepatic toxicity. *Drug Metab. Rev.* **2021**, 1–9. [CrossRef]
64. Islam, M.S.; Afrin, S.; Jones, S.I.; Segars, J. Selective Progesterone Receptor Modulators-Mechanisms and Therapeutic Utility. *Endocr. Rev.* **2020**, *41*, 643–694. [CrossRef]
65. Middleton, E., Jr.; Kandaswami, C.; Theoharides, T.C. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, *52*, 673–751. [PubMed]
66. Feng, X.L.; Zhan, X.X.; Zuo, L.S.; Mo, X.F.; Zhang, X.; Liu, K.Y.; Li, L.; Zhang, C.X. Associations between serum concentration of flavonoids and breast cancer risk among Chinese women. *Eur. J. Nutr.* **2021**, *60*, 1347–1362. [CrossRef] [PubMed]
67. Gates, M.A.; Tworoger, S.S.; Hecht, J.L.; De Vivo, I.; Rosner, B.; Hankinson, S.E. A prospective study of dietary flavonoid intake and incidence of epithelial ovarian cancer. *Int. J. Cancer* **2007**, *121*, 2225–2232. [CrossRef] [PubMed]
68. Wang, L.; Tu, Y.C.; Lian, T.W.; Hung, J.T.; Yen, J.H.; Wu, M.J. Distinctive antioxidant and antiinflammatory effects of flavonols. *J. Agric. Food Chem.* **2006**, *54*, 9798–9804. [CrossRef] [PubMed]
69. Orhan, I.; Kupeli, E.; Terzioglu, S.; Yesilada, E. Bioassay-guided isolation of kaempferol-3-O-beta-D-galactoside with anti-inflammatory and antinociceptive activity from the aerial part of Calluna vulgaris L. *J. Ethnopharmacol.* **2007**, *114*, 32–37. [CrossRef]
70. Park, M.J.; Lee, E.K.; Heo, H.S.; Kim, M.S.; Sung, B.; Kim, M.K.; Lee, J.; Kim, N.D.; Anton, S.; Choi, J.S.; et al. The anti-inflammatory effect of kaempferol in aged kidney tissues: The involvement of nuclear factor-kappaB via nuclear factor-inducing kinase/IkappaB kinase and mitogen-activated protein kinase pathways. *J. Med. Food* **2009**, *12*, 351–358. [CrossRef]
71. Chuwa, A.H.; Sone, K.; Oda, K.; Tanikawa, M.; Kukita, A.; Kojima, M.; Oki, S.; Fukuda, T.; Takeuchi, M.; Miyasaka, A.; et al. Kaempferol, a natural dietary flavonoid, suppresses 17beta-estradiol-induced survivin expression and causes apoptotic cell death in endometrial cancer. *Oncol. Lett.* **2018**, *16*, 6195–6201. [CrossRef]
72. Hu, G.; Liu, H.; Wang, M.; Peng, W. IQ Motif Containing GTPase-Activating Protein 3 (IQGAP3) Inhibits Kaempferol-Induced Apoptosis in Breast Cancer Cells by Extracellular Signal-Regulated Kinases 1/2 (ERK1/2) Signaling Activation. *Med. Sci. Monit.* **2019**, *25*, 7666–7674. [CrossRef]
73. Li, Y.; Ding, Z.; Wu, C. Mechanistic Study of the Inhibitory Effect of Kaempferol on Uterine Fibroids In Vitro. *Med. Sci. Monit.* **2016**, *22*, 4803–4808. [CrossRef]
74. Horinaka, M.; Yoshida, T.; Shiraishi, T.; Nakata, S.; Wakada, M.; Sakai, T. The dietary flavonoid apigenin sensitizes malignant tumor cells to tumor necrosis factor-related apoptosis-inducing ligand. *Mol. Cancer Ther.* **2006**, *5*, 945–951. [CrossRef]
75. Mafuvadze, B.; Benakanakere, I.; Hyder, S.M. Apigenin blocks induction of vascular endothelial growth factor mRNA and protein in progestin-treated human breast cancer cells. *Menopause* **2010**, *17*, 1055–1063. [CrossRef] [PubMed]
76. Cook, M.T.; Liang, Y.; Besch-Williford, C.; Goyette, S.; Mafuvadze, B.; Hyder, S.M. Luteolin inhibits progestin-dependent angiogenesis, stem cell-like characteristics, and growth of human breast cancer xenografts. *Springerplus* **2015**, *4*, 444. [CrossRef] [PubMed]
77. Cook, M.T.; Liang, Y.; Besch-Williford, C.; Hyder, S.M. Luteolin inhibits lung metastasis, cell migration, and viability of triple-negative breast cancer cells. *Breast Cancer* **2017**, *9*, 9–19. [CrossRef] [PubMed]

78. Manchope, M.F.; Calixto-Campos, C.; Coelho-Silva, L.; Zarpelon, A.C.; Pinho-Ribeiro, F.A.; Georgetti, S.R.; Baracat, M.M.; Casagrande, R.; Verri, W.A., Jr. Naringenin Inhibits Superoxide Anion-Induced Inflammatory Pain: Role of Oxidative Stress, Cytokines, Nrf-2 and the NO-cGMP-PKG-KATP Channel Signaling Pathway. *PLoS ONE* **2016**, *11*, e0153015. [CrossRef] [PubMed]
79. Lee, M.H.; Yoon, S.; Moon, J.O. The flavonoid naringenin inhibits dimethylnitrosamine-induced liver damage in rats. *Biol. Pharm. Bull.* **2004**, *27*, 72–76. [CrossRef] [PubMed]
80. Hostetler, G.L.; Ralston, R.A.; Schwartz, S.J. Flavones: Food Sources, Bioavailability, Metabolism, and Bioactivity. *Adv. Nutr.* **2017**, *8*, 423–435. [CrossRef]
81. Mabry, T.; Markham, K.R.; Thomas, M.B. *The Systematic Identification of Flavonoids*; Springer: Berlin/Heidelberg, Germany, 2012.
82. Mafuvadze, B.; Benakanakere, I.; Lopez Perez, F.R.; Besch-Williford, C.; Ellersieck, M.R.; Hyder, S.M. Apigenin prevents development of medroxyprogesterone acetate-accelerated 7,12-dimethylbenz(a)anthracene-induced mammary tumors in Sprague-Dawley rats. *Cancer Prev. Res.* **2011**, *4*, 1316–1324. [CrossRef]
83. Mafuvadze, B.; Liang, Y.; Besch-Williford, C.; Zhang, X.; Hyder, S.M. Apigenin induces apoptosis and blocks growth of medroxyprogesterone acetate-dependent BT-474 xenograft tumors. *Horm. Cancer* **2012**, *3*, 160–171. [CrossRef] [PubMed]
84. Horwitz, K.B.; Sartorius, C.A. Progestins in hormone replacement therapies reactivate cancer stem cells in women with preexisting breast cancers: A hypothesis. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 3295–3298. [CrossRef]
85. Yin, F.; Giuliano, A.E.; Law, R.E.; Van Herle, A.J. Apigenin inhibits growth and induces G2/M arrest by modulating cyclin-CDK regulators and ERK MAP kinase activation in breast carcinoma cells. *Anticancer Res.* **2001**, *21*, 413–420. [PubMed]
86. Lee, H.H.; Jung, J.; Moon, A.; Kang, H.; Cho, H. Antitumor and Anti-Invasive Effect of Apigenin on Human Breast Carcinoma through Suppression of IL-6 Expression. *Int. J. Mol. Sci.* **2019**, *20*, 3143. [CrossRef] [PubMed]
87. Hyder, S.M. Sex-steroid regulation of vascular endothelial growth factor in breast cancer. *Endocr. Relat. Cancer* **2006**, *13*, 667–687. [CrossRef]
88. Meyer, H.; Bolarinwa, A.; Wolfram, G.; Linseisen, J. Bioavailability of apigenin from apiin-rich parsley in humans. *Ann. Nutr. Metab.* **2006**, *50*, 167–172. [CrossRef]
89. Chen, D.; Landis-Piwowar, K.R.; Chen, M.S.; Dou, Q.P. Inhibition of proteasome activity by the dietary flavonoid apigenin is associated with growth inhibition in cultured breast cancer cells and xenografts. *Breast Cancer Res.* **2007**, *9*, R80. [CrossRef]
90. Dean, M.; Austin, J.; Jinhong, R.; Johnson, M.E.; Lantvit, D.D.; Burdette, J.E. The Flavonoid Apigenin Is a Progesterone Receptor Modulator with In Vivo Activity in the Uterus. *Horm. Cancer* **2018**, *9*, 265–277. [CrossRef]
91. Fidelis, Q.C.; Faraone, I.; Russo, D.; Aragao Catunda, F.E., Jr.; Vignola, L.; de Carvalho, M.G.; de Tommasi, N.; Milella, L. Chemical and Biological insights of *Ouratea hexasperma* (A. St.-Hil.) Baill.: A source of bioactive compounds with multifunctional properties. *Nat. Prod. Res.* **2019**, *33*, 1500–1503. [CrossRef]
92. Park, S.; Lim, W.; You, S.; Song, G. Ameliorative effects of luteolin against endometriosis progression in vitro and in vivo. *J. Nutr. Biochem.* **2019**, *67*, 161–172. [CrossRef]
93. Zaidun, N.H.; Thent, Z.C.; Latiff, A.A. Combating oxidative stress disorders with citrus flavonoid: Naringenin. *Life Sci.* **2018**, *208*, 111–122. [CrossRef]
94. Pereira-Caro, G.; Borges, G.; van der Hooft, J.; Clifford, M.N.; Del Rio, D.; Lean, M.E.; Roberts, S.A.; Kellerhals, M.B.; Crozier, A. Orange juice (poly)phenols are highly bioavailable in humans. *Am. J. Clin. Nutr.* **2014**, *100*, 1378–1384. [CrossRef]
95. Kanaze, F.I.; Bounartzi, M.I.; Georgarakis, M.; Niopas, I. Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. *Eur. J. Clin. Nutr.* **2007**, *61*, 472–477. [CrossRef] [PubMed]
96. Martinez, R.M.; Pinho-Ribeiro, F.A.; Steffen, V.S.; Caviglione, C.V.; Vignoli, J.A.; Barbosa, D.S.; Baracat, M.M.; Georgetti, S.R.; Verri, W.A., Jr.; Casagrande, R. Naringenin Inhibits UVB Irradiation-Induced Inflammation and Oxidative Stress in the Skin of Hairless Mice. *J. Nat. Prod.* **2015**, *78*, 1647–1655. [CrossRef] [PubMed]
97. Pinho-Ribeiro, F.A.; Zarpelon, A.C.; Fattori, V.; Manchope, M.F.; Mizokami, S.S.; Casagrande, R.; Verri, W.A., Jr. Naringenin reduces inflammatory pain in mice. *Neuropharmacology* **2016**, *105*, 508–519. [CrossRef] [PubMed]
98. Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb. Perspect. Biol.* **2009**, *1*, a001651. [CrossRef]
99. Ghosh, S.; Karin, M. Missing pieces in the NF-kappaB puzzle. *Cell* **2002**, *109*, S81–S96. [CrossRef]
100. Liu, X.; Wang, W.; Hu, H.; Tang, N.; Zhang, C.; Liang, W.; Wang, M. Smad3 specific inhibitor, naringenin, decreases the expression of extracellular matrix induced by TGF-beta1 in cultured rat hepatic stellate cells. *Pharm. Res.* **2006**, *23*, 82–89. [CrossRef]
101. Rosenberg, R.S.; Grass, L.; Jenkins, D.J.; Kendall, C.W.; Diamandis, E.P. Modulation of androgen and progesterone receptors by phytochemicals in breast cancer cell lines. *Biochem. Biophys. Res. Commun.* **1998**, *248*, 935–939. [CrossRef]
102. Gehm, B.D.; McAndrews, J.M.; Chien, P.Y.; Jameson, J.L. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 14138–14143. [CrossRef]
103. Park, S.; Lim, W.; Bazer, F.W.; Song, G. Naringenin induces mitochondria-mediated apoptosis and endoplasmic reticulum stress by regulating MAPK and AKT signal transduction pathways in endometriosis cells. *Mol. Hum. Reprod.* **2017**, *23*, 842–854. [CrossRef] [PubMed]

SCIENTIFIC REPORTS

OPEN

Advanced 3D Imaging of Uterine Leiomyoma's Morphology by Propagation-based Phase-Contrast Microtomography

Received: 13 March 2019
Accepted: 10 July 2019
Published online: 22 July 2019

Alessandra Giuliani[1], Stefania Greco[2], Serena Pacilè[3], Alessandro Zannotti[1,2], Giovanni Delli Carpini[1], Giuliana Tromba[3], Stefano Raffaele Giannubilo[1], Andrea Ciavattini[1] & Pasquapina Ciarmela[2]

Uterine leiomyoma is the most common benign smooth muscle tumor in women pelvis, originating from the myometrium. It is caused by a disorder of fibrosis, with a large production and disruption of extracellular matrix (ECM). Medical treatments are still very limited and no preventative therapies have been developed. We supposed that synchrotron-based phase-contrast microtomography (PhC-microCT) may be an appropriate tool to assess the 3D morphology of uterine leiomyoma, without the use of any contrast agent. We used this technique to perform the imaging and the quantitative morphometric analysis of healthy myometrium and pathologic leiomyomas. The quantitative morphometric analysis of collagen bundles was coupled to the Roschger approach. This method, previously only used to evaluate mineralized bone density distribution, was applied here to study the fibrosis mass density distribution in healthy and pathologic biopsies from two patients. This protocol was shown to be powerful in studying uterine leiomyomas, detecting also small signs of the ECM alteration. This is of paramount importance not only for the follow-up of the present study, i.e. the investigation of different compounds and their possible therapeutic benefits, but also because it offers new methodologic possibilities for future studies of the ECM in soft tissues of different body districts.

Uterine leiomyoma (myoma or fibroid) is the most common benign smooth muscle tumor in women pelvis causing significant morbidity in a large segment of reproductive-aged women[1,2], including significant impact on the reproductive healthy status and on the pregnancy outcome of affected patients[3,4].

Fibroids remain the leading indication for hysterectomy[5,6] especially during the peri-menopause period, although the assumption that leiomyoma symptoms will resolve with the onset of the menopause is too simplistic and may not be always valid[7]. Medical treatments for leiomyoma are still very limited and no preventative therapies have been developed[8].

The precise pathogenesis of uterine leiomyoma is not well understood. Genetic alterations, epigenetic mechanisms, steroids, growth factors, cytokines and chemokines provide the clue of initiators and promoters of leiomyoma growth[9]. Uterine myoma is thought to be a consequence of myofibroblasts activation and extracellular matrix (ECM) production, following an improper inflammatory response inside the myometrium[10–12]. Excessive accumulation of ECM components including collagens, fibronectin and proteoglycans are the major structural part of leiomyoma tissue that are abnormally orientated modifying mechanical stress on cells and leading to activation of internal mechanical signaling and dynamic reciprocity[13–15]. The ECM stiffness causes the rigid structure of the leiomyoma and it is thought to be a cause of abnormal bleeding and pelvic pressure and pain[16].

The role of inflammation and reparative processes as well as the effect of some potential therapeutic compounds (tranilast, strawberry extracts, omega-3 fatty acids, genistein, natural fitoterapeutics, vitamin D) on uterine leiomyoma are currently under *in vitro* investigation[17–29].

[1]Department of Clinical Sciences, Università Politecnica delle Marche, Ancona, Italy. [2]Department of Experimental and Clinical Medicine, Università Politecnica delle Marche, Ancona, Italy. [3]Elettra Sincrotrone Trieste S.C.p.A, Trieste, Italy. Correspondence and requests for materials should be addressed to A.G. (email: a.giuliani@univpm.it) or P.C. (email: p.ciarmela@univpm.it)

At the moment, structural laboratory studies of myometrial and fibrotic tissue are limited to the two-dimensional (2D) morphological investigation. Only an advanced three-dimensional (3D) characterization of the fibrotic tissue structure can enhance our knowledge of the tissue architecture, significantly supporting the development of an effective therapeutic solution. For example, the *in-vivo* efficiency of therapeutic compounds could be hampered, even if delivered locally, by their inability to reach the leiomyoma cells due to the fibrotic hard and perhaps not permeable tissue architecture. Therefore, it would be essential to evaluate the whole 3D tissue structure.

Nowadays, X-ray tomography (CT) is not the method of choice for the characterization of pelvic masses; indeed, uterine fibroids are detected incidentally by CT scans, often performed for other reasons. Medical ultrasonography (USG) is usually the initial investigation tool to examine the female pelvis, performing both transabdominal and transvaginal scans. Magnetic resonance imaging (MRI) is the favourite method for an accurate characterization of pelvic masses. Indeed, MRI was shown to be more sensitive in identifying uterine fibroids than USG, and it has the advantage, respect to CT, that it does not involve the use of ionizing radiation[30,31].

However, up to now, literature has not definitely clarified if the discrimination between healthy and pathologic myometrium is just a matter of stiffness or there are also any density variations. For instance, the typical CT images show fibroids that may appear morphologically complex, with hypodense, though they may be isointense, and rarely hyper intense areas. Calcification is seen in approximately 4% of fibroids: it is typically dense and amorphous, sometimes confined to the periphery of the fibroids. As such, the CT appearance of leiomyomas cannot reliably be distinguished from uterine or cervical neoplasms[32].

X-ray phase-contrast imaging (XRPCI) techniques may constitute a potential powerful tool for non-invasive myometrium imaging, in healthy and pathologic conditions and without the use of any contrast agent. Indeed, differently from the X-ray absorption-based imaging, where the contrast originates from attenuation mismatches between different tissues inside a sample, in the XRPCI the contrast is due to the phase-shift δ of the refractive index $n = 1 - \delta + i\beta$, describing the interaction of the X-ray beam throughout the material[33,34]. This δ value, in non-mineralized biological tissues like the myometrium, can be up to three orders of magnitude larger than attenuation values β[35,36], achieving an highly increased contrast, as observed investigating several organs/tissues, including brain[37,38], vessels[39–41], kidney[42], neuronal system[43], cartilage[44,45] and breast tissues[46,47]. Moreover, ten years ago, synchrotron radiation-based high resolution phase-contrast tomography (SR-PhC-microCT) was also used, in a pioneering way, for the study of the ECM organization within polymeric scaffolds[48].

No XRPCI studies, to the proponents' best knowledge, have been performed so far on the uterine myometrium and leiomyomas without the use of a contrast agent. Thus, in the present demonstrative study, we aimed to test the use of the propagation-based phase-contrast imaging to discriminate healthy and pathologic uterine tissues.

Results

Taking into account that the slight nominal mismatch between collagen ($1.41\,g/cm^3$)[49] and muscle ($1.055 \div 1.112\,g/cm^3$)[50] mass densities would have prevented, most likely, a reliable analysis by absorption-based imaging, all the 15 biopsies were investigated by PhC-microCT and subsequently reconstructed exploiting the Paganin's method[51] for the phase retrieval processing.

Representative 2D slices of healthy myometrium (Ctr) and leiomyoma (L) tissues are shown in Fig. 1 (panels a–d) for both patients P1 and P2. Stack-sequences of 2D axial slices (subvolumes, each with a final volume of $600 \times 600 \times 400\,\mu m^3$) have been reported for representative samples of P1 in Movies 1 and 2 in the Supplementary Material section. 3D reconstructions of the same volumes are shown in Fig. 1 (panels e,f), where all the tissues but the collagen phase have been made virtually transparent. Morphological dissimilarities were clearly found between Ctr and L samples of the same patient (P-Ctr vs. P-L) and between pathologic tissues of the two patients (P1-L vs. P2-L). The analysis was also able to reveal differences between the healthy tissues of the two patients (P1-Ctr vs. P2-Ctr).

In agreement with previous literature, mainly based on histologic findings, the pathologic biopsies revealed an higher amount of collagen than the healthy myometrium. Moreover, distribution and orientation of the collagen bundles were very varied in the different samples, suggesting the need for a detailed morphometric analysis.

The quantitative analysis was performed with an approach based on two levels of investigation.

In the first level, the application of the Mixture Modeling algorithm[52] to the histograms (based on an unsigned 8-bit scale of grey) of the full set of samples indicated a threshold of 110 between muscle and collagen grey levels, achieving the collagen morphometric quantification, as detailed in Table 1 and illustrated in Fig. 2.

The specific surface (CollS/CollV) and the mean distance between the collagen bundles (Sp) in the leiomyoma were lower than in the healthy myometrium. Coherently, the specific volume (CollV/TV), the mean thickness (Th) and the mean number (Nr) of collagen bundles were higher than in the healthy myometrium.

Unexpectedly, three other results were obtained, as detailed in Table 1:

(1) although the number of samples analyzed was comparable, the standard deviation of data obtained for P1-Ctr was always higher than for data related to P2-Ctr;
(2) the Nr was higher in P1-Ctr than in P2-Ctr;
(3) the Connectivity Density (Conn.D) was comparable between P1-Ctr and P1-L but lower in P2-Ctr than in P2-L.

In general, these three results, revealed large structural inhomogeneity inside myometrial and leiomyoma tissues.

Moreover, up to now, literature has not definitely clarified if the discrimination between healthy and pathologic myometrium is just a matter of stiffness or there are also density variations. Consequently, we achieved the

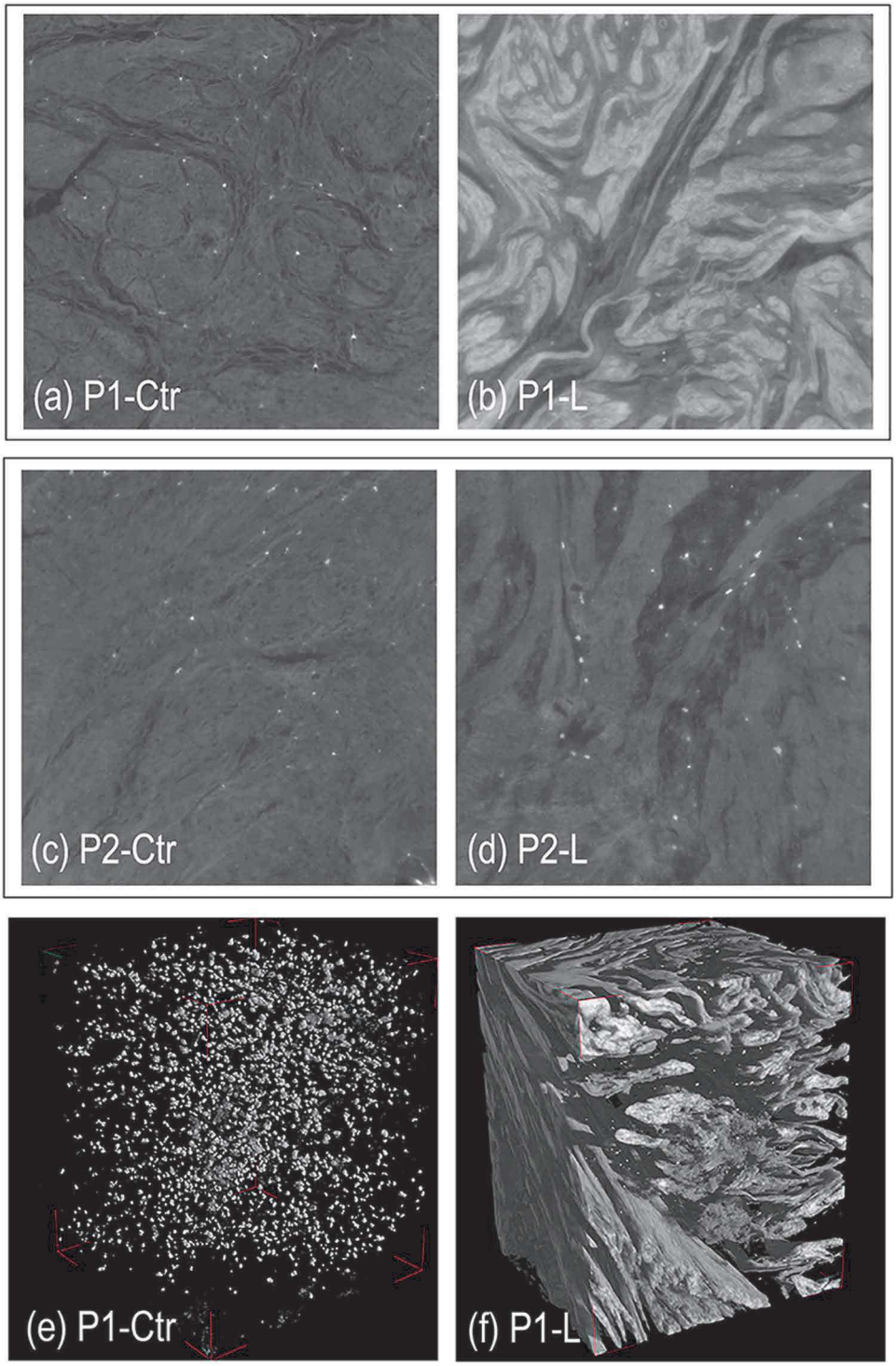


Figure 1. (**a**–**d**) Representative 2D slices of healthy myometrium (Ctr) and pathologic (L) tissues. (**a**,**b**) Patient 1 (P1): healthy myometrium (**a**: P1-Ctr) and leiomyoma (**b**: P1-L) tissues; (**c**,**d**) Patient 2 (P2): healthy myometrium (**c**: P2-Ctr) and leiomyoma (**d**: P2-L) tissues. Morphological dissimilarities were found not only between Ctr and L samples of the same patient and between pathologic tissues of the two patients (P1-L vs. P2-L) but also between the healthy tissues of the two patients (P1-Ctr vs. P2-Ctr). (**e**,**f**) Representative 3D volumes of healthy myometrium (**e**: P1-Ctr) and pathologic (**f**: P1-L) tissues: all the tissues but the collagen phase have been made virtually transparent. The bright spots that we found in all the samples were due, most likely, to the presence of blood clots residues, not removed by washing because of the structure tortuosity.

second level of the quantitative analysis. It was based on the approach designed by Roschger[53] and, to date, it was succesfully used to evaluate the mineral density distribution in bone (BMDD)[54,55]. Indeed, the Roschger method delivered fundamental descriptors of the mineral density distribution of the bone matrix throughout a sample:

	P1-Ctr	P1-L	P2-Ctr	P2-L
CollS/CollV [mm^{-1}]	388 ± 213	63 ± 20	422 ± 117	200 ± 71
CollV/TV [%]	15.0 ± 24.4	70.5 ± 10.9	3.1 ± 2.1	32.6 ± 15.8
Th [μm]	7.8 ± 6.8	35.4 ± 13.1	5.0 ± 1.2	11.4 ± 4.9
Nr [mm^{-1}]	13.0 ± 10.7	21.1 ± 3.8	6.0 ± 3.2	28.7 ± 4.4
Sp [μm]	145.9 ± 125.2	13.9 ± 3.7	211.3 ± 126.4	23.9 ± 7.1
DA	0.378 ± 0.081	0.632 ± 0.089	0.399 ± 0.166	0.378 ± 0.081
Conn.D [$\times 10^{-7}$ $pixel^{-3}$]	234.6 ± 368.5	259.4 ± 52.5	45.0 ± 58.3	741.9 ± 472.2

Table 1. Collagen three-dimensional morphometric analysis in the retrieved biopsies. Mean values ± standard deviation. P1-Ctr: heathy myometrium in patient 1; P1-L: leiomyoma in patient 1; P2-Ctr: healthy myometrium in patient 2; P2-L: leiomyoma in patient 2.

thus, it was shown to play a fundamental role in testing if either diseases and/or treatments might be of significant biological and clinical relevance[53].

Thus, starting from the rational that the leiomyoma pathology was shown to be connected to the excessive accumulation of collagen, the study of fibrosis mass density distribution by the Roschger's approach was supposed to help in clarifying if distribution deviations from healthy conditions were involved. We tested the Roschger method descriptors (Fig. 3a) to evaluate, for the first time to the authors' knowledge, the apparent uterine mass density distribution (MDD^r). Similarly to the previous studies, absolute values of mass density could not be retrieved because they might have been biased by the used constant delta-over-beta ratio in the Paganin phase retrieval[56]. However, being the different biopsies comparable in terms of size and composition, the quantitative comparison of the relative difference in mass density distribution between the samples is feasible. Thus, hereinafter the superscript r will denote relative values for all mass density distribution parameters.

The quantitative data obtained in the investigated biopsies were reported in the box plots of the Fig. 3b–f. Median values of the relative mass density distribution in P1-Ctr were found lower than in P1-L; on the contrary, the fwhm and the high (99.5th percentile) values were higher in the leiomyoma in comparison to the healthy controls for both the patients. However, as expected after the morphometric analysis of collagen, the fwhm and the high values of P1-Ctr were higher than those obtained in P2-Ctr. Therefore, it must be concluded that some or all the biopsies derived from patient 1, clinically considered non-pathological tissue portions, were actually composed by very heterogenic samples, in terms of stiffness and density distribution.

Discussion

The role of the extracellular matrix (ECM) in the biomechanics of the human uterus has just been begun to be studied in recent years. The ECM does not only surround cells, but its rigidity stresses them mechanically, producing signals that depend on the amount of collagen, its cross-linking and hydration. Therefore, the ECM can induce the so-called mechano-transduction process, namely cells convert a mechanical stimulus into an electrochemical activity. Indeed, the etiology of uterine fibroids indicates that its growth is due to an increase not only of the cell number but also of the ECM amount, both promoted by endocrine and autocrine growth factors. In turns, the alterations in ECM volume and distribution can modify mechanical stress on cells, leading to activation of specific cell's signaling that contribute to leiomyoma growth[16,57]. Therefore, alterations of morphology and/or composition of the ECM can be important signals of the onset and of the evolution of this pathology.

Previous studies showed an impaired expression of collagen, fibronectin and proteoglycans in leiomyoma compared to normal myometrium[58–61]: in particular, leiomyoma was shown to contain an abnormal collagen fibril structure and orientation, suggesting that the well-regulated fibril formation in myometrium is altered in leiomyomas. In fact, an important study showed that the interaction of two or more different types of fibrillar collagen chains may interact and result in the formation of heterotypic fibrils, missing in leiomyomas, that in myometrium would assist in regular fibril formation in normal uterine tissue. In this context, alterations in collagen may play a role in the pathogenesis of leiomyomas[62]. Thus, the study of specific collagen parameters, such as the size of collagen bundles, orientation and interconnectivity, turns out to be particularly interesting because its morphometry should determine specific cellular behaviors. This is similar to what happens in bone sites, although the interpretation of what morphometric measurements mean for collagen masses in the myometrium must be related to the specific myometrial tissue and its function.

Therefore, we have focused our analysis on the study of collagen. The propagation-based phase-contrast microCT was used, for the first time to the authors' knowledge, to evaluate the collagen amount and distribution in intramural fibroids excised from the uterus of two fertile women.

In our study, we observed higher volumes, thickness and number of collagen bundles in the leiomyoma compared to normal adjacent myometrium. In the case of patient 1, the interconnectivity of the collagen structure was also increased in the pathological tissue compared to the control. These data obtained on collagen networks justify the increase in the rigidity of the pathological tissue compared to the healthy one and they are in agreement with the observation performed with conventional 2D techniques, like histology and electron microscopy, in several studies found in literature.

The specific surface (CollS/CollV), defined as the surface area per unit volume, is an architectural parameter that can contribute to determine type and properties of a material. This measure is strongly dependent from shape: if volume is held constant, matrix of different shapes will typically adopt different specific surface values. Interestingly, we found that collagen specific surface in leiomyoma is lower than in normal counterpart. Certainly,

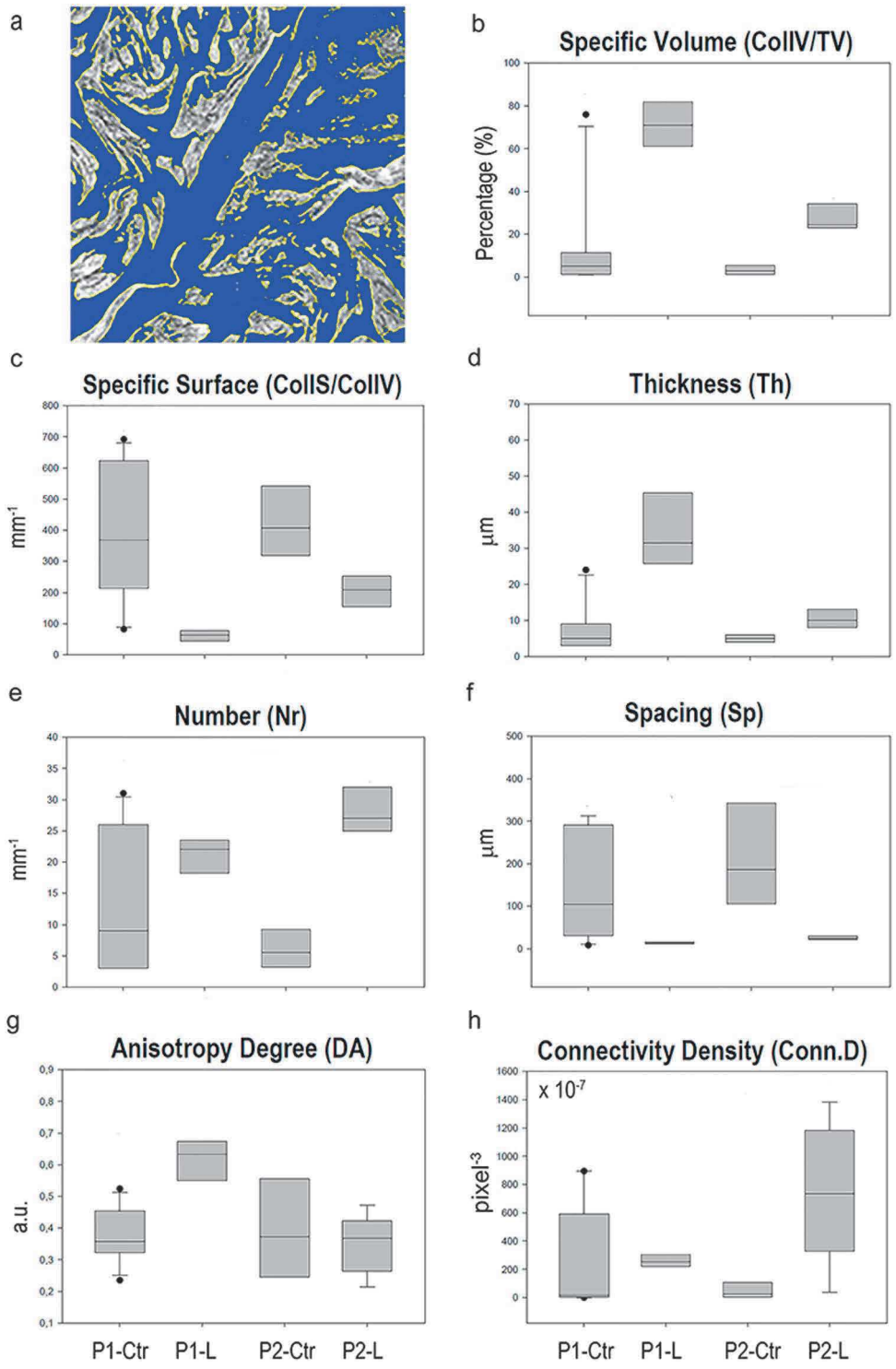


Figure 2. 1st level analysis: study of the collagen morphometric quantification. (**a**) Sampling 2D slice of a pathologic biopsy where the histogram has been segmented according to the application of the Mixture Modeling algorithm. Blue phase: smooth muscle; graded grey phase: collagen bundles. (**b**–**h**) Box-plots graphically depicting groups of the extracted collagen morphometric parameters through their quartiles, as a function of the selected groups of samples P1-Ctr, P1-L, P2-Ctr and P2-L.

this is due to the fragmentation of collagen in myometrium versus the aggregation of collagen in leiomyoma. To the authors' knowledge, collagen specific surface has never been measured in myometrium or leiomyoma tissue, although it could represent an important index to study, due to relevant clinical implications. The presence of focal nodal collagen distribution in the myometrium, as in the case of uterine fibroids, could alter the normal

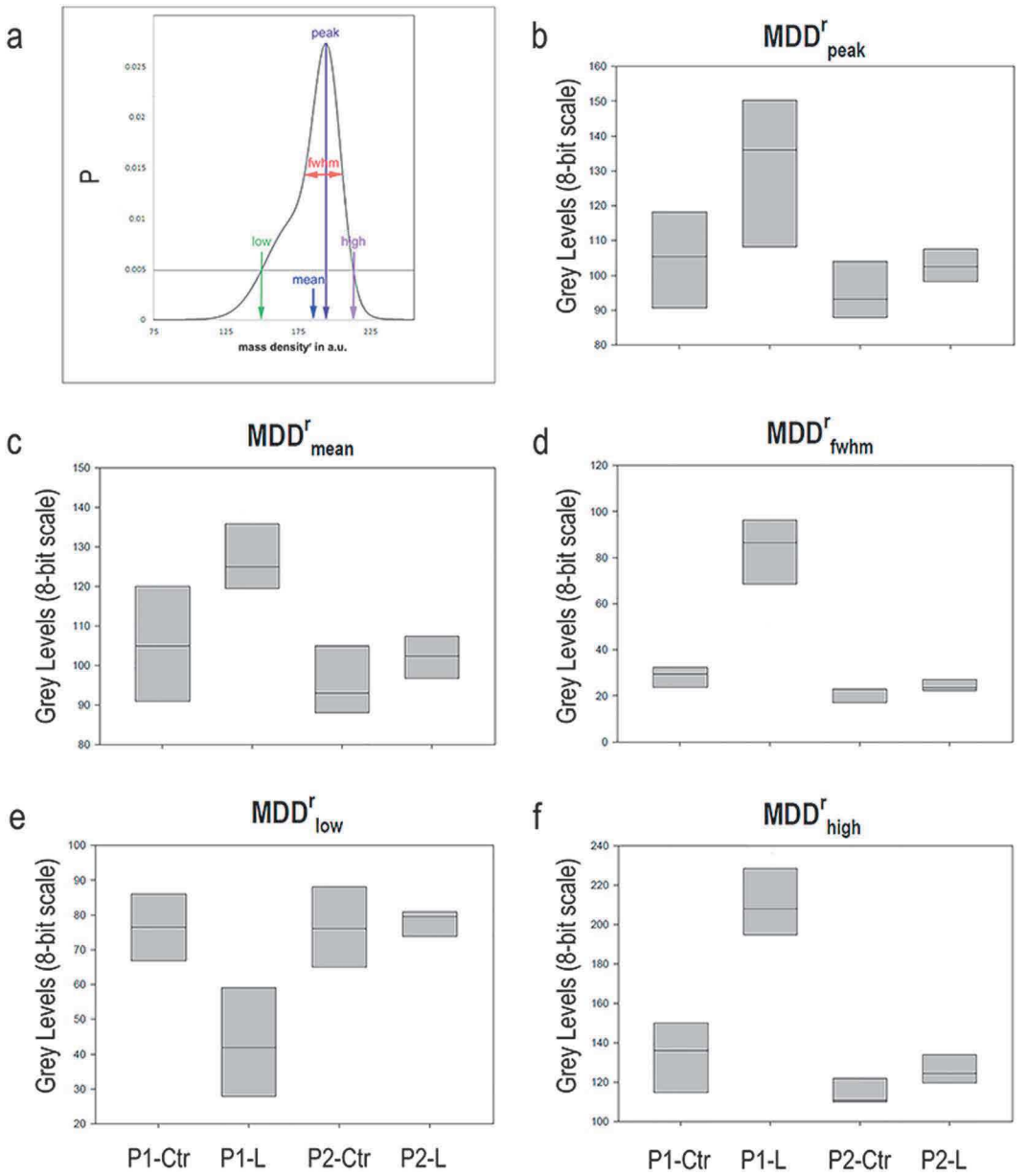


Figure 3. 2nd level analysis: study of the Relative Mass Density Distribution (MDD^r). (**a**) The parameters derived from the profile fitting are indicated. (**b**–**f**) Box-plots graphically depicting the extracted MDD^r parameters through their quartiles as a function of the selected groups of samples P1-Ctr, P1-L, P2-Ctr and P2-L.

uterine peristalsis through mechanical interference. Indeed, it was reported that patients with intramural fibroids may present abnormal uterine peristalsis during the mid-luteal phase. Since uterine peristalsis is involved in normal reproductive processes, this condition seems to reduce the possibility of obtaining spontaneous pregnancy[63]. Moreover, the study from Yoshino and colleagues highlighted how myomectomy reduced the frequency of abnormal peristalsis in all patients[64]. Collagen specific surface may become a useful parameter to perform further studies, i.e. comparing normal myometrial tissues versus fibroid tissues, as well as to study different type of fibroids (i.e. large vs small or pre-menopausal vs post-menopausal or benign vs malignant).

Although the present study should be considered prevalently as a set-up of a new study methodology, we consider appropriate to point out that the structural properties of fibroids and even of healthy myometrium tissues differ widely within the same tissue and among the different patients.

We and others have recently observed that the amount of collagen inside tissues within and between biopsies is heterogeneous mainly in leiomyoma, but also in myometrial tissue. For example, morphometric analysis of Masson's trichrome stained tissue reported values of collagen in leiomyoma ranging from 4.7% to 31,3% in our previous study[11]; from 37% to 77% in Jayes[65] study and from 3.9% up to 70% in a large study of brazilian cases ($n = 150$) performed by Da Silva[66]. Flake and colleagues categorized uterine leiomyoma in 4 different phases on the basis of percent collagen content ranging from none (phase 1) up to 72% (>50%, phase 4) independently of

the tumor size[67]. The collagen content in myometrium ranged from 0.3% to 23% in our previous samples[11] and from 1.6% up to 20.70% in Da Silva's study[66]. Interestingly, the leiomyoma tissues appeared inhomogeneous also in stiffness, quantified through rheometry by measuring complex shear moduli of the tissues. Fibroid tissues, with stiffness of 8014 ± 798 Pa (mean ± SEM), were stiffer than myometrial samples (3630 ± 276 Pa)[65]. With the same indication, evidence of biomechanical and biochemical heterogeneity in uterine fibroids has just been reported. The authors observed heterogeneity in structure, collagen content, and stiffness highlighting that fibroid regions differ in biochemical status and they suggest that these differences might be associated with variations in local pressure, biomechanical signaling, and altered growth[68].

All this opens new prospective to understand uterine fibroids and to explain how it is possible that, despite of the same hormonal milieu, different fibroids of the same subject have a different fate and degree of growth. These differences in fibroids structure could also explain the heterogeneous effect of fibroids of the same dimensions, number, and localization on fertility, on response to medical therapy, and on pregnancy outcomes.

Therefore, the present and all these recent studies, provide novel evidences that structural properties of uterine leiomyoma and, even if less pronounced, also of the myometrium are widely heterogeneous. The variability within and among tissues should be considered in order to characterize the samples, to design and conduct studies to understand the pathobiology as well as to test potential treatments.

Moreover, it has to be stressed that physical, topological, and biochemical composition of the ECM is not only tissue-specific, but it is also markedly heterogeneous: ECM is a highly dynamic structure that is constantly being remodeled, as it happens in bone sites. For instance, collagen density distributions in human knee ligaments were documented to quantify differences in density within and between these ligaments[69]. Very recently, heterogeneity in structure, collagen content, and stiffness in uterine fibroids was reported[68]. Thus, it is interesting to evaluate if this heterogeneity is expressed also in terms of density distributions, as it was shown in human knee ligaments.

In our study, we performed the analysis of fibroid mass density distribution by the Roschger's approach. We found that the full width at half maximum and the 99.5th percentile values were significantly higher in the leiomyoma in comparison to the healthy adjacent controls for both the patients. This means that, in pathological biopsies, not only the amount of collagen is greater (as found out by the morphometric analysis) but also its density distribution is wider than in controls, with presence of highly fibrotic and high-density areas.

Another remark that deserves to be made concerns the density variability of healthy myometrial tissue.

Indeed, using the Roschger approach we showed that, comparing healthy myometrial tissues, the full width at half maximum and the 99.5th percentile values of patient 1 were higher than the respective ones obtained in patient 2, confirming that some tissue portions clinically classified as healthy, showed not only differences in ECM volume but also in density distribution.

On the other hand, it should not be surprising to realize that myometrium of fibromatous uterus is not a homogeneous tissue; it presents structural and functional alterations and consequently it cannot be considered completed healthy. For example, just consider that in the myometrium surrounding the leiomyoma there is accumulation of inflammatory cells[11]. Furthermore, irregular thickening of the endomyometrial junctional zone due to inordinate proliferation of the inner myometrium, junctional zone hyperplasia, is a common MR finding in women suffering from menstrual dysfunction[70,71].

In this context, a textural analysis would have been additionally informative; however, its application in this demonstrative study would have been challenging and perhaps not completely reliable. Indeed, in order to have a reliable textural analysis, the pixel size should be decreased to at least 500–600 nm, in order to fully resolve the texture also in the healthy myometrium (in Fig. 1e the spotty-signal reveals only the larger nodes of the collagen structure).

In conclusion, the propagation-based phase-contrast microCT was shown to be a powerful method in studying uterine leiomyomas, detecting also small signs of the ECM alteration.

In general, the PhC-microCT was shown to provide 3D images of intact tissues as well as a wide range of numerical indices that can be calculated and used to identify mismatches between different tissues. This is of fundamental importance with a view to follow up these studies in various directions: first of all, to visualize the 3D structure of the ECM in myometrium and pathologic tissues (different forms of leiomyoma, as well as leiomyosarcoma); secondly, to test *ex-vivo* in toto tissue the efficacy of innovative therapeutic treatments for leiomyomas; more generally, to offer new methodological possibilities for future studies on ECM in soft tissues of different body districts.

Methods

Sample collection and Permissions. Samples of myometrial tissue and leiomyoma were excised from two fertile women submitted to hysterectomy. The patients were Caucasians (age: 49 years and 43) and the position of the leiomyomas was intramural: the former with a single mass of 10 cm, the latter with two masses of 4 cm and 2 cm. Both patients displayed good general condition; none of them had a history of myomectomy or uterine surgery, had received medical therapy or oral contraceptives in the previous three months, or had evidence of genital tract infection, endometriosis, or ovarian disease. Patients gave their informed consent and the permission of the Human Investigation Committee was granted (Ethics Committee of Marche Region, Prot. N 2015 0486OR).

All experiments were performed in accordance with relevant guidelines and regulations.

The samples, immediately after hysterectomy, were collected in Hanks' Balanced Salt Solution (HBSS) (Euroclone, Milan, Italy) and transferred to the laboratory for washing them with Dulbecco's PBS (Invitrogen, Life Technologies, Carlsbad, CA, USA) in order to remove excess of blood and cutting them into small pieces.

In the former patient (P1), four pieces were taken from the fibrotic tissue (P1-L) and other four from the healthy myometrium (P1-Ctr); in the latter patient (P2), four pieces were taken from the leiomyoma (P2-L) and other three from the healthy myometrium (P2-Ctr).

Synchrotron Radiation microCT examination. The SR-microCT experiments were performed at the SYRMEP beamline of the ELETTRA Synchrotron Facility (Basovizza, TS, Italy).

SR-microCT in its simplest form is based on the inversion of the Radon transform of the experimental projections. After the so-called flat fielding of the projection, the filtered back projection (FBP) algorithm is applied to the corrected data in order to reconstruct the stack of 2D slices[72]. The conventional x-ray imaging approach is based on the discrimination of the different attenuation properties of the elements composing the imaged object, that are related to β, the complex part of the index of refraction $n = 1 - \delta + i\beta$. In this case, image contrast is generated by differences in x-ray absorption. However, because of the coherence of SR, also the electron density, related to the phase shift term δ, might be exploited leading to phase-contrast imaging[33]. With this approach, applicable only if X-ray wavefield has high coherence characteristics, the setup is sensitive to detect not only the absorption differences but also the phase shifts occurring to x-rays crossing the sample. This approach becomes fundamental when the discrimination between two materials having similar electron densities or negligible X-ray absorption is required. Indeed, the reconstruction of δ distribution improves the image contrast, thus simplifies the image segmentation and the subsequent quantitative analysis.

We used this last method to investigate the 3D morphology of the fibrotic tissue and the mass density distribution in the biopsies retrieved from patients affected by leiomyoma.

The scans were performed using the pink SR beam provided by the machine, filtered by 0.5 mm Silicon and corresponding to an average energy of 19 keV; we used 0.2 s of exposure time per projection over a total range of 180°; the sample-detector distance was set to 100 mm, resulting in 1 μm^3 isotropic voxel size in the reconstructed 3D images. The tomographic reconstruction was performed using the SYRMEP Tomo Project (STP) open source software[72]. Including the intensity of the recorded radiographs a phase contrast signal, a phase retrieval algorithm, exploiting the Paganin's method[51], was applied to the (flat-corrected) projection data in order to reconstruct the decrement δ of the refractive index n. In the Paganin's method, the phase is retrieved by simply assuming a linear relationship between the absorption index β and the refractive index decrement δ. The approximation is valid for homogenous samples and propagation distances in the near field regime[51], conditions fulfilled for the considered samples and the adopted scans geometries. The δ/β ratio was set to 100.

Afterwards, the commercial software VG Studio MAX 1.2 (Volume Graphics, Heidelberg, Germany) was used to generate 3D images, where grey levels were proportional to the phase distribution. Optimal image quality was achieved by setting the Scatter HQ algorithm with an oversampling factor of 5.0.

Image analysis. The presence of different phases within the biopsies translated into different peaks in the gray-level scale. Thus, the volume of each phase was obtained by multiplying the volume of a voxel by the number of voxels underlying the peak associated with the relevant phase. The Mixture Modeling algorithm, implemented as plugin in the NIH ImageJ software (https://imagej.net/Mixture_Modeling_Thresholding and https://imagej.nih.gov/ij/plugins/mixture-modeling.html)[73], was chosen to threshold the portion of the histograms related to the two adjacent main tissues, namely the smooth muscle and the fibrotic collagen-based phase. The Mixture Modeling algorithm is a histogram-based technique that assumes that the histogram distribution is represented by two Gaussian curves. It calculates the image threshold as the intersection of these two Gaussians, finding a threshold that is, in several cases, very close to real world data. In our histogram data, the Gaussian curve on the left was representative of the smooth muscle, the second curve on the right of the collagen-phase. Indeed, in this range of densities should be included also the endothelial signal but the thickness of vessels, if excluding the pregnancy period, is negligible, not affecting the segmentation process. Therefore, we analyzed in each pathological biopsy, with the aforementioned algorithm, a dozen slices, uniformly selected in the axial direction: the selected threshold value was the average of all those obtained. Notably, for the selection of this threshold value, control biopsies were not examined because they were considered unreliable due to their low collagen content. An example of histogram segmented by the Mixture Modeling algorithm has been reported for a representative sample of P1-L in Fig. 4 of the Supplementary Material section.

For each biopsy, several subvolumes were analyzed: each of them was a 3D portion fully included in the sample bulk and the complete set of them allowed to achieve the complete sample mapping.

The quantitative analysis was performed in two steps.

In the first step, the morphometric evaluation of the fibrotic collagen-based phase was made using the structural indices usually measured for bone samples[74]: the collagen specific volume (CollV/TV, expressed as a percentage), its specific surface (CollS/CollV, per millimeter), the mean collagen bundles thickness (Th, expressed in micrometers), the mean collagen bundles number (Nr, per millimeter) and the mean collagen bundles spacing (Sp, expressed in micrometers). Furthermore, as collagen bundles could vary their orientation depending on the pathology, we also extracted information about the anisotropy of the collagen structure, i.e. the presence of preferential orientation(s). The anisotropy degree index (DA) measures the similarity of a fabric to a uniform distribution and varies between 0, representing all observations confined to a single plane or axis, and 1, corresponding to the perfect isotropy. The DA analysis was performed using the BoneJ Plugin[75] of the ImageJ software[73], version 3. The morphometric analysis was also extended to the inclusion of a descriptor for the interconnectivity of the collagen bundles: its connectivity density (Conn.D – $pixel^{-3}$) does not carry information about positions or size of connections, but it is a simple global measure of connectivity that gives higher values for better-connected structures, i.e. more entangled fibrotic tissues, and lower values for poorly connected ones, i.e. well-oriented bundles.

In the second step, the reconstructed complex refractive index distribution was exploited[54]; indeed, it is linearly related to the mass density and was assessed computing the relative mass density distribution (MDD^r) of each sample, as shown in Fig. 3a. In each slice-image, the individual grey level voxels reflect the average density found in the corresponding volume elements of the investigated sample region: consequently, the acquisition of grey

level voxel images provided the information on the local variation of density throughout the tissue. This variation in density is best described and quantified by a frequency distribution (histogram), as shown in the Fig. 3a. In order to compare different mass density distributions between biopsies, a reduction of the huge amount of histogram data to only few characteristic parameters is necessary. Briefly, the MDD^r was calculated within the overall tissue domain and was normalized by the area under the curve.

The absolute values of mass density could not be retrieved because the reconstructed complex refractive index might be biased by the chosen δ/β ratio used in the Paganin phase retrieval processing[56]. Thus, being the different samples comparable in terms of size and composition, the superscript r was used to denote relative values for all mass density distribution parameters. The Roschger approach[53] was used, for the first time to the authors' knowledge, to study tissues different from bone.

Five parameters were extracted from the MDD^r profile of each sample: MDD^r_{peak}, the peak position of the histogram, which indicates the most frequently measured density (density with the highest number of pixels); MDD^r_{mean}, the mean relative mass density obtained from the integrated area under the curve; MDD^r_{fwhm}, the full width at half maxima of the distribution, describing the variation in density; MDD^r_{low}, the percentage of the area that has a density below the 5th percentile of the reference range; and the MDD^r_{high}, the percentage of the area that has a density above the 95^{th} percentile of the reference range. The threshold of $P = 0.005$ was arbitrarily chosen because it was considered a good compromise between maintaining good sensitivity for low and high values in the MDD^r and minimizing potential artifacts due to partial volume effect in the evaluation of MDD^r_{low}. This post-processing calculation of the MDD^r parameters was performed using the PeakFit software (Systat Software, San Jose, CA).

References

1. Ciarmela, P. *et al*. Growth factors and myometrium: biological effects in uterine fibroid and possible clinical implications. *Human Reproduction Update* **17**(6), 772–790 (2011).
2. Day Baird, D. *et al*. High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence. *American Journal of Obstetrics and Gynecology* **188**(1), 100–107 (2003).
3. Ciavattini, A. *et al*. Number and size of uterine fibroids and obstetric outcomes. *J Matern Fetal Neonatal Med* **28**(4), 484–8 (2015).
4. Ciavattini, A. *et al*. Growth trend of small uterine fibroids and human chorionic gonadotropin serum levels in early pregnancy: an observational study. *Fertil Steril* **105**(5), 1255–1260 (2016).
5. Cardozo, E. R. *et al*. The estimated annual cost of uterine leiomyomata in the United States. *American journal of obstetrics and gynecology* **206**(3), 211. e1–211. e9 (2012).
6. Downes, E. *et al*. The burden of uterine fibroids in five European countries. *European Journal of Obstetrics, Gynecology, and Reproductive Biology* **152**(1), 96–102 (2010).
7. Ciarmela, P. *et al*. Management of leiomyomas in perimenopausal women. *Maturitas* **78**(3), 168–73. (2014).
8. Islam, M. S. *et al*. Uterine leiomyoma: available medical treatments and new possible therapeutic options. *Journal of Clinical Endocrinology & Metabolism* **98**(3), 921–934 (2013).
9. Islam, M. S. *et al*. Complex networks of multiple factors in the pathogenesis of uterine leiomyoma. *Fertility and Sterility* **100**(1), 178–193 (2013).
10. Leppert, P., M. Fouany & J. H. Segars Understanding uterine fibroids, in Fibroids, J.H. Segars, Editor. 2013, John Wiley & Sons, Ltd: Oxford. p. 1–10.
11. Protic, O. *et al*. Possible involvement of inflammatory/reparative processes in the development of uterine fibroids. *Cell & Tissue Research* **364**, 415–427 (2016).
12. Protic, O. *et al*. Activin A in Inflammation, Tissue Repair, and Fibrosisossible Role as Inflammatory and Fibrotic Mediator of Uterine Fibroid Development and Growth. *Semin Reprod Med* **35**(6), 499–509 (2017).
13. Leppert, P. C., Jayes, F. L. & Segars, J. H. The extracellular matrix contributes to mechanotransduction in uterine fibroids. *Obstet Gynecol Int* **2014**, 783289 (2014).
14. Thorne, J. T. *et al*. Dynamic reciprocity between cells and their microenvironment in reproduction. *Biol Reprod* **92**(1), 25 (2015).
15. Rafique, S., Segars, J. H. & Leppert, P. C. Mechanical Signaling and Extracellular Matrix in Uterine Fibroids. *Semin Reprod Med* **35**(6), 487–493 (2017).
16. Islam, M. S. *et al*. Extracellular matrix in uterine leiomyoma pathogenesis: a potential target for future therapeutics. *Hum Reprod Update* **24**(1), 59–85 (2018).
17. Islam, M. S. *et al*. Use of dietary phytochemicals to target inflammation, fibrosis, proliferation, and angiogenesis in uterine tissues: Promising options for prevention and treatment of uterine fibroids? *Molecular nutrition & food research* **58**(8), 1667–1684 (2014).
18. Islam, M. S. *et al*. Tranilast, an orally active antiallergic compound, inhibits extracellular matrix production in human uterine leiomyoma and myometrial cells. *Fertility and sterility* **102**(2), 597–606 (2014).
19. Islam, M. S. *et al*. Antiproliferative effect of tranilast on human myometrial and leiomyoma cells. *Biological and Biomedical Reports* **2**(5), 321–327 (2012).
20. Islam, M. S. *et al*. Dietary phytochemicals for possible preventive and therapeutic option of uterine fibroids: Signaling pathways as target. *Pharmacological Reports* **69**(2017), 57–70 (2016).
21. Islam, M. S. *et al*. Molecular targets of dietary phytochemicals for possible prevention and therapy of uterine fi broids: Focus on fi brosis. *Critical Reviews in Food Science and Nutrition* **57**(17), 3583–3600 (2017).
22. Islam, M. S. *et al*. An anthocyanin rich strawberry extract induces apoptosis and ROS while decreases glycolysis and fibrosis in human uterine leiomyoma cells. *Oncotarget* **8**(14), 23575–23587 (2017).
23. Islam, M. S. *et al*. Omega-3 fatty acids modulate the lipid profile, membrane architecture, and gene expression of leiomyoma cells. *J Cell Physiol* **233**(9), 7143–7156 (2018).
24. Giampieri, F. *et al*. Romina: A powerful strawberry with *in vitro* efficacy against uterine leiomyoma cells. *J Cell Physiol* (2018).
25. Castro, L. *et al*. A High Concentration of Genistein Induces Cell Death in Human Uterine Leiomyoma Cells by Autophagy. *Expert Opin Environ Biol*, **5**(Suppl 1) (2016).
26. Di, X. *et al*. A high concentration of genistein down-regulates activin A, Smad3 and other TGF-β pathway genes in human uterine leiomyoma cells. *Experimental & molecular medicine* **44**(4), 281–292 (2012).
27. Di, X. *et al*. A low concentration of genistein induces estrogen receptor-alpha and insulin-like growth factor-I receptor interactions and proliferation in uterine leiomyoma cells. *Hum Reprod* **23**(8), 1873–83 (2008).
28. Moore, A. B. *et al*. Stimulatory and inhibitory effects of genistein on human uterine leiomyoma cell proliferation are influenced by the concentration. *Hum Reprod* **22**(10), 2623–2631 (2007).
29. Ciavattini, A. *et al*. Hypovitaminosis D and "small burden" uterine fibroids: Opportunity for a vitamin D supplementation. *Medicine (Baltimore)* **95**(52), e5698 (2016).

30. Hossain, M. Z. *et al*. A Comparative Study of Magnetic Resonance Imaging and Transabdominal Ultrasonography for the Diagnosis and Evaluation of Uterine Fibroids. *Mymensingh Med J* **26**(4), 821–827 (2017).
31. Levens, E. D. *et al*. Magnetic resonance imaging and transvaginal ultrasound for determining fibroid burden: implications for research and clinical care. *Am J Obstet Gynecol* **200**(5), 537 e1–7 (2009).
32. Ascher, S. M. & Silverman, P. M. Applications of computed tomography in gynecologic diseases. *Urol Radiol* **13**(1), 16–28 (1991).
33. Bravin, A., Coan, P. & Suortti, P. X-ray phase-contrast imaging: from pre-clinical applications towards clinics. *Phys Med Biol* **58**(1), R1–35 (2013).
34. Snigirev, A. *et al*. On the possibilities of x-ray phase contrast microimaging by coherent high-energy synchrotron radiation. *Review of Scientific Instruments* **66**(12), 5486–5492 (1995).
35. Lewis, R. A. *et al*. X-ray refraction effects: application to the imaging of biological tissues. *Br J Radiol* **76**(905), 301–8 (2003).
36. Momose, A. *et al*. Phase-contrast X-ray computed tomography for observing biological soft tissues. *Nat Med* **2**(4), 473–5 (1996).
37. Croton, L. C. P. *et al*. *In situ* phase contrast X-ray brain CT. *Sci Rep* **8**(1), 11412 (2018).
38. Pinzer, B. R. *et al*. Imaging brain amyloid deposition using grating-based differential phase contrast tomography. *Neuroimage* **61**(4), 1336–46 (2012).
39. Giuliani, A. *et al*. Synchrotron Phase Tomography: An Emerging Imaging Method for Microvessel Detection in Engineered Bone of Craniofacial Districts. *Front Physiol* **8**, 769 (2017).
40. Cedola, A. *et al*. X-Ray Phase Contrast Tomography Reveals Early Vascular Alterations and Neuronal Loss in a Multiple Sclerosis Model. *Sci Rep* **7**(1), 5890 (2017).
41. Vagberg, W. *et al*. Cellular-resolution 3D virtual histology of human coronary arteries using x-ray phase tomography. *Sci Rep* **8**(1), 11014 (2018).
42. Velroyen, A. *et al*. X-ray phase-contrast tomography of renal ischemia-reperfusion damage. *PLoS One* **9**(10), e109562 (2014).
43. Bukreeva, I. *et al*. Quantitative 3D investigation of Neuronal network in mouse spinal cord model. *Sci Rep* **7**, 41054 (2017).
44. Coan, P. *et al*. Characterization of osteoarthritic and normal human patella cartilage by computed tomography X-ray phase-contrast imaging: a feasibility study. *Invest Radiol* **45**(7), 437–44 (2010).
45. Horng, A. *et al*. Cartilage and soft tissue imaging using X-rays: propagation-based phase-contrast computed tomography of the human knee in comparison with clinical imaging techniques and histology. *Invest Radiol* **49**(9), 627–34. (2014).
46. Grandl, S. *et al*. Visualizing typical features of breast fibroadenomas using phase-contrast CT: an *ex-vivo* study. *PLoS One* **9**(5), e97101 (2014).
47. Pacile, S. *et al*. Clinical application of low-dose phase contrast breast CT: methods for the optimization of the reconstruction workflow. *Biomed Opt Express* **6**(8), 3099–112 (2015).
48. Albertini, G. *et al*. Organization of extracellular matrix fibers within polyglycolic acid-polylactic acid scaffolds analyzed using X-ray synchrotron-radiation phase-contrast micro computed tomography. *Tissue Eng Part C Methods* **15**(3), 403–11 (2009).
49. Noda, H. Partial specific volume of collagen. *J Biochem* **71**(4), 699–703 (1972).
50. Ward, S. R. & Lieber, R. L. Density and hydration of fresh and fixed human skeletal muscle. *J Biomech* **38**(11), 2317–20 (2005).
51. Paganin, D. *et al*. Simultaneous phase and amplitude extraction from a single defocused image of a homogeneous object. *J Microsc* **206**(Pt 1), 33–40 (2002).
52. Schindelin, J. *et al*. Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**(7), 676–82 (2012).
53. Roschger, P. *et al*. Bone mineralization density distribution in health and disease. *Bone* **42**(3), 456–66 (2008).
54. Hesse, B. *et al*. Alterations of mass density and 3D osteocyte lacunar properties in bisphosphonate-related osteonecrotic human jaw bone, a synchrotron microCT study. *PLoS One* **9**(2), e88481 (2014).
55. Giuliani, A. *et al*. Bisphosphonate-related osteonecrosis of the human jaw: A combined 3D assessment of bone descriptors by histology and synchrotron radiation-based microtomography. *Oral Oncol* **82**, 200–202 (2018).
56. Weitkamp, T. *et al*. ANKAphase: software for single-distance phase retrieval from inline X-ray phase-contrast radiographs. *J Synchrotron Radiat* **18**(Pt 4), 617–29. (2011).
57. Walker, C. L. & Stewart, E. A. Uterine fibroids: the elephant in the room. *Science* **308**(5728), 1589–1592 (2005).
58. Leppert, P. C., Catherino, W. H. & Segars, J. H. A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays. *Am J Obstet Gynecol* **195**(2), 415–420 (2006).
59. Stewart, E. A. *et al*. Relative overexpression of collagen type I and collagen type III messenger ribonucleic acids by uterine leiomyomas during the proliferative phase of the menstrual cycle. *Journal of Clinical Endocrinology & Metabolism* **79**(3), 900–906 (1994).
60. Norian, J. M. *et al*. Transforming growth factor beta3 regulates the versican variants in the extracellular matrix-rich uterine leiomyomas. *Reproductive Sciences* **16**(12), 1153–1164 (2009).
61. Islam, M. S. *et al*. Role of activin-A and myostatin and their signaling pathway in human myometrial and leiomyoma cell function. *Journal of Clinical Endocrinology & Metabolism* **99**(5), E775–E785 (2014).
62. Leppert, P. C. *et al*. Comparative ultrastructure of collagen fibrils in uterine leiomyomas and normal myometrium. *Fertility and Sterility* **82**(Suppl 3), 1182–1187 (2004).
63. Yoshino, O. *et al*. Decreased pregnancy rate is linked to abnormal uterine peristalsis caused by intramural fibroids. *Hum Reprod* **25**(10), 2475–9 (2010).
64. Yoshino, O. *et al*. Myomectomy decreases abnormal uterine peristalsis and increases pregnancy rate. *J Minim Invasive Gynecol* **19**(1), 63–7 (2012).
65. Jayes, F. L. *et al*. Loss of stiffness in collagen-rich uterine fibroids after digestion with purified collagenase Clostridium histolyticum. *Am J Obstet Gynecol* **215**(5), 596 e1–596 e8 (2016).
66. da Silva, A. P. *et al*. Histopathological and Digital Morphometrical Evaluation of Uterine Leiomyoma in Brazilian Women. *Obstet Gynecol Int* **2016**, 2968410 (2016).
67. Flake, G. P. *et al*. The natural history of uterine leiomyomas: light and electron microscopic studies of fibroid phases, interstitial ischemia, inanosis, and reclamation. *Obstet Gynecol Int* **2013**, 528376 (2013).
68. Jayes, F. L. *et al*. Evidence of biomechanical and collagen heterogeneity in uterine fibroids. *PLoS One* **14**(4), e0215646 (2019).
69. Mommersteeg, T. J. *et al*. Nonuniform distribution of collagen density in human knee ligaments. *J Orthop Res* **12**(2), 238–45 (1994).
70. Brosens, J. J., Barker, F. G. & de Souza, N. M. Myometrial zonal differentiation and uterine junctional zone hyperplasia in the non-pregnant uterus. *Hum Reprod Update* **4**(5), 496–502 (1998).
71. Ciavattini, A. *et al*. Uterine Fibroids: Pathogenesis and Interactions with Endometrium and Endomyometrial Junction. *Obstetrics and Gynecology International* **2013**, 11 (2013).
72. Brun, F. *et al*. SYRMEP Tomo Project: a graphical user interface for customizing CT reconstruction workflows. *Adv Struct Chem Imaging* **3**(1), 4 (2017).
73. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**(7), 671–5 (2012).
74. Parfitt, A. M. *et al*. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* **2**(6), 595–610 (1987).
75. Doube, M. *et al*. BoneJ: Free and extensible bone image analysis in ImageJ. *Bone* **47**(6), 1076–9 (2010).

Acknowledgements
The authors acknowledge the ELETTRA Synchrotron Facility User Office for kindly providing beam time for the propagation-based phase-contrast microCT investigations.

Author Contributions
A. Giuliani and P. Ciarmela designed research; G. Delli Carpini, S.R. Giannubilo and A. Ciavattini selected biopsies and data on patients; S. Pacilè and G. Tromba contributed with synchrotron-based microCT experiments and related analytic tools; A. Giuliani, S. Greco, S. Pacilè, A. Zannotti and P. Ciarmela performed research and data analysis; A. Giuliani and P. Ciarmela wrote the paper; while S. Greco, S. Pacilè, A. Zannotti, G. Delli Carpini, G. Tromba, S.R. Giannubilo and A. Ciavattini revised it critically for important intellectual content; ALL gave the final approval of the version to be published.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-47048-0.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019

RBMO

ELSEVIER

REVIEW

Uterine fibroid vascularization: from morphological evidence to clinical implications

BIOGRAPHY

Dr Ciarmela is Associate Professor of Human Anatomy at Università Politecnica delle Marche, Italy. Her research focus is uterine physiology, pathophysiology and the role of growth factors. The aim of her research is to understand the pathogenesis of uterine fibroids and to develop potential therapeutic agents.

Pasquapina Ciarmela[1,*], Giovanni Delli Carpini[2], Stefania Greco[1], Alessandro Zannotti[1,2], Nina Montik[2], Luca Giannella[2], Lucia Giuliani[2], Camilla Grelloni[2], Francesca Panfoli[2], Michela Paolucci[2], Gloria Pierucci[2], Federica Ragno[2], Pamela Pellegrino[1], Felice Petraglia[3], Andrea Ciavattini[2]

KEY MESSAGE

Microscopic and macroscopic vascular aspects of uterine fibroids need further investigation to understand the pathogenesis of the disease and to develop new treatments. Vascularization is important for understanding clinical manifestations, for predicting the effectiveness of non-surgical treatments and to enable differential diagnosis to be made with benign conditions or malignancy.

ABSTRACT

Uterine fibroids are the most common cause of solid pelvic tumours, occurring in 20–30% of fertile women and presenting clinical complications that seriously affect women's health. They commonly cause severe symptoms, such as heavy, prolonged menstrual bleeding and anaemia. The study of microscopic and macroscopic vascular aspects of uterine fibroids is important for understanding the clinical manifestations of uterine fibroids, for predicting the effectiveness of alternative treatments to surgery, i.e. uterine artery embolization, for improving surgery outcomes and for carrying out a differential diagnosis with other benign conditions, e.g. adenomyosis, or malignancy, e.g. leiomyosarcoma, and to develop new therapeutic approaches. In this review, current knowledge of how the vascular network and angiogenesis are implied in the formation of uterine fibroids and in the pathogenesis of related symptoms is explored, and evidence on the role of ultrasound in evaluating fibroid vascularization is summarized. This review combines anatomical, morphological and biomolecular information related to angiogenic mechanisms with diagnostic and clinical information, highlighting the various interconnections. Uterine and fibroid vascularization need further investigation to gain a deeper understanding of the pathogenetic elements that lead to the formation of uterine fibroids and their clinical manifestations.

[1] Department of Experimental and Clinical Medicine, Università Politecnica delle Marche, Ancona 60126, Italy
[2] Gynecologic Section, Department od Odontostomatologic and Specialized Clinical Sciences, Università Politecnica delle Marche, Ancona 60126, Italy
[3] Department of Biomedical, Experimental and Clinical Sciences, Division of Obstetrics and Gynecology, University of Florence, Careggi University Hospital Florence, Italy

© 2021 Published by Elsevier Ltd on behalf of Reproductive Healthcare Ltd.
*Corresponding author. E-mail address: p.ciarmela@univpm.it (P. Ciarmela). https://doi.org/10.1016/j.rbmo.2021.09.005
1472-6483/© 2021 Published by Elsevier Ltd on behalf of Reproductive Healthcare Ltd.
Declaration: The authors report no financial or commercial conflicts of interest.

KEYWORDS

Abnormal uterine bleeding
Doppler ultrasound
Leiomyoma
Vascularization

INTRODUCTION

Uterine fibroids are the most frequent benign uterine tumours, with an age-specific cumulative incidence of about 70% among white women and over 80% among black women (*Baird et al., 2003*). Even if asymptomatic in most cases, they can be associated with symptoms that can significantly affect quality of life, such as abnormal uterine bleeding (AUB), heavy uterine bleeding, pelvic pain, pressure symptoms or infertility (*Ciavattini et al., 2017*; *Rakotomahenina et al., 2017*; *Critchley et al., 2020*). In particular, AUB and associated iron deficiency anaemia may have the most significant effect on the physical, social and emotional health of affected women (*Critchley et al., 2020*). In the USA, the annual direct costs of uterine fibroids and AUB are estimated to range from $4.1 to $9.4 billion (*Cardozo et al., 2012*).

Different mechanisms have been proposed to explain the association between uterine fibroids and AUB. These may interfere with myometrial contractions (especially of the inner junctional zone), cause endometrial ulcerations, compression of the venous plexus, determine abnormal paracrine signalling or alter the haemostatic regulation of the endometrium. In addition, uterine fibroids and the surrounding myometrium often present rich vascularity, which could be implicated in the pathogenesis of AUB (Ikhena *et al*., 2018).

MACROSCOPIC UTERINE AND FIBROID VASCULARIZATION

The main blood supply of the uterus is provided by the uterine artery, which arises from the first or second branch of the anterior division of the internal iliac artery (hypogastric artery), and it is usually dilated in the presence of uterine fibroids. During its initial course, the uterine artery is found lateral and superior to the ureter for about 2.5 cm and then crosses the ureter anteriorly to its medial side to reach the cervix of the uterus. The relationship between the uterine artery is remembered by the phrase 'water (ureter) under the bridge (uterine artery)' (FIGURE 1). The uterine artery presents three segments: a descending segment, which runs parallel to the lateral pelvic wall; a transverse segment, which crosses the ureter at the level of the cervix; and an ascending segment along the margin of the uterus at the medial edge of the broad ligament, up to the uterine cornua where it joins the ovarian artery via the ovarian branch and the tubal branch (*Pelage et al., 2005*; *Bhatt et al., 2006*) (FIGURE 2). Branches of the uterine artery are the cervicovaginal artery and arcuate or intramural arteries that penetrate the outer third of the myometrium and generate a 'spoke wheel', with anastomosis between left and right uterine arteries (*Pelage et al., 2005*; *Bhatt et al., 2006*; *Secil et al., 2008*). Radial branches run through the myometrium generating the basal and spiral arterioles within the endometrium. The terminal branches of the uterine artery are the fundus uteri arteries and the artery for the round ligament (*Pelage et al., 2005*; *Bhatt et al., 2006*).

The arterial pattern presented in FIGURE 2 is the most common (around 90% of woman), although alternative blood flow supplies can be present. In about 50% of cases, the ovaries receive the entire blood supply through the ovarian artery. In fact, in about 56% of cases, the ovaries receive the blood supply from the ovarian and uterine artery, 40% of cases come from the ovarian artery and 4% of cases from the uterine artery (*Standring, 2008*) (FIGURE 3).

In most cases, the main peripheral blood supply of uterine fibroid origin from the uterine artery is often distorted and enlarged. Arteries supplying the fibroid periphery are usually larger than those supplying the normal myometrium and generate a rich perifibroid plexus (*Secil et al., 2008*). Fibroid parenchyma is hypovascular, with small centripetal arteries supplying the internal portion. Pedunculated fibroids present a feeding artery through the stalk. Additional blood supply may arise in 5–10% of cases from the ovarian arteries, more frequently in women with large subserosal fibroids, in women who have had previous pelvic surgery or in those with ovarian or tubal pathologies (*Secil et al., 2008*).

Greyscale ultrasound characteristics of uterine fibroids

Fibroids are usually homogeneously hypoechoic nodular areas, with sharp, well-defined margins at the greyscale ultrasound examination (*Oh et al., 2019*). The ecostructure is influenced by the different relationship between fibrous and muscular components, by possible degenerative phenomena, e.g. hyaline, cystic, myxomatous, lipomatous and calcific degeneration, and by infectious or ischaemic events. In larger fibroids, the evolution towards degenerative-necrotic phenomena is more frequent owing to inadequate vascular supply. In these cases, irregular anechoic areas are found inside the fibroid. A massive colliquation can result in a completely cystic ultrasound image and create diagnostic-differential difficulties with an adnexal mass. The thickening of the connective matrix surrounding the fibroid constitutes the so-called 'pseudocapsule', and its identification is crucial for ultrasound diagnosis of fibroids. The pseudocapsule is represented by a hypoechoic zone interposed between the fibroid and the surrounding myometrium, sometimes presenting characteristics of hyperechogenicity caused by calcification phenomena of the myometrium (*Tinelli et al., 2014*).

INTRATISSUTAL VASCULARIZATION IN THE MYOMETRIUM AND FIBROIDS

The microscopic anatomy of the vasculature of fibroids and the myometrium in patients with uterine fibromatosis has been studied with different techniques, ranging from Doppler ultrasound, for clinical assessment, to immunohistochemistry and electron microscope for the morphological study of tissues. The attention to this aspect derives from the awareness that, as in all expansive lesions, an increase in cellularity is generated by an increase in vascularization through neo-angiogenic stimulation and that, when this does not occur, hypoxic conditions and tissue necrosis are generated (*Konarska et al., 2016*). The vascularization of fibroids derives from branches of the uterine arteries that can undergo alterations caused by the increased demand for blood flow. A diffuse uterine hypervascularization is visible with multiple fibroids. The reported increased size and tortuosity of these vessels, is due to the fact that, in addition to normal myometrium, they must also compensate for the presence of fibroids neoformations (*Pelage et al., 2005*). The capillaries present in fibroids, on the contrary, are of normal appearance, different from those typical in malignant tumours, which are irregular and tortuous because of an important

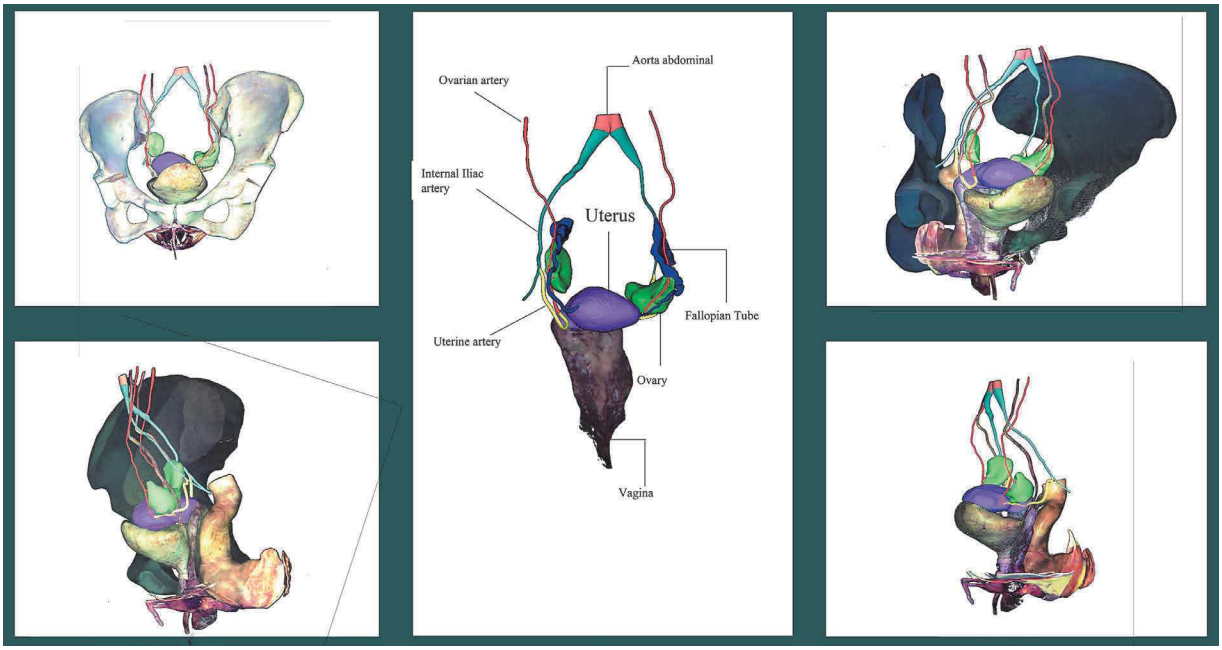


FIGURE 1 The uterus and its vascularization. The virtual dissection table 3D anatomy platform (Anatomage) was used to process the images and was supplied by the Faculty of Medicine and Surgery, Università Politecnica della Marche, Ancona, Italy. The images show the abdominal aorta (pink), the ovarian artery (red) and the uterine artery (yellow), which is the first or second branch of the anterior division of the internal iliac artery (green).

and rapid proangiogenic stimulus (*Walocha et al., 2003*).

Extensive studies show that fibroids have a mainly peripheral vascularization surrounding the tumour, the so-called 'perifibromal vascular capsule', whereas the tumour itself is relatively hypovascularized. Small centripetal vessels originate from the perifibromal plexus that spread into the fibroid (*Pelage et al., 2005*). The presence of this 'capsule' or 'vascular pseudocapsule' is demonstrated by Doppler ultrasounds and by the detection of increased activity of various vascular growth factors in this site (*Bereza et al., 2013*). The vascular system of intramural fibroid was described by *Walocha et al. (2003)* who analysed the vascularization of fibroid tissues by scanning electron microscopy by the observation of vascular corrosion casting of injected resin fills of all blood vessels, including cappilaries of uterine fibromatosis (FIGURE 4A). The observation showed the existence of two different types of vascular arrangement: one in the form of a dense peripheral 'vascular capsule' and a hypovascularized core, and the other consisting of hypovascularization with lateral branches that separate 'foci of intensive tumour regression'. In these areas, multiple tumours are characterized by the presence of intratumoral septa of connective and vascular tissue, which separate the foci with increased proliferation. The vascular patterns observed in Walocha's study of fibroids of various sizes allow us to propose a development pathway of the vascular system in these tumours. In fact, small fibroids (1–3 mm) are almost avascular, being surrounded by a relatively dense myometrial vascular network composed mainly of capillaries, and containing a few larger vessels, both arteries and veins (*Walocha et al., 2003*). Fibroids with larger dimensions (up to 1 cm), however,

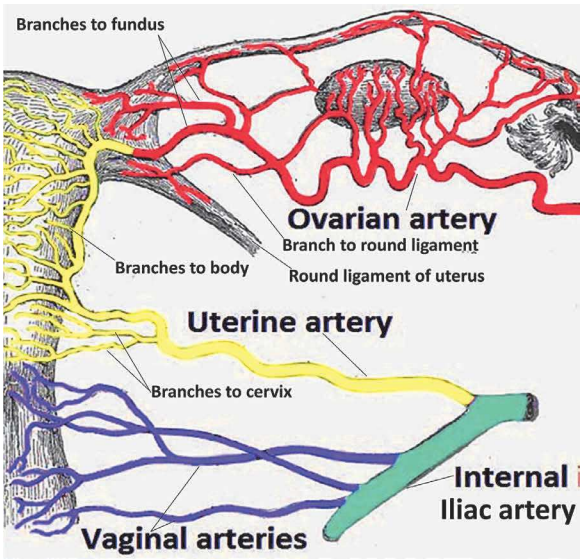


FIGURE 2 The origin of the uterine artery, branches and related anastomoses. In this image, the uterine artery and its branches (yellow) show a transverse segment that crosses the ureter at the level of the cervix, a descending segment that runs parallel to the lateral pelvic wall, an ascending segment along the edge of the uterus at the medial border of the broad ligament, where it joins the ovarian artery indicated in red, and the terminal branches of the fundus artery and the artery of the round ligament of the uterus. The vaginal artery is represented by two or three branches that anastomose with the branches of the uterine artery (purple).

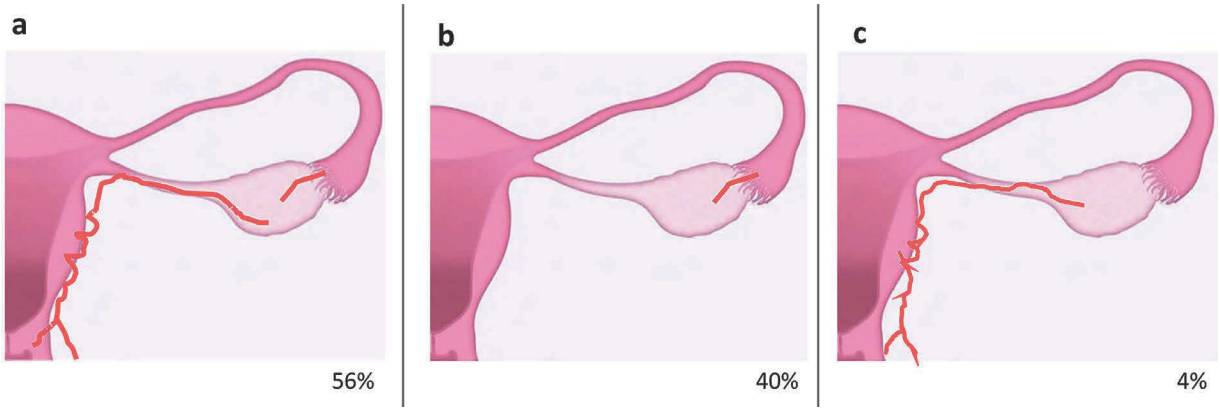


FIGURE 3 Arterial vascularization of the female genital organ. (A–C) Variants of ovarian vascularization.

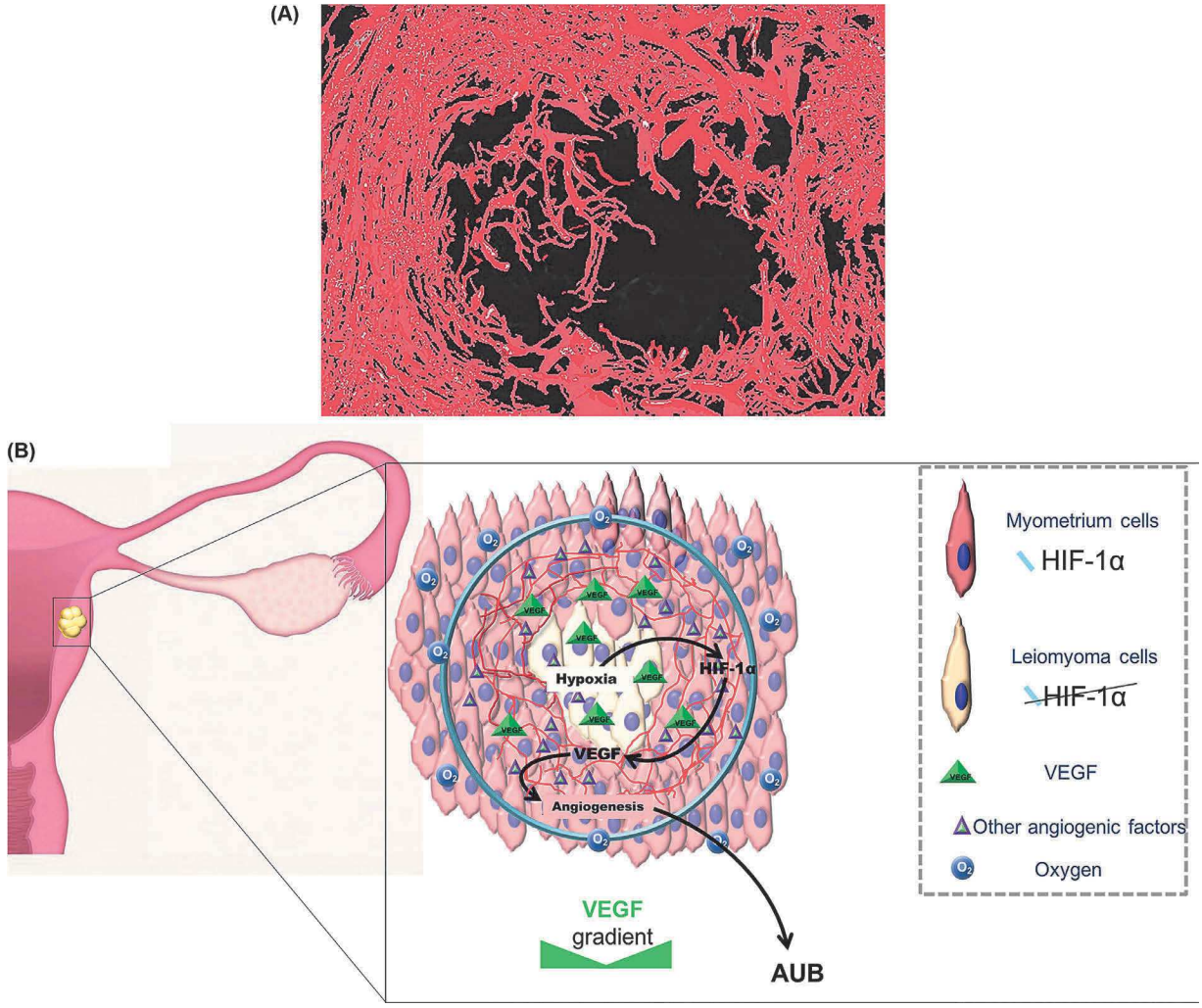


FIGURE 4 (A) The square shows the vascular architecture of a fibroid characterized by a vascularized area surrounding the avascular 'core'. This image is representative and reproduces the scanning electron microscopy observation of the vascular corrosion casting (adapted from *Walocha et al., 2003*); (B) A possible explanation of the vascularization structure of fibroid. This tissue shows an avascular core inside a vascularized capsule (red lines). Even if fibroids are hypoxic, they do not express hypoxia inducible factor 1α (HIF-1α). On the other hand, HIF-1α (light blue lines) is over-expressed by the myometrium cells (dark pink in the figure) next to fibroid cells (light pink area). In addition to this, several angiogenic factors, such as basic fibroblast growth and adrenomedullin (both represented by the small purple and light green triangles in the figure) and also vascular endothelial growth factor (VEGF) (dark green triangles in the figure) are expressed by myometrium cells and also by fibroid cells. In particular, a VEGF concentration gradient establishes within the fibroid. This gradient increases from the inner part to the peripheral zone of the fibroid. The HIF-1α, expressed only by the fibroid cells, could lead the action of these angiogenic factors. All this may explain the vascularized capsule growing around the avascular fibroid. Moreover, this condition can account for the abnormal uterine bleeding phenomenon. The black arrows show the whole process from hypoxia to abnormal uterine bleeding. Oxygen is also represented (light blue).

are characterized by a greater density of surrounding vessels and by few small blood vessels (capillaries, arterioles and small arteries) that invade the lesion. A feature of larger (>1 cm) fibroids is the presence of a 'vascular capsule', a dense vascular network at the border between the tumour and the surrounding myometrium, often separated from the unchanged myometrial tissue by a narrow avascular clust. Capillaries, arterioles and venules, tend to form parallel arrays, and are the predominant components of this network. This vascular capsule arises from small fibromatous foci, which compress the pre-existing blood vessels, inducing the regression of the vessels, leading to the formation of avascular regions within these. Subsequently, the density of the blood vessels increases near adjacent fibroids and, as the fibroid increases in size, new blood vessels penetrate the area in which the fibroid is present, leading to the formation of the 'vascular capsule'. The small avascular areas that are observed in larger fibroids could probably represent foci of necrosis or neo-fibroids (*Walocha et al., 2003*). Furthermore, several studies have shown that the vascularization of these tumours largely depended on the size and position of the fibroid and on secondary degenerative changes (*Kurjak et al., 1992*; *Sosic et al., 1996*). The differences in vascular density between the myometrium and the fibroid can represent an important difference in angiogenesis and vascular remodelling. These differences in angiogenesis may be the result of changes in the balance between factors that promote or inhibit angiogenesis. *Fay (1993)* studied the vascular density of fibroids by scanning electron microscopy and by immunohistochemistry of factor VIII, an important factor involved in the coagulation cascade that forms a part of von Willebrand factor complex, and with reduced expression in smaller, less mature blood vessels. The factor VIII seemed to be lower or similar to that of myometrial tissue. Furthermore, both techniques showed and confirmed the presence in all fibroids of a greater density of peripheral vessels and, therefore, the presence of the 'vascular capsule'. Furthermore, it has been observed that some large fibroids do not produce excessive collagen deposition, unlike small fibroids, which, on the contrary, are capable of producing large amounts of collagen, resulting in interstitial ischaemia. The formation of a fibroid seems to follow this sequential pattern, in which more balanced progressive growth initially occurs as a result of the proliferation of transformed myocytes and the production and deposition of collagen, until the tumours evolve as a result of the accumulation of collagen in the stroma with secondary interstitial ischaemia. An inverse relationship exists between the percentage of collagenous tumour matrix and the density of microvessels, which can lead to myocyte atrophy and eventual tumour involution, through interstitial ischaemia and with vascular ischaemia. *Flake et al. (2013)* hypothesized that many fibroids undergo progressive involution, largely owing to excessive production of collagen in the interstitial matrix, thus increasing the distance between tumour myocytes and their blood supply. As the smooth muscle cells of the blood vessels of fibroids are similar to the phenotypic transformation changes of tumour myocytes, the blood vessels of fibroids also become progressively more fibrotic and hyalinized. Therefore, tumour myocytes are subject to a reduced supply of essential nutrients and oxygen because of vascular and interstitial ischaemia. If vascular and interstitial ischaemia develop within these tumours with increased collagen deposition, the proliferative capacity of myocytes may decrease, as the diffusion of nutrients and oxygen is hindered by thickened fibrotic vessels and by fibrotic interstitium. Furthermore, if the angiogenesis rate is not equivalent to, or higher than, the fibrogenesis rate, the tumour myocytes would have the additional stress of a greater distance between myocytes and capillaries (reduced microvascular density). A consequence of the increased diffusion distance between myocytes and blood vessels is the density of the fibrotic interstitial tissue and vessel walls, and a reduction in the supply of nutrients and oxygen to the cells. Decreased blood flow could cause necrosis, and a lower degree of ischaemia or hypoxia could induce an apoptotic reaction, and both types of cell death could be seen in fibroids (*Flake et al., 2013*).

IMPLICATION OF ANGIOGENESIS IN FIBROID PATHOGENESIS

The pathogenesis of fibroids is unclear. Extensive studies have established that ovarian steroids (oestrogen and progesterone) significantly affect the growth of fibroids, and their actions are partly mediated by local production of growth factors. In particular, steroids may modulate the production of different growth factors. These include epidermal growth factor, platelet-derived growth factor, insulin-like growth factor-1, transforming growth factor alfa, transforming growth factor beta, vascular endothelial growth factor (VEGF), acidic fibroblast growth factor, basic fibroblast growth factor, activin-A and myostatin (*Sozen et al., 2002*; *Ciarmela et al., 2011*), cytokines (interleukin [IL]: IL-1, IL-6, IL-11, IL-13, IL-15, tumour necrosis factor alfa, granulocyte-macrophage colony-stimulating factor and erythropoietin) as well as chemokines and their receptors (macrophage inflammatory proteins alfa, macrophage inflammatory proteins beta, RANTES, eotaxin, eotaxin-2, IL-8, chemokine receptor [CCR] CCR1, CCR3, CCR5, CXCR1, CXCR2, monocyte chemoattractant protein-1 (*Sozen et al., 1998*; *Syssoev et al., 2008*), which are involved in fibroid biology. Furthermore, it would seem that the pathogenesis is also linked to vascularization and angiogenesis; in fact, they seem to play an important role in the development and growth of fibroids (*Leppert et al., 2013*), even if the mechanism is not clear. Fibroids are considered benign tumours differing from malignant ones in angiogenesis and consequent vascularization. In fact, the fibroids show reduced vascularization compared with malignant tumours. It is known that hypoxia inducible factor 1α (HIF-1α) causes the tissue to adapt to hypoxia (*Vincent et al., 2002*). Interestingly, *Mayer et al. (2010)* demonstrated that HIF-1α, is not expressed in fibroid tissue, although it is overexpressed in leiomyosarcoma (malignant countpart). Such findings could represent an explanation of the vascular architecture of the fibroid, consisting of an avascular core inside a vascularized capsule (*Tal et al., 2014*). It has, however, been shown that the fibroid is a hypoxic tissue (*Mayer et al., 2008*) and, therefore, should be highly vascularized (*Tal et al., 2014*). The suppression of HIF-1α expression (*Mayer et al., 2010*) in fibroid tissue suggests an inadequate response of fibroid tissue to hypoxia (*Tal et al., 2014*). This may lead to a not sufficient angiogenic response in fibroids so that they appear avascular (*Tal et al., 2014*). These results are compatible with the findings of *Carmeliet et al. (1998)*, who showed that disruption

of HIF-1α impairs the formation of large blood vessels, interfering with vascular function so that, within the tumour mass, a hypoxic microenvironment establishes. It is known that most fibroids arise within the areas of myometrial hyperplasia, which are characterized by hypoxia and by an overexpression of the HIF-1α (*Cramer et al., 2009*). Fibroids are hypoxic, but do not express the HIF-1α, suggesting that the lack of expression of the HIF-1α could lead to reduce vascularization in the fibroid tissue. Therefore, the hypoxia of the fibroid tissue could drive the vascularization from the periphery of the tumour into the surrounding myometrium making the vascular capsule responsible for the blood supply to the growing tumour. The aberrant response of hypoxia and the lack of activation of the HIF-1α in fibroid tissue suggests that this factor could be involved in the pathophysiology of the fibroid, and reduced vascularization, growth, invasion and metastasis of the tumour. In addition to this, the formation of the vascularized capsule surrounding the avascular fibroid that could be interconnected with the vascular density in the normal myometrium encompassing the tumour may, in turn, provide an explanation about the menorrhagia that afflicts women with uterine fibroids and it also may explain the recurrent bleeding during myomectomy (*Tal et al., 2014*) (**FIGURE 4B**).

Another possible explanation for the avascular structure of fibroids could be represented by genetic aberrations that lead to an insufficient response to the stimuli of pro-angiogenic factors (*Tal et al., 2014*). In fact, fibroids are characterized by cytogenetic anomalies in about 40% of cases (*Levy et al., 2012*) and by an abnormal pattern of DNA methylation (*Yamagata et al., 2009*). A further explanation could be that the upregulation of angiogenic inhibitors reduce the microvascularization of fibroids through their counteracting action against angiogenic promoters (*Leppert et al., 2013*). In support of this hypothesis, it has been shown that collagen 4A2, the precursor of the angiogenic inhibitor canstatin (*Kamphaus et al., 2000*), is upregulated in fibroid tissue compared with myometrial tissue (*Weston et al., 2003*). Other studies suggest that fibroids overexpress and release angiogenic factor that can stimulate angiogenesis of adjacent myometrium, forming the vascular capsule that surround the avascular fibroid (*Hague et al., 2000*; *Walocha et al., 2003*). This hypothesis is supported by *Wei et al. (2006)*, who showed an increasing concentration gradient of VEGF from the centre of the fibroid to its peripheral area. Furthermore, it has been shown that, in myometrial tissue affected by fibromatosis compared with healthy myometrial tissue, an overexpression of several growth factors is involved in angiogenesis, such as VEGF, basic fibroblast growth and adrenomedullin (ADM) (*Hague et al., 2000*). In particular, it has been shown that the vascular density of the fibroid and the adjacent myometrium correlates with the expression of the angiogenic factor ADM induced by hypoxia (*Hague et al., 2000*). Therefore, ADM could be an important molecule involved in the development of the well-vascularized capsule surrounding the avascular core of the fibroid (*Hague et al., 2000*). Furthermore, it has been shown that fibroids with larger dimensions (>1 cm), even if with a lower vascular density compared with healthy myometrium, are more vascularized than small fibroids (1–3 mm) (*Walocha et al., 2003*). Fibroids, which are characterized by overexpression of the high mobility group AT-hook 2 (HMGA2), representing about 10–15% of cases (*Mehine et al., 2016*; *Makinen et al., 2017*), are usually larger than fibroids without HMGA2 overexpression (*Gross et al., 2003*; *Griffin et al., 2019*). Fibroid variant that usually presents HMGA2 overexpression, such as the intravascular leiomyomatosis (*Ordulu et al., 2016*), the hydropic fibroid (*Griffin et al., 2019*) and the lipoleiomyoma (*Pedeutour et al., 2000*), usually have a high cellular and hypervascular appearance (*Li et al., 2020*) with high vessel density and increased vessel proliferation (*Griffin et al., 2019*). Indeed, fibroids showing overexpressed HMGA2 have been shown to be characterized by increased expression and secretion of angiogenic factors, such as IGF2, bFGF, tumour necrosis factor alfa, VEGFR1 and VEGFR2 compared with MED12 fibroids (*Li et al., 2020*). As HMGA2 expression has been shown to correlate with all these upregulated genes, it has been suggested that HMGA2 overexpression is associated with up-regulation of angiogenesis factors (*Li et al., 2020*). Angiogenesis factors may play an important role in the pathophysiology of fibroids, particularly in abnormal vascularization, survival and growth of fibroids (*Leppert et al., 2013*). *Konarska et al. (2016)* addressed this topic by attempting to clarify how pro-angiogenic factors play a role in the growth of fibroids. Although a high expression of pro-angiogenic factors would expect a flourishing tumour neo-angiogenesis, this does not happen in the fibroma but rather in the surrounding healthy myometrium. This seems to be caused by chromosomal alterations, induced by cell replication, which make tumour cells insensitive to these factors, as well as to oestrogenic hormonal stimuli. The latter are favourable to neovascularization and have less action on the fibroma because there are fewer receptors (*Konarska et al., 2016*). In the study by *Casey et al. (2000)*, analysis of immunohistochemical vascular factors and microscopic microvessels counting, was used to show that the myometrium is increasingly vascularized compared with fibromas of any size: small fibromas with CD31; large fibromas in both central and peripheral areas using factor VIII; and any fibromal or peripheral areas using lectin *Ulex europaeus*. The myometrium has larger vessels and large fibroids compared with small ones using CD34, factor VIII and *U. europaeus* lectin.

The myometrium responds to the presence of proangiogenic factors produced by small fibroids, which increase the vessels and hmicrovascular density. This can generate capillaries that feed the small fibroids, promoting their growth and increasing their size. Probably symptoms such as menorrhagia or increased risk of bleeding during myomectomy surgery depend on this increased myometrial vascular density. Alternatively, the fibroids may have reduced angiogenic response or increased angiogenesis inhibitors, such that the myometrium responds by increasing peri-fibroid vascular growth and blood flow (*Casey et al., 2000*).

The excessive bleeding that may occur mainly with menstruation in women with uterine fibroids depends on a complex interaction of different factors. First, the amount of menstrual bleeding seems to be related to the location of the fibroids, with submucosal fibroids that are more likely to disrupt endometrial integrity rather that subserosal fibroids (*Ikhena et al., 2018*). In addition, fibroids may increase the surface area of the endometrium, and, consequently, the amount of bleeding (*Critchley et al., 2020*). Large fibroids may interfere with

myometrial contractions that normally occur during menstruation to reduce bleeding and with the vasoconstriction of the spiral arteriole, through an abnormal expression of endothelin receptors A and a consequent alterated effect of the endothelin 1– endothelin receptors A axis and also endothelin 1, 2, 3 isoforms action via endothelin receptors B, which play an important role in the vasoconstriction of uterine vessels (*Salamonsen et al., 1999*; Tanfin *et al*., 2012). Moreover, fibroids also seem to increase the levels of prostaglandin F2 alpha, altering the inflammatory milieu of normal endometrium during menstruation, with subsequent disordered contractility and increased blood loss (*Kitaya et al., 2010*).

ULTRASOUND STUDY OF UTERINE AND FIBROID VASCULARIZATION

Ultrasound is the primary imaging modality used to evaluate patients in whom uterine fibroids are suspected (*Van den Bosch et al., 2015*). Doppler ultrasound can be used to study uterine and fibroid vascularization (*Van den Bosch et al., 2015*). Colour Doppler, power Doppler, pulsed-wave Doppler and three-dimensional power Doppler are reported to be effective for the visualization of the fibroid flow pattern and the study of Doppler parameters (*Fleischer, 2003*; *Stepniak et al., 2017*). Contrast-enhanced ultrasound (CEUS) is an additional tool that can be used for evaluating the macro- and the micro-vascularization of the uterus and fibroids (*Stoelinga et al., 2018*; *Chhabra et al., 2020*).

Acquisition technique

Doppler ultrasound examination of the uterus and fibroids can be carried out using transabdominal or transvaginal probes. Transvaginal probes perform better than transabdominal probes for the examination of pelvic organs, and the use of transabdominal probes should be considered as complementary in the case of large lesions extending beyond the small pelvis (*Van den Bosch et al., 2015*).

The paracervical vascular plexus can be identified in a side-to-side (lateral-to-lateral) scan of the uterus. The uterine artery can then be visualized at the junction between the uterine body and the cervix, where it ascends into the uterine body; at approximately 2 cm from the cervix, the uterine artery crosses the ureter to become medial to it. With their descending cervical and ascending uterine branches, the arcuate arteries can be visualized in the outer myometrium, often close to the uterine border. Doppler waveforms of uterine artery typically present a high-velocity, high-resistance pattern, and an identifiable dicrotic notch (*Bhatt et al., 2006*; *Secil et al., 2008*; *Idowu et al., 2017*).

The acquisition should be optimized by adjusting specific settings. In particular, the colour box should be kept small, covering the area of interest, improving frame rate and colour resolution; the focal zone should be placed where vessels are expected; the angle of insonation should be less than 60° (30–60°); the pulse repetition frequency should be set to low values to evaluate slow flow vessels, i.e. such as those of fibroids, with adjustments to obtain a colour or a spectrum just over the aliasing limit; the colour and spectral gain should be set just below the noise level until all colour artifacts disappear; the colour and spectral wall filter should be set between 50–100 Hz; the sample gate size for pulsed-wave doppler should be set to about two-thirds of the vessel lumen; for the evaluation of artery supplying fibroids, the gate sample could be set to 1 mm (*Fleischer, 2003*; *Bhatt et al., 2006*; *Secil et al., 2008*; *Van den Bosch et al., 2015*; *Idowu et al., 2018*).

Colour Doppler and power Doppler

Colour Doppler converts the Doppler measurements into an array of colours and is combined with standard ultrasound to show the speed and direction of blood flow through a blood vessel. Pulsed-wave Doppler is used to acquire images that are difficult to obtain using colour Doppler and provides greater blood flow details. Power Doppler is less angle-dependent (*Stepniak et al., 2017*) and superior to colour Doppler for detecting small vessels with low blood-flow velocities but provides no information about flow direction. Pulsed-wave Doppler, however, is more susceptible to movement artifacts than colour Doppler and requires a longer acquisition time.

The fibroid vascular pattern includes a circumferential rim of vascularity in the pseudocapsule and a pronounced intralesional flow in most cases (*Sladkevicius et al., 1996*; *Fleischer, 2003*). A few vessels may arise from the peripheral rim and penetrate the centre of the fibroid (*Fleischer, 2003*). It is reported that about one-half of the fibroids may only present peripheral vascularity with decreased or avascular core (**FIGURE 5A**) (*Weintraub et al., 2002*), and only

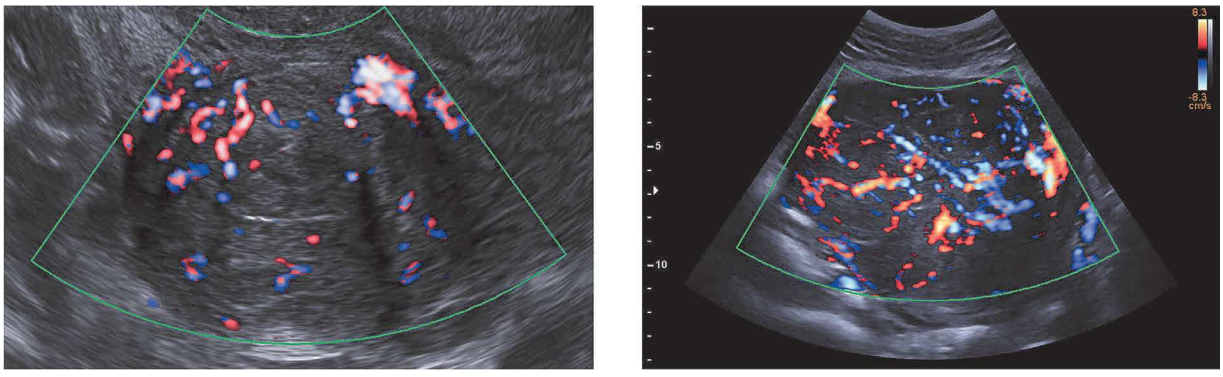


FIGURE 5 Power-Doppler imaging of uterine vascularization. (A) Typical fibroid vascularizazion, with peripheral flow (colour score 2) and avascular core (colour score 1); (B) atypical fibroid vascularization, with increased central flow (colour score 3). Colour score: (1) no flow, (2) minimal flow, (3) moderate flowh and (4) high vascular flow. Images obtained from the Gynaecologic Section, DISCO, Università Politecnica delle Marche, Ancona, Italy.

a small fraction (about 2–5%) may be avascular (*Idowu et al., 2018*). Colour Doppler and power Doppler can also help the differential diagnosis between a pedunculated subserosal fibroid and ovarian masses by identifying the 'vascular bridging sign' or the 'vascular pedicle sign'. 'Vascular bridging sign' is defined as the presence of multiple vessels originating from the uterine arteries feeding the uterine fibroid that confirms the uterine origin of the evidenced mass, whereas, in the case of ovarian neoformation, it is absent (*Bhatt et al., 2006*). 'Vascular pedicle sign' may indicate the existence of a solitary vessel originating from the uterine artery that supplies a subserosal fibroid through its peduncle (or stalk), demonstrating a common source of blood supply between the mass and the uterus (*Bhatt et al., 2006*).

In clinical practice, the degree of vascularization should be reported using a subjective colour score that includes the percentage of vascularization and the colour hue. A colour score of 1 represents no colour and a score of 4 abundant colour signals. The score may be assigned separately to circumferential and intralesional vascularity (*Van den Bosch et al., 2015*).

Pulsed-wave Doppler

Pulsed-wave Doppler can be useful in evaluating blood flow of the uterine arteries of patients with fibroid and arteries supplying fibroids, distinguished in peripheral (or perifibroid) and intrafibroid (or core). The most frequently studied pulsed-wave Doppler parameters are the

Resistance Index, defined as:

> Peak systolic velocity – end-diastolic velocity/peak systolic velocity; and

Pulsatility Index, defined as:

> Peak systolic velocity – end-diastolic velocity/(Peak systolic velocity + end-diastolic velocity)/2.

Uterine vascularity seems to be globally increased in women with uterine fibroids, as expressed by lower values of Resistance Index (0.60–0.77) and Pulsatility Index (1.67-2.50) of uterine arteries of affected women compared with controls (Resistance Index 0.82–0.84, Pulsatility Index 1.97–3.40) (*Alatas et al., 1997*; *Alcázar et al., 1997*).

Fibroid vascularization present low values of Resistance Index and Pulsatility Index both on vessels surrounding the lesion (fibroid border/wall/pseudocapsule) and in the inner part (fibroid center/core): Resistance Index 0.54–0.60 /Pulsatility Index 0.81–1.10 and Resistance Index 0.44–0.50/Pulsatility Index 0.66–0.80, respectively (*Weiner et al., 1993*). The interpretation of these parameters demonstrates a low impedance to flow and a high velocity (*Huang et al., 1996*), which increases local vascularity and blood flow (*Sosic et al., 1996*; *Farmakides et al., 1998*).

Available evidence suggests that those parameters may be negatively correlated with fibroid size and volume (*Huang et al., 1996*), with increased vascularization for greater lesions (*Idowu et al., 2018*). Submucosal and subserosal fibroids may present lower Resistance Index values than intramural fibroids (*Sosic et al., 1996*). Moreover, Resistance Index and Pulsatility Index may also be lower in cases of necrosis and secondary degenerative or inflammatory changes (*Kurjak et al., 1992*). The association with the possibility of fibroid growth seems to be less clear as some investigators have reported a higher possibility of growth over time in cases of higher vascularization, and others have reported no association between fibroid vascularization and proliferative histopathological parameters (*Huang et al., 1996*; *Testa et al., 2003*).

Three-dimensional power Doppler

The combination of three-dimensional (3D) imaging and power Doppler has been reported to improve the assessment of the vascularity of a volume (*Alcazar, 2008*), and it has proven effective and reproducible in the evaluation of fibroid vascularization, with an inter-observer agreement between 0.56 and 0.89 (*Nieuwenhuis et al., 2015*). A 3D grayscale and a 3D power Doppler volume of the region of interest can be acquired and stored. A manual or automatic contour is then traced and analysed with dedicated software (VOCAL), which generates three vascular indices of the selected volume, the vascularization index, the flow Index and vascularization flow index (VFI). Vascularization Index is defined as the number of colour voxels divided by the total number of colour and grey voxels, and reflects the proportion of blood vessels within the tissue. Flow Index is determined as the average colour value of all colour voxels, indicating the average flow velocity. Vascularization Index and Flow Index are unit-less and expressed as a value ranging between 0 and 100 (*Stepniak et al., 2017*). The VFI is the average colour value of all grey and colour voxels within the volume, a product of the Vascularization Index and the Flow Index divided by 100 (*Nieuwenhuis et al., 2015*; *Stepniak et al., 2017*).

Those indices may be determined for the total volume of fibroid, for the 'shell' volume (peripheral portion of the fibroid), and for the most vascularized portion of the fibroid, subjectively identified and reconstructed as a sphere (*Minsart et al., 2012*). The most vascularized area is usually localized at the periphery of the fibroid rather than at the centre, which is often hypovascularized and the site of necrosis (*Minsart et al., 2012*). The limitations of this technique are that the Vascularization Index decreases as the distance between the probe and the vessels increases. Moreover, the sample size, the machine settings and the patients' parameters, such as heart pulsatility, may interfere with volume acquisition and determination of vascular indices (*Stepniak et al., 2017*).

A positive correlation between the fibroid cellular activity and the Vascularization Index or VFI of the most vascularized portion of the fibroid has been described, as well as a negative correlation between histopathological evidence of ischaemic necrosis and Vascularization Index or VFI of the total fibroid volume (*Minsart et al., 2012*).

Vascularization Index also seems to be related to fibroid growth over time, with a 7.0 cm^3 increase in fibroid volume over time for each 1% increase of Vascularization Index at baseline (*Nieuwenhuis et al., 2018*).

Response to treatment

Doppler ultrasound can provide clinically useful information for evaluating the response to conservative treatments for uterine fibroids, such as medical treatment (gonadotrophin-releasing hormone analogues or ulipristal acetate) or uterine artery embolization (UAE), in which the vascular component plays a crucial role (*Stepniak et al., 2017*). Patients with uterine fibroids treated with gonadotrophin releasing hormone

analogues (leuprolide acetate, triptorelin, buserelin or goserelin) show an increase in pulsed-Doppler parameters after 3–6 months of treatment for uterine arteries and fibroid-supplying arteries compared with baseline (Resistance Index 0.52–0.73 versus 0.87–1.05 for uterine artery and Resistance Index 0.48–0.55 versus 0.71–0.91 for fibroid artery; Pulsatility Index 1.48 versus 2.31 for uterine artery and Pulsatility Index 0.90–0.96 versus 1.48–1.60 for fibroid artery), demonstrating a reduction in blood flow,which seems to contribute to symptom control and fibroid volume shrinkage (*Creighton et al., 1994*; *Aleem et al., 1995*; *Matta et al., 1988*; *Kanelopoulos et al., 2003*). Also, the analysis of 3D Power doppler parameters of fibroid vascularization after 3 months of treatment with leuprolide acetate confirms a reduction in fibroid blood flow (shell Vascularization Index 4.30 versus 0.93 and inner Vascularization Index 6.34 versus 1.28) (*Frijlingh et al., 2020*).

Baggio et al. (2018) analysed the effect of ulipristal acetate by studying uterine and fibroid vascularization. They found an increase in the Resistance Index and Pusatility Index of the fibroid core artery after 3 months of treatment (Resistance Index 0.71 versus 0.95 and Pulsatility Index 1.31 versus 1.41). A non-significant increase in the Pulsatility Index and Resistance Index of uterine arteries was noted, assuming that the effect of ulipristal acetate in fibroid shrinkage was mainly caused by the reduction of fibroid vascularization rather than the effect on the entire uterine blood flow (*Baggio et al., 2018*). The reduction of fibroid vascularization after 3 months of treatment with ulipristal acetate was also assessed by 3D Power Doppler, with a reduction of Vascularization Index (4.16 versus 0.54), Flow Index (26.10 versus 16.30) and VFI (0.82 versus 0.12) (*Czuczwar et al., 2015*). These results, however, were not confirmed in the study by *Frjlingh et al. (2020)*, in which the difference in 3D Power doppler parameters did not reach statistical significance (*Frijlingh et al., 2020*).

The response to UAE can be evaluated by assessing the vascularization parameters before the procedure, in the hours after the procedure and up to 3–6 months, with an accuracy comparable to that of arteriography (*Muniz et al., 2002*). The subjective assessment of fibroid vascularity (hypervascular, isovascular or hypovascular compared with normal myometrium) by colour Doppler shows the most significant reduction in vascularity 1 day after the procedure (*Fleischer et al., 2000*; *Weintraub et al., 2002*), and a slight increase at 6 months; moreover, hypervascularized fibroids seem to have the greatest reduction in size after the procedure (*Fleischer et al., 2000*). The reduction in fibroid vascularity after UAE seems to precede the decrease of fibroid volume at 3 months; indeed, fibroids without detectable vascularization are those with the most significant volume reduction (*Tranquart et al., 2002*). The Resistance Index of uterine arteries may predict volume reduction at 3 months after UAE as it is reported that a Resistance Index value between 0.82 and 0.88 immediately after the procedure is associated with a 38–61% reduction in fibroid volume at 3 months (*Naguib et al., 2012*). In addition, 3D power Doppler fibroid vascularization parameters seem to reduce after 3 months from UAE (Vascularization Index 1.44 versus 0.12, Flow Index 24.01 versus 10.44 and VFI 0.24 versus 0.01) (*Czuczwar et al., 2015*).

Conservative treatments

Other conservative treatments are available for uterine fibroids, such as radiofrequency ablation, magnetic resonance-guided high-intensity focused ultrasound, and ultrasound-guided high intensity focused ultrasound. Radiofrequency ablation can be delivered by a laparoscopic, transvaginal or transcervical approach into the uterine fibroid to induce coagulative necrosis (*Bradley et al., 2019*). Magnetic resonance-guided high-intensity focused ultrasound is a non-invasive procedure that destroys uterine fibroid by thermal ablation, combining magnetic resonance imaging and high-intensity focused ultrasound (*Pron, 2015*). In the case of ulatrasound-guided high intensity focused ultrasound, ultrasound is used to focus high intensity ultrasound in the target (*Zhang et al., 2015*). Magnetic resonance or ultrasound are used to visualize the patient anatomy, map the volume of tissue to be treated, allow treatment adjustment and enable treatment results to be evluated. The Doppler ultrasound study is of limited use, considering that those techniques rely less on the vascularity of fibroids in their therapeutic mechanism, and the degree of response to treatment is better evaluated by measuring the size of uterine fibroids after treatment.

Differential diagnosis

In clinical practice, Doppler ultrasound may help the differential diagnosis between uterine fibroids and other conditions, such as endometrial polyps (in the case of submucosal fibroids), adenomyosis or leiomyosarcomas (LMS).

The power Doppler examination of uterine intracavitary lesions can highlight some typical features that can distinguish endometrial polyps from submucosal fibroids. Endometrial polyps present in most cases a single feeding vessel originating from the base and going through the central part of the endometrium (pedicle artery sign or single-vessel pattern) (*Alcazar et al., 2003*; *Timmerman et al., 2003*), whereas submucosal fibroids have a 'rim-like pattern' of vascularization, with circular or semicircular vessels surrounding the focal endocavitary lesion (*Cil et al., 2010*).

Adenomyosis is characterized by the presence of endometrial glands in the thickness of the myometrium and can be associated with symptoms such as pelvic pain, AUB and infertility, similar to those of uterine fibroids. The distinction between the two entities is essential for the correct treatment choice (*Chiang et al., 1999*; *Exacoustos et al., 2011*; *Van den Bosch et al., 2015*). At the Doppler examination, the vascularization of adenomyosis is typically diffuse, with multiple scattered vessels and a translesional flow (*Chiang et al., 1999*; *Exacoustos et al., 2011*; *Sharma et al., 2015*; *Van den Bosch et al., 2015*). In contrast, fibroids typically had a circumferential flow, with peripheral vessels and outer feeding vessels (*Chiang et al., 1999*; *Van den Bosch et al., 2015*). Pulsatility Index and Resistance Index are generally higher in adenomyosis compared with uterine fibroids (Pulsatility Index 1.5 versus 0.9 and Resistance Index 0.9 versus 0.5) (*Sharma et al., 2015*). Also, 3D power Doppler may be useful in discriminating fibroids from adenomyosis, with Vascularization Index, Flow Index and VFI usually higher in the case of fibroids (*Cheng et al., 2012*; *Elkattan et al., 2016*).

Surgical planning and preoperative diagnosis are of crucial importance

when approaching patients with uterine masses, as a misdiagnosis of LMS as benign fibroid can result in inadequate treatment. For example, conservative treatment such as UAE or mini-invasive approaches with morcellation of malignant masses can significantly worsen a patient's prognosis (*Ludovisi et al., 2019*; *Sun et al., 2019*).

At the subjective assessment of vascularity, LMS mostly present moderate (colour score 3) or rich (colour score 4) vascularization in the inner part of the lesion (colour score 3–4 in 55.1–87.5% of cases) and the circumference (colour score 3–4 in 40.8–100% of cases) (**FIGURE 5B**) (*Exacoustos et al., 2007*; *Ludovisi et al., 2019*). The vascularity of LMS is increased in the central portion compared with the circumference, an opposite condition to that found in fibroids, in which the peripheral vascularity is increased (*Exacoustos et al., 2007*). Up to one-third of LMS, however, may have an absent or minimal vascularization that can be explained by necrotic phenomena occurring in the centre of the lesion (*Ludovisi et al., 2019*). The finding of a marked vascularization of a myometrial neoformation presents high sensitivity (up to 100%) for a malignant diagnosis, but a limited positive predictive value (19–44%), which can be improved by combining other sonographic features, such as lesion diameter (>8 cm) or cystic degeneration, reaching values of 57–60% (*Exacoustos et al., 2007*).

Doppler flow indices of LMS typically demonstrate low impedance to flow (Resistance Index 0.41–0.49) (*Szabo et al., 1997*; *Aviram et al., 2005*) and high peak systolic velocity (sensitivity of 80% and false-positive rate of 2.4% for LMS diagnosis if velocity is greater than 41 cm/s (*Hata et al., 1997*). These findings, however, are of limited use in clinical practice as significant overlap occurs with uterine fibroids (*Amant et al., 2009*; *Sun et al., 2019*).

In summary, LMS may present typical vascularization features that are not specific enough when considered alone for the differential diagnosis of benign conditions. Doppler findings should be evaluated in conjunction with other ultrasound features (tumour dimension, inhomogeneous echogenicity, internal irregular cystic areas, absence of shadows and absence of calcifications) (*Ludovisi et al., 2019*), symptoms (AUB) and laboratory examinations (neutrophil-to-lymphocyte ratio), number of platelets and lactate dehydrogenase (*Kohler et al., 2019*; *Zhang et al., 2020*).

Contrast-enhanced ultrasound

Contrast-enhanced ultrasound is an imaging technique that allows the visualization of the macro- and micro-vasculature using an intravenous contrast agent composed of gas-filled microbubbles (1.5–5 μm) stabilized by a protein or lipid shell (*Stoelinga et al., 2018*; *Chhabra et al., 2020*). *Stoelinga et al. (2018)* reported CEUS features of normal myometrium, endometrium and uterine fibroids. In normal myometrium and endometrium, the contrast enhancement is firstly observed in the outer layer of the myometrium, subsequently in the inner layer and finally in the endometrium. On the other hand, the wash-out occurs first in the endometrium and then in the myometrium. Uterine fibroids present a contrast wash-in that begins from the pseudo-capsule (allowing a clear definition of fibroid compared with the surrounding myometrium) and continues through branched vessels with a heterogeneous enhancement of the inner part of the lesion; areas of absence of vascularization can be detected inside the fibroid parenchyma. Fibroid wash-out begins from the centre and gradually involves the remaining part and the adjacent myometrium (*Stoelinga et al., 2018*). The performance of CEUS is similar to magnetic resonance in the study of uterine fibroids, and can be proposed as an alternative for patients with contraindications to magnetic resonance. Indeed, high levels of correlation between CEUS and magnetic resonance have been reported for the number of identified fibroids, for fibroid size (91% of agreement), fibroid location (93% of agreement) and the presence of enhancement (100% of agreement) (*Chhabra et al., 2020*). The systematic review by *Torkzaban et al. (2021)* has highlighted that CEUS performs similarly to magnetic resonance in evaluating the therapeutic effect of high-intensity focused ultrasound ablation or percutaneous microwave ablation, and it is helpful in determining the end point of the UAE procedure and in the follow-up after UAE (*Torkzaban et al., 2021*). *Li et al. (2021)* proposed that CEUS may help distinguish between different types of fibroids (common uterine fibroids, cellular uterine fibroid, hysteromyoma with hyaline degeneration and uterine leiomyosarcomas) by evaluating specific CEUS parameters, such as enhanced time, enhanced level, enhanced morphology, rise time, peak intensity, time to peak and mean transit time (*Li et al., 2021*).

Magnetic resonance

Even if ultrasound is the imaging tecnique of choice for the evaluation of uterine fibroids, magnetic resonance may be useful for further characterization or differential diagnosis in the case of coexisting pelvic disease, severe uterine abnormalities, very large masses or when leiomyosarcoma is suspected. Fibroids without sign of degeneration are well-delineated round or ovoid lesions homogeneously hypointense on T2-weighted imaging (T2WI) related to the outer myometrium and isointense on T1-weighted imaging (T1WI), with heterogeneous and variable enhancement (*Nougaret et al., 2020*). It has been reported that magnetic resonance has a sensitivity of 94.1% and a specificity of 68.7% for uterine fibroids identification (*Murase et al., 1999*). Magnetic resonance may also be helpful in distinguishing between cellular fibroids and lipoleiomyomas, the first with higher signal intensity on T2WI and avid enhancement (*Deshmukh et al., 2012*), and the latter with high signal intensity of the fat components on T1WI and T2WI with loss of signal intensity on fat-saturated sequences (*DeMulder et al., 2018*). Magnetic resonance may also identify typical features of fibroid degeneration (hyaline, cystic, myxoid, haemorrhagic or calcific) (*Nougaret et al., 2020*). No specific magnetic resonance features have been identified for the diagnosis of uterine leiomysarcoma, making differential diagnosis difficult; however, an association between a diagnosis of leiomyosarcoma and the following features have been reported: identification of nodular borders, haemorrhage (high signal intensity on T1WI), T2-weighted (T2W) dark areas, central areas of non-enhancement, rapid early enhancement of the solid components or restricted diffusion (*Bolan et al., 2016*; *Lakhman et al., 2017*; *Fujii et al., 2021*).

CONCLUSIONS

Uterine and fibroid vascularization are crucial aspects that need to be

further investigated to gain a deeper understanding of the pathogenetic elements that lead to the formation of uterine fibroids and their clinical manifestations. The ultrasound study of uterine and fibroid vascularization may be carried out in different ways to obtain multiple clinical information and provide individualized treatment. In particular, an ultrasound scan may help to evaluate the response to conservative treatment and accurately plan the surgical approach, intensifying the prevention procedures in patients at high-risk for blood loss. Regarding the differential diagnosis with malignant pathology, the evaluation of vascularization alone may not be enough to discriminate between benign and malignant conditions, but it should be related to other clinical features. Future research should focus on new ultrasonographic techniques and new applications of the existing techniques to improve our diagnostic and therapeutic capacity. Because the contribution of fibroids to heavy menstrual bleeding or AUB also depends on localization, it will be interesting to focus on submucosal or intramural fibroids and compare their vascularization and cellularity with subserosal fibroids, and, in the same patients, the thickness of endometrial layer. In addition, as the differential diagnosis with myosarcoma is a current clinical question, it is worth investigating different techniques for imaging vascularization in these patients compared with benign fibroids.

REFERENCES

Alatas, G., Aksoy, E., Akarsu, C., Yakin, K., Bahceci, M. **The effect of uterine volume on uterine artery Doppler velocimetry in the myomatous state.** Gynecol. Obstet. Invest. 1997; 43: 55–59. doi:10.1159/000291820

Alcazar, J.L. **Three-dimensional power Doppler derived vascular indices: what are we measuring and how are we doing it?** Ultrasound Obstet. Gynecol. 2008; 32: 485–487. doi:10.1002/uog.6144

Alcazar, J.L., Castillo, G., Minguez, J.A., Galan, M.J. **Endometrial blood flow mapping using transvaginal power Doppler sonography in women with postmenopausal bleeding and thickened endometrium.** Ultrasound Obstet. Gynecol. 2003; 21: 583–588. doi:10.1002/uog.143

Alcázar, J.L., Griffioen, M., Jurado, M. **Uterine artery blood flow in women with uterine myomas.** European Journal of Ultrasound 1997; 5: 165–169. doi:10.1016/S0929-8266(97)00016-5

Aleem, F.A., Predanic, M. **The hemodynamic effect of GnRH agonist therapy on uterine leiomyoma vascularity: a prospective study using transvaginal color Doppler sonography.** Gynecol. Endocrinol. 1995; 9: 253–258. doi:10.3109/09513599509160454

Amant, F., Coosemans, A., Debiec-Rychter, M., Timmerman, D., Vergote, I. **Clinical management of uterine sarcomas.** Lancet Oncol. 2009; 10: 1188–1198. doi:10.1016/S1470-2045(09)70226-8

Aviram, R., Ochshorn, Y., Markovitch, O., Fishman, A., Cohen, I., Altaras, M.M., Tepper, R. **Uterine sarcomas versus leiomyomas: gray-scale and Doppler sonographic findings.** J. Clin. Ultrasound 2005; 33: 10–13. doi:10.1002/jcu.20075

Baggio, S., Pomini, P., Galeone, F., Presti, F., Santi, L., Raffaelli, R., Franchi, M. **Influence of Ulipristal Acetate Therapy on Uterine Fibroid-Related Symptoms and on Uterine and Fibroid Volumes and Vascularity Indices Assessed by Ultrasound.** J. Ultrasound Med. 2018; 37: 2215–2223. doi:10.1002/jum.14573

Baird, D.D., Dunson, D.B., Hill, M.C., Cousins, D., Schectman, J.M. **High cumulative incidence of uterine leiomyoma in black and white women: ultrasound evidence.** American Journal of Obstetrics and Gynecology 2003; 188: 100–107

Bereza, T., Lis, G., Mitus, J., Sporek, M., Chmielewski, P., Kolber, W., Mazur, M., Goncerz, G., Kuniewicz, M. **Blood vessels of the intratumoral septa in uterine leiomyomata.** Folia Med. Cracov. 2013; 53: 99–106

Bhatt, S., Dogra, V.S. **Doppler Imaging of the Uterus and Adnexae.** Ultrasound Clinics, Elsevier 2006; 1: 201–221. doi:10.1016/j.cult.2005.09.010

Bolan, C., Caserta, M.P. **MR imaging of atypical fibroids.** Abdom Radiol (NY) 2016; 41: 2332–2349. doi:10.1007/s00261-016-0935-0

Bradley, L.D., Pasic, R.P., Miller, L.E. **Clinical Performance of Radiofrequency Ablation for Treatment of Uterine Fibroids: Systematic Review and Meta-Analysis of Prospective Studies.** J. Laparoendosc. Adv. Surg. Tech. A 2019; 29: 1507–1517. doi:10.1089/lap.2019.0550

Cardozo, Eden R., Clark, Andrew D., Banks, Nicole K., Henne, Melinda B., Stegmann, Barbara J., Segars, James H. **The estimated annual cost of uterine leiomyomata in the United States.** American journal of obstetrics and gynecology 2012; 206: 211

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C.J., Ratcliffe, P., Moons, L., Jain, R.K., Collen, D., Keshert, E. **Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis.** Nature 1998; 394: 485–490. doi:10.1038/28867

Casey, R., Rogers, P.A., Vollenhoven, B.J. **An immunohistochemical analysis of fibroid vasculature.** Hum. Reprod. 2000; 15: 1469–1475. doi:10.1093/humrep/15.7.1469

Cheng, Y.M., Chen, Y., Hsu, Y.Y., Cheng, Y.C., Cheng, M.J., Hsu, Y.C. **Preoperative three-dimensional power doppler ultrasonographic evaluation of adenomyosis and uterine leiomyoma.** Digest Journal of Nanomaterials and Biostructures 2012; 7: 621–627

Chhabra, P., Daugherty, R., LeNoir, A.M., Grilli, C., Makai, G., Patel, N., DeMauro, C. **Comparison of Contrast-Enhanced Ultrasound Versus Magnetic Resonance Imaging in the Detection and Characterization of Uterine Leiomyomas.** J. Ultrasound Med. 2020. doi:10.1002/jum.15495

Chiang, C.H., Chang, M.Y., Hsu, J.J., Chiu, T.H., Lee, K.F., Hsieh, T.T., Soong, Y.K. **Tumor vascular pattern and blood flow impedance in the differential diagnosis of leiomyoma and adenomyosis by color Doppler sonography.** J. Assist. Reprod. Genet. 1999; 16: 268–275. doi:10.1023/a:1020371614069

Ciarmela, P., Islam, M.S., Reis, F.M., Gray, P.C., Bloise, E., Petraglia, F., Vale, W., Castellucci, M. **Growth factors and myometrium: biological effects in uterine fibroid and possible clinical implications.** Human Reproduction Update 2011; 17: 772–790

Ciavattini, A., Clemente, N., Delli Carpini, G., Saccardi, C., Borgato, S., Litta, P. **Laparoscopic uterine artery bipolar coagulation plus myomectomy vs traditional laparoscopic myomectomy for "large" uterine fibroids: comparison of clinical efficacy.** Arch. Gynecol. Obstet. 2017; 296: 1167–1173. doi:10.1007/s00404-017-4545-z

Cil, A.P., Tulunay, G., Kose, M.F., Haberal, A. **Power Doppler properties of endometrial polyps and submucosal fibroids: a preliminary observational study in women with known intracavitary lesions.** Ultrasound Obstet. Gynecol. 2010; 35: 233–237. doi:10.1002/uog.7470

Cramer, S.F., Mann, L., Calianese, E., Daley, J., Williamson, K. **Association of seedling myomas with myometrial hyperplasia.** Hum. Pathol. 2009; 40: 218–225. doi:10.1016/j.humpath.2008.07.004

Creighton, S., Bourne, T.H., Lawton, F.G., Crayford, T.J., Vyas, S., Campbell, S., Collins, W.P. **Use of transvaginal ultrasonography with color Doppler imaging to determine an appropriate treatment regimen for uterine fibroids with a GnRH agonist before surgery: a preliminary study.** Ultrasound Obstet. Gynecol. 1994; 4: 494–498. doi:10.1046/j.1469-0705.1994.04060494.x

Critchley, H.O.D., Babayev, E., Bulun, S.E., Clark, S., Garcia-Grau, I., Gregersen, P.K., Kilcoyne, A., Kim, J.J., Lavender, M., Marsh, E.E., Matteson, K.A., Maybin, J.A., Metz, C.N., Moreno, I., Silk, K., Sommer, M., Simon, C., Tariyal, R., Taylor, H.S., Wagner, G.P., Griffith,

L.G. **Menstruation: science and society.** Am. J. Obstet. Gynecol. 2020; 223: 624–664. doi:10.1016/j.ajog.2020.06.004

Czuczwar, P., Wozniak, S., Szkodziak, P., Milart, P., Wozniakowska, E., Wrona, W., Paszkowski, T. **Influence of ulipristal acetate therapy compared with uterine artery embolization on fibroid volume and vascularity indices assessed by three-dimensional ultrasound: prospective observational study.** Ultrasound Obstet. Gynecol. 2015; 45: 744–750. doi:10.1002/uog.14668

DeMulder, D., Ascher, S.M. **Uterine Leiomyosarcoma: Can MRI Differentiate Leiomyosarcoma From Benign Leiomyoma Before Treatment?** AJR Am. J. Roentgenol. 2018; 211: 1405–1415. doi:10.2214/AJR.17.19234

Deshmukh, S.P., Gonsalves, C.F., Guglielmo, F.F., Mitchell, D.G. **Role of MR imaging of uterine leiomyomas before and after embolization.** Radiographics 2012; 32: E251–E281. doi:10.1148/rg.326125517

Elkattan, E., Kamel, R., Elghazaly, H., Elariki, E. **Can Three-dimensional (3D) power Doppler and uterine artery Doppler differentiate between fibroids and adenomyomas?** Middle East Fertility Society Journal 2016; 21: 46–51

Exacoustos, C., Brienza, L., Di Giovanni, A., Szabolcs, B., Romanini, M.E., Zupi, E., Arduini, D. **Adenomyosis: three-dimensional sonographic findings of the junctional zone and correlation with histology.** Ultrasound Obstet. Gynecol. 2011; 37: 471–479. doi:10.1002/uog.8900

Exacoustos, C., Romanini, M.E., Amadio, A., Amoroso, C., Szabolcs, B., Zupi, E., Arduini, D. **Can gray-scale and color Doppler sonography differentiate between uterine leiomyosarcoma and leiomyoma?** J. Clin. Ultrasound 2007; 35: 449–457. doi:10.1002/jcu.20386

Farmakides, G., Stefanidis, K., Paschopoulos, M., Mamopoulos, M., Lolis, D. **Uterine artery Doppler velocimetry with leiomyomas.** Arch. Gynecol. Obstet. 1998; 262: 53–57. doi:10.1007/s004040050227

Fay, P.J. **Factor VIII structure and function.** Thromb. Haemost. 1993; 70: 63–67

Flake, G.P., Moore, A.B., Sutton, D., Kissling, G.E., Horton, J., Wicker, B., Walmer, D., Robboy, S.J., Dixon, D. **The natural history of uterine leiomyomas: light and electron microscopic studies of fibroid phases, interstitial ischemia, inanosis, and reclamation.** Obstet. Gynecol. Int. 2013; 2013528376. doi:10.1155/2013/528376

Fleischer, A.C. **Color Doppler sonography of uterine disorders.** Ultrasound Q 2003; 19: 179–189. doi:10.1097/00013644-200312000-00004

Fleischer, A.C., Donnelly, E.F., Campbell, M.G., Mazer, M.J., Grippo, D., Lipsitz, N.L. **Three-dimensional color Doppler sonography before and after fibroid embolization.** J. Ultrasound Med. 2000; 19: 701–705. doi:10.7863/jum.2000.19.10.701

Frijlingh, M., De Milliano, I., Hehenkamp, W.J.K., Huirne, J.A.F. **Differences in fibroid vascularity after three months of pre-treatment with leuprolide acetate or ulipristal acetate: A pilot study.** Eur. J. Obstet. Gynecol. Reprod. Biol. 2020; 245: 186–192. doi:10.1016/j.ejogrb.2019.08.002

Fujii, S., Mukuda, N., Ochiai, R., Yunaga, H., Murakami, A., Gonda, T., Kishimoto, M., Yamaji, D., Ishibashi, M. **MR imaging findings of unusual leiomyoma and malignant uterine myometrial tumors: what the radiologist should know.** Jpn. J. Radiol. 2021; 39: 527–539. doi:10.1007/s11604-021-01096-7

Griffin, B.B., Ban, Y., Lu, X., Wei, J.J. **Hydropic leiomyoma: a distinct variant of leiomyoma closely related to HMGA2 overexpression.** Hum. Pathol. 2019; 84: 164–172. doi:10.1016/j.humpath.2018.09.012

Gross, K.L., Neskey, D.M., Manchanda, N., Weremowicz, S., Kleinman, M.S., Nowak, R.A., Ligon, A.H., Rogalla, P., Drechsler, K., Bullerdiek, J., Morton, C.C. **HMGA2 expression in uterine leiomyomata and myometrium: quantitative analysis and tissue culture studies.** Genes Chromosomes Cancer 2003; 38: 68–79

Hague, S., Zhang, L., Oehler, M.K., Manek, S., MacKenzie, I.Z., Bicknell, R., Rees, M.C. **Expression of the hypoxically regulated angiogenic factor adrenomedullin correlates with uterine leiomyoma vascular density.** Clin. Cancer Res. 2000; 6: 2808–2814

Hata, K., Hata, T., Maruyama, R., Hirai, M. **Uterine sarcoma: can it be differentiated from uterine leiomyoma with Doppler ultrasonography? A preliminary report.** Ultrasound Obstet. Gynecol. 1997; 9: 101–104. doi:10.1046/j.1469-0705.1997.09020101.x

Huang, S.C., Yu, C.H., Huang, R.T., Hsu, K.F., Tsai, Y.C., Chou, C.Y. **Intratumoral blood flow in uterine myoma correlated with a lower tumor size and volume, but not correlated with cell proliferation or angiogenesis.** Obstet. Gynecol. 1996; 87: 1019–1024. doi:10.1016/0029-7844(96)00073-7

Idowu, B.M., Ibitoye, B.O. **Doppler sonography of perifibroid and intrafibroid arteries of uterine leiomyomas.** Obstet. Gynecol. Sci. 2018; 61: 395–403. doi:10.5468/ogs.2018.61.3.395

Idowu, B.M., Ibitoye, B.O., Adetiloye, V.A. **Uterine Artery Doppler Velocimetry of Uterine Leiomyomas in Nigerian Women.** Rev. Bras. Ginecol. Obstet. 2017; 39: 464–470. doi:10.1055/s-0037-1604489

Ikhena, D.E., Bulun, S.E. **Literature Review on the Role of Uterine Fibroids in Endometrial Function.** Reprod. Sci. 2018; 25: 635–643. doi:10.1177/1933719117725827

Kamphaus, G.D., Colorado, P.C., Panka, D.J., Hopfer, H., Ramchandran, R., Torre, A., Maeshima, Y., Mier, J.W., Sukhatme, V.P., Kalluri, R. **Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth.** J. Biol. Chem. 2000; 275: 1209–1215. doi:10.1074/jbc.275.2.1209

Kanelopoulos, N., Dendrinos, S., Oikonomou, A., Panagopoulos, P., Markussis, V. **Doppler-ultrasound as a predictor of uterine fibroid response to GnRH therapy.** Int. J. Gynaecol. Obstet. 2003; 82: 41–87. doi:10.1016/s0020-7292(03)00127-9

Kitaya, K., Yasuo, T. **Leukocyte density and composition in human cycling endometrium with uterine fibroids.** Hum. Immunol. 2010; 71: 158–163. doi:10.1016/j.humimm.2009.11.014

Kohler, G., Vollmer, M., Nath, N., Hessler, P.A., Dennis, K., Lehr, A., Koller, M., Riechmann, C., Bralo, H., Trojnarska, D., Lehnhoff, H., Krichbaum, J., Krichbaum, M., Evert, K., Evert, M., Zygmunt, M., Kaderali, L. **Benign uterine mass-discrimination from leiomyosarcoma by a preoperative risk score: a multicenter cohort study.** Arch. Gynecol. Obstet. 2019; 300: 1719–1727. doi:10.1007/s00404-019-05344-0

Konarska, M., Wrona, A.N., Aleksandrovych, V., Bereza, T., Sajewicz, M., Gach-Kuniewicz, B., Lis, M., Komnata, K., Paziewski, M., Maleszka, A., Depukat, P., Solewski, B., Warchol, L. **Angiogenesis and pro-angiogenic factors in uterine fibroids - facts and myths.** Folia Med. Cracov. 2016; 56: 37–43

Kurjak, A., Kupesic-Urek, S., Miric, D. **The assessment of benign uterine tumor vascularization by transvaginal color Doppler.** Ultrasound Med. Biol. 1992; 18: 645–649. doi:10.1016/0301-5629(92)90079-p

Lakhman, Y., Veeraraghavan, H., Chaim, J., Feier, D., Goldman, D.A., Moskowitz, C.S., Nougaret, S., Sosa, R.E., Vargas, H.A., Soslow, R.A., Abu-Rustum, N.R., Hricak, H., Sala, E. **Differentiation of Uterine Leiomyosarcoma from Atypical Leiomyoma: Diagnostic Accuracy of Qualitative MR Imaging Features and Feasibility of Texture Analysis.** Eur. Radiol. 2017; 27: 2903–2915. doi:10.1007/s00330-016-4623-9

Leppert, P., Fouany, M., Segars, J.H **Understanding uterine fibroids.** Segars J.H. Fibroids John Wiley & Sons, Ltd. Oxford 2013: 1–10

Levy, G., Hill, M.J., Beall, S., Zarek, S.M., Segars, J.H., Catherino, W.H. **Leiomyoma: genetics, assisted reproduction, pregnancy and therapeutic advances.** Journal of assisted reproduction and genetics 2012; 29: 703–712

Li, Y., Qiang, W., Griffin, B.B., Gao, T., Chakravarti, D., Bulun, S., Kim, J.J., Wei, J.J. **HMGA2-mediated tumorigenesis through angiogenesis in leiomyoma.** Fertil. Steril. 2020; 114: 1085–1096. doi:10.1016/j.fertnstert.2020.05.036

Li, Z., Zhang, P., Shen, H., Ding, B., Wang, H., Li, J., Shen, Y. **Clinical value of contrast-enhanced ultrasound for the differential diagnosis of specific subtypes of uterine leiomyomas.** J. Obstet. Gynaecol. Res. 2021; 47: 311–319. doi:10.1111/jog.14527

Ludovisi, M., Moro, F., Pasciuto, T., Di Noi, S., Giunchi, S., Savelli, L., Pascual, M.A., Sladkevicius, P., Alcazar, J.L., Franchi, D., Mancari, R., Moruzzi, M.C., Jurkovic, D., Chiappa, V., Guerriero, S., Exacoustos, C., Epstein, E., Fruhauf, F., Fischerova, D., Fruscio, R., Ciccarone, F., Zannoni, G.F., Scambia, G., Valentin, L., Testa, A.C. **Imaging in gynecological disease (15): clinical and ultrasound characteristics of uterine sarcoma.** Ultrasound Obstet. Gynecol. 2019; 54: 676–687. doi:10.1002/uog.20270

Makinen, N., Kampjarvi, K., Frizzell, N., Butzow, R., Vahteristo, P. **Characterization of MED12, HMGA2, and FH alterations reveals molecular variability in uterine smooth muscle tumors.** Mol. Cancer 2017; 16: 101. doi:10.1186/s12943-017-0672-1

Matta, W.H., Stabile, I., Shaw, R.W., Campbell, S. **Doppler assessment of uterine blood flow changes in patients with fibroids receiving the gonadotropin-releasing hormone agonist Buserelin.** Fertil. Steril. 1988; 49: 1083–1085. doi:10.1016/s0015-0282(16)59966-x

Mayer, Arnulf, Höckel, Michael, Wree, Alexander, Leo, Cornelia, Horn, Lars-Christian, Vaupel, Peter **Lack of hypoxic response in uterine leiomyomas despite severe tissue hypoxia.** Cancer research 2008; 68: 4719–4726

Mayer, Arnulf, Hoeckel, Michael, von Wallbrunn, Angelika, Horn, Lars-Christian, Wree, Alexander, & Vaupel, Peter. (2010). HIF-mediated hypoxic response is missing in severely hypoxic uterine leiomyomas. In Eiji Takahashi & Duane F Bruley (Eds.), *Oxygen*

Transport to Tissue XXXI (Vol. 662, pp. 399-405): Springer.

Mehine, M., Kaasinen, E., Heinonen, H.R., Makinen, N., Kampjarvi, K., Sarvilinna, N., Aavikko, M., Vaharautio, A., Pasanen, A., Butzow, R., Heikinheimo, O., Sjoberg, J., Pitkanen, E., Vahteristo, P., Aaltonen, L.A. **Integrated data analysis reveals uterine leiomyoma subtypes with distinct driver pathways and biomarkers.** Proc. Natl. Acad. Sci. U S A, 2016; 113: 1315–1320. doi:10.1073/pnas.1518752113

Minsart, A.F., Ntoutoume Sima, F., Vandenhoute, K., Jani, J., Van Pachterbeke, C **Does three-dimensional power Doppler ultrasound predict histopathological findings of uterine fibroids? A preliminary study.** Ultrasound Obstet. Gynecol. 2012; 40: 714–720. doi:10.1002/uog.11155

Muniz, C.J., Fleischer, A.C., Donnelly, E.F., Mazer, M.J. **Three-dimensional color Doppler sonography and uterine artery arteriography of fibroids: assessment of changes in vascularity before and after embolization.** J. Ultrasound Med. 2002; 21: 129–133. doi:10.7863/jum.2002.21.2.129

Murase, E., Siegelman, E.S., Outwater, E.K., Perez-Jaffe, L.A., Tureck, R.W. **Uterine leiomyomas: histopathologic features, MR imaging findings, differential diagnosis, and treatment.** Radiographics 1999; 19: 1179–1197. doi:10.1148/radiographics.19.5.g99se131179

Naguib, N.N., Nour-Eldin, N.E., Serag-Eldin, F., Mazloum, Y.Z., Agameya, A.F., Abou-Seif, S., Etaby, A.N., Lehnert, T., Gruber-Rouh, T., Zangos, S., Ackermann, H., Vogl, T.J. **Role of uterine artery Doppler in the management of uterine leiomyoma by arterial embolization.** Ultrasound Obstet. Gynecol. 2012; 40: 452–458. doi:10.1002/uog.11074

Nieuwenhuis, L.L., Betjes, H.E., Hehenkamp, W.J., Heymans, M.W., Brolmann, H.A., Huirne, J.A. **The use of 3D power Doppler ultrasound in the quantification of blood vessels in uterine fibroids: feasibility and reproducibility.** J. Clin. Ultrasound 2015; 43: 171–178. doi:10.1002/jcu.22208

Nieuwenhuis, L.L., Keizer, A.L., Stoelinga, B., Twisk, J., Hehenkamp, W., Brolmann, H., Huirne, J. **Fibroid vascularisation assessed with three-dimensional power Doppler ultrasound is a predictor for uterine fibroid growth: a prospective cohort study.** BJOG 2018; 125: 577–584. doi:10.1111/1471-0528.14608

Nougaret, S., Sbarra, M., Robbins, J. **Imaging Spectrum of Benign Uterine Disease and Treatment Options.** Radiol. Clin. North Am. 2020; 58: 239–256. doi:10.1016/j.rcl.2019.10.004

Oh, J., Park, S.B., Park, H.J., Lee, E.S. **Ultrasound Features of Uterine Sarcomas.** Ultrasound Q 2019; 35: 376–384. doi:10.1097/RUQ.0000000000000454

Ordulu, Z., Nucci, M.R., Dal Cin, P., Hollowell, M.L., Otis, C.N., Hornick, J.L., Park, P.J., Kim, T.M., Quade, B.J., Morton, C.C. **Intravenous leiomyomatosis: an unusual intermediate between benign and malignant uterine smooth muscle tumors.** Mod. Pathol. 2016; 29: 500–510. doi:10.1038/modpathol.2016.36

Pedeutour, F., Quade, B.J., Sornberger, K., Tallini, G., Ligon, A.H., Weremowicz, S., Morton, C.C. **Dysregulation of HMGIC in a uterine lipoleiomyoma with a complex rearrangement including chromosomes 7, 12, and 14.** Genes Chromosomes Cancer 2000; 27: 209–215

Pelage, J.P., Cazejust, J., Pluot, E., Le Dref, O., Laurent, A., Spies, J.B., Chagnon, S., Lacombe, P. **Uterine fibroid vascularization and clinical relevance to uterine fibroid embolization.** Radiographics 2005; 25: S99–117. doi:10.1148/rg.25si055510

Pron, G. **Magnetic Resonance-Guided High-Intensity Focused Ultrasound (MRgHIFU) Treatment of Symptomatic Uterine Fibroids: An Evidence-Based Analysis.** Ont. Health Technol. Assess Ser. 2015; 15: 1–86

Rakotomahenina, H., Rajaonarison, J., Wong, L., Brun, J.L. **Myomectomy: technique and current indications.** Minerva Ginecol. 2017; 69: 357–369. doi:10.23736/S0026-4784.17.04073-4

Salamonsen, L.A., Marsh, M.M., Findlay, J.K. **Endometrial endothelin: regulator of uterine bleeding and endometrial repair.** Clin. Exp. Pharmacol. Physiol. 1999; 26: 154–157. doi:10.1046/j.1440-1681.1999.03012.x

Secil, M., Dogra, V.S. **Color Flow Doppler Evaluation of Uterus and Ovaries and Its Optimization Techniques.** Ultrasound Clinics, Elsevier 2008; 3: 461–482. doi:10.1016/j.cult.2008.07.004

Sharma, K., Bora, M.K., Venkatesh, B.P., Barman, P., Roy, S.K., Jayagurunathan, U., Sellamuthu, E., Moidu, F. **Role of 3D Ultrasound and Doppler in Differentiating Clinically Suspected Cases of Leiomyoma and Adenomyosis of Uterus.** J. Clin. Diagn. Res. 2015; 9: QC08–QC12. doi:10.7860/JCDR/2015/12240.5846

Sladkevicius, P., Valentin, L., Marsal, K. **Transvaginal Doppler examination of uteri with myomas.** J. Clin. Ultrasound 1996; 24: 135–140. doi:10.1002/(SICI)1097-0096(199603)24:3<135::AID-JCU5>3.0.CO;2-I

Sosic, A., Skupski, D.W., Streltzoff, J., Yun, H., Chervenak, F.A. **Vascularity of uterine myomas: assessment by color and pulsed Doppler ultrasound.** Int. J. Gynaecol. Obstet. 1996; 54: 245–250. doi:10.1016/0020-7292(96)02722-1

Sozen, I., Arici, A. **Interactions of cytokines, growth factors, and the extracellular matrix in the cellular biology of uterine leiomyomata.** Fertility and Sterility 2002; 78: 1–12

Sozen, I., Olive, D.L., Arici, A. **Expression and hormonal regulation of monocyte chemotactic protein-1 in myometrium and leiomyomata.** Fertility and Sterility 1998; 69: 1095–1102

Standring, S. 2008 **Gray's Anatomy. The Anatomical Basis of Clinical Practice.** Churchill Livingstone Elsevier

Stepniak, A., Czuczwar, P. **3D Power Doppler vascular indices as a novel technique in assessing the outcome of minimally invasive techniques in uterine fibroids treatment.** Prz Menopauzalny 2017; 16: 118–121. doi:10.5114/pm.2017.72755

Stoelinga, B., Dooper, A.M.C., Juffermans, L.J.M., Postema, A.W., Wijkstra, H., Brolmann, H.A.M., Huirne, J.A.F. **Use of Contrast-Enhanced Ultrasound in the Assessment of Uterine Fibroids: A Feasibility Study.** Ultrasound Med. Biol. 2018; 44: 1901–1909. doi:10.1016/j.ultrasmedbio.2018.03.030

Sun, S., Bonaffini, P.A., Nougaret, S., Fournier, L., Dohan, A., Chong, J., Smith, J., Addley, H., Reinhold, C. **How to differentiate uterine leiomyosarcoma from leiomyoma with imaging.** Diagn. Interv. Imaging 2019; 100: 619–634. doi:10.1016/j.diii.2019.07.007

Syssoev, K.A., Kulagina, N.V., Chukhlovin, A.B., Morozova, E.B., Totolian, A.A. **Expression of mRNA for chemokines and chemokine receptors in tissues of the myometrium and uterine leiomyoma.** Bulletin of Experimental Biology and Medicine 2008; 145: 84–89

Szabo, I., Szantho, A., Papp, Z. **Uterine sarcoma: diagnosis with multiparameter sonographic analysis.** Ultrasound Obstet. Gynecol. 1997; 10: 220–221. doi:10.1046/j.1469-0705.1997.10030220.x

Tal, Reshef, Segars, James H **The role of angiogenic factors in fibroid pathogenesis: potential implications for future therapy.** Human Reproduction Update 2014; 20: 194–216

Testa, A.C., Pomini, F., Fattorossi, A., Battaglia, A., Ferrandina, G., Mansueto, D., Mastromarino, C., Scambia, G., Caruso, A. **Doppler velocimetry and cytofluorimetric analysis in uterine myomas.** Gynecol. Obstet. Invest. 2003; 56: 139–142. doi:10.1159/000073772

Timmerman, D., Verguts, J., Konstantinovic, M.L., Moerman, P., Van Schoubroeck, D., Deprest, J., van Huffel, S. **The pedicle artery sign based on sonography with color Doppler imaging can replace second-stage tests in women with abnormal vaginal bleeding.** Ultrasound Obstet. Gynecol. 2003; 22: 166–171. doi:10.1002/uog.203

Tinelli, A., Mynbaev, O.A., Mettler, L., Hurst, B.S., Pellegrino, M., Nicolardi, G., Kosmas, I., Malvasi, A. **A combined ultrasound and histologic approach for analysis of uterine fibroid pseudocapsule thickness.** Reprod. Sci. 2014; 21: 1177–1186. doi:10.1177/1933719114537719

Torkzaban, M., Machado, P., Gupta, I., Hai, Y., Forsberg, F. **Contrast-Enhanced Ultrasound for Monitoring Non-surgical Treatments of Uterine Fibroids: A Systematic Review.** Ultrasound Med. Biol. 2021; 47: 3–18. doi:10.1016/j.ultrasmedbio.2020.09.016

Tranquart, F., Brunereau, L., Cottier, J.P., Marret, H., Gallas, S., Lebrun, J.L., Body, G., Herbreteau, D., Pourcelot, L. **Prospective sonographic assessment of uterine artery embolization for the treatment of fibroids.** Ultrasound Obstet. Gynecol. 2002; 19: 81–87. doi:10.1046/j.0960-7692.2001.00535.x

Van den Bosch, T., Dueholm, M., Leone, F.P., Valentin, L., Rasmussen, C.K., Votino, A., Van Schoubroeck, D., Landolfo, C., Installe, A.J., Guerriero, S., Exacoustos, C., Gordts, S., Benacerraf, B., D'Hooghe, T., De Moor, B., Brolmann, H., Goldstein, S., Epstein, E., Bourne, T., Timmerman, D. **Terms, definitions and measurements to describe sonographic features of myometrium and uterine masses: a consensus opinion from the Morphological Uterus Sonographic Assessment (MUSA) group.** Ultrasound Obstet. Gynecol. 2015; 46: 284–298. doi:10.1002/uog.14806

Vincent, K.A., Feron, O., Kelly, R.A. **Harnessing the response to tissue hypoxia: HIF-1 alpha and therapeutic angiogenesis.** Trends Cardiovasc. Med. 2002; 12: 362–367. doi:10.1016/s1050-1738(02)00186-x

Walocha, J.A., Litwin, J.A., Miodonski, A.J. **Vascular system of intramural leiomyomata revealed by corrosion casting and scanning electron microscopy.** Hum. Reprod. 2003; 18: 1088–1093. doi:10.1093/humrep/deg213

Wei, J.J., Zhang, X.M., Chiriboga, L., Yee, H., Perle, M.A., Mittal, K. **Spatial differences in**

biologic activity of large uterine leiomyomata. Fertil. Steril. 2006; 85: 179–187. doi:10.1016/j.fertnstert.2005.07.1294

Weiner, Z., Beck, D., Rottem, S., Brandes, J.M., Thaler, I. **Uterine artery flow velocity waveforms and color flow imaging in women with perimenopausal and postmenopausal bleeding. Correlation to endometrial histopathology.** Acta Obstet. Gynecol. Scand. 1993; 72: 162–166. doi:10.3109/00016349309013365

Weintraub, J.L., Romano, W.J., Kirsch, M.J., Sampaleanu, D.M., Madrazo, B.L. **Uterine artery embolization: sonographic imaging findings.** J. Ultrasound Med. 2002; 21: 633–637. doi:10.7863/jum.2002.21.6.633

Weston, G., Trajstman, A.C., Gargett, C.E., Manuelpillai, U., Vollenhoven, B.J., Rogers, P.A. **Fibroids display an anti-angiogenic gene expression profile when compared with adjacent myometrium.** Mol. Hum. Reprod. 2003; 9: 541–549. doi:10.1093/molehr/gag066

Yamagata, Y., Maekawa, R., Asada, H., Taketani, T., Tamura, I., Tamura, H., Ogane, J., Hattori, N., Shiota, K., Sugino, N. **Aberrant DNA methylation status in human uterine leiomyoma.** Molecular human reproduction 2009; 15: 259–267

Zhang, G., Yu, X., Zhu, L., Fan, Q., Shi, H., Lang, J. **Preoperative clinical characteristics scoring system for differentiating uterine leiomyosarcoma from fibroid.** BMC Cancer 2020; 20: 514. doi:10.1186/s12885-020-07003-z

Zhang, L., Zhang, W., Orsi, F., Chen, W., Wang, Z. **Ultrasound-guided high intensity focused ultrasound for the treatment of gynaecological diseases: A review of safety and efficacy.** Int. J. Hyperthermia 2015; 31: 280–284. doi:10.3109/02656736.2014.996790

Received 17 June 2021; received in revised form 24 August 2021; accepted 10 September 2021.

Received: 3 December 2017 | Accepted: 12 February 2018
DOI: 10.1002/jcp.26537

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

Omega-3 fatty acids modulate the lipid profile, membrane architecture, and gene expression of leiomyoma cells

Md Soriful Islam[1,2] | Clara Castellucci[3] | Rosamaria Fiorini[4] | Stefania Greco[1] | Riccardo Gagliardi[5] | Alessandro Zannotti[1] | Stefano R. Giannubilo[6] | Andrea Ciavattini[6] | Natale G. Frega[7] | Deborah Pacetti[7] | Pasquapina Ciarmela[1,8]

[1] Department of Experimental and Clinical Medicine, Università Politecnica delle Marche, Ancona, Italy

[2] Biotechnology and Microbiology Laboratory, Department of Botany, University of Rajshahi, Rajshahi, Bangladesh

[3] Department of Clinical and Molecular Sciences, Università Politecnica delle Marche, Ancona, Italy

[4] Department of Life and Environmental Sciences, Università Politecnica delle Marche, Ancona, Italy

[5] Eureka srl – Lab Division, R&D Department, Chiaravalle (AN), Italy

[6] Department of Clinical Science, Università Politecnica delle Marche, Ancona, Italy

[7] Department of Agricultural, Food and Environmental Sciences, Università Politecnica delle Marche, Ancona, Italy

[8] Department of Information Engineering, Università Politecnica delle , Marche, Ancona, Italy

Correspondence
Pasquapina Ciarmela, PhD, Faculty of Medicine, Department of Experimental and Clinical Medicine, Università Politecnica delle Marche, via Tronto 10/a, 60020 Ancona, Italy.
Email: p.ciarmela@univpm.it
Deborah Pacetti, PhD, Department of Agricultural, Food and Environmental Sciences, Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy.
Email: d.pacetti@univpm.it

Uterine leiomyomas (fibroids or myomas) are the most common benign tumors of premenopausal women and new medical treatments are needed. This study aimed to determine the effects of omega-3 fatty acids on the lipid profile, membrane architecture and gene expression patterns of extracellular matrix components (collagen1A1, fibronectin, versican, or activin A), mechanical signaling (integrin β1, FAK, and AKAP13), sterol regulatory molecules (ABCG1, ABCA1, CAV1, and SREBF2), and mitochondrial enzyme (CYP11A1) in myometrial and leiomyoma cells. Myometrial tissues had a higher amount of arachidonic acid than leiomyoma tissues while leiomyoma tissues had a higher level of linoleic acid than myometrial tissues. Treatment of primary myometrial and leiomyoma cells with eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) reduced the monounsaturated fatty acid (MUFA) content and increased the polyunsaturated fatty acid (PUFA) content in both cell types. Myometrial and leiomyoma cell membranes were in the liquid-crystalline phase, but EPA- and DHA-treated cells had decreased membrane fluidity. While we found no changes in the mRNA expression of ECM components, EPA and DHA treatment reduced levels of ABCG1, ABCA1, and AKAP13 in both cell types. EPA and DHA also reduced FAK and CYP11A1 expression in myometrial cells. The ability of omega-3 fatty acids to remodel membrane architecture and downregulate the expression of genes involved in mechanical signaling and lipid accumulation in leiomyoma cells offers to further investigate this compound as preventive and/or therapeutic option.

KEYWORDS
ABCG1 and ABCA1, lipid profile, mechanical signaling, omega-3 fatty acids, uterine leiomyoma

1 | INTRODUCTION

Uterine leiomyomas (fibroids or myomas), originating from myometrial smooth muscle cells of the uterus, are the most common benign tumors of fertile women (Bulun, 2013; Protic et al., 2016). Uterine leiomyomas affect about 77% of women of reproductive-age and approximately 25% of reproductive-age women bear clinically apparent tumors (Buttram & Reiter, 1981; Cramer & Patel, 1990). The incidence and severity of symptoms typically depend on the size, number, and location of the fibroids (Buttram & Reiter, 1981). The common symptoms associated with uterine leiomyomas are irregular and excessive menstrual bleeding, which often causes anaemia, pain in

Md Soriful Islam, Clara Castellucci, and Rosamaria Fiorini contributed equally to this work.

 © 2018 Wiley Periodicals, Inc.

the back of the legs, pelvic pain or pressure, bowel and bladder dysfunctions, pressure sensation in the lower abdomen, pain during sexual intercourse, infertility, and recurrent abortion (Buttram & Reiter, 1981; Evans & Brunsell, 2007; Marsh & Bulun, 2006; Stewart, 2001). Uterine leiomyomas are currently the most common indication for hysterectomy in the world. Despite their high prevalence, significant associated health problems and huge economic impact on the healthcare system, relatively little is understood about the etiology and pathophysiology of uterine leiomyomas and effective therapeutic strategies are lacking. The prevalence of uterine leiomyoma is over three-fold higher in black women compared to white women (Marshall et al., 1997). In addition, age (late reproductive years), heredity, nulliparity, obesity, polycystic ovary syndrome, diabetes, hypertension, oral contraceptives, and hormone replacement therapy are major risk factors associated with this tumor (Flake, Andersen, & Dixon, 2003; Okolo, 2008; Walker & Stewart, 2005).

To date, genetic and epigenetic factors, sex steroids, growth factors, cytokines, chemokines, inflammation, and extracellular matrix (ECM) components are known factors involved in the pathogenesis of leiomyoma (Ciarmela et al., 2011; Gallagher & Morton, 2016; Islam, Akhtar, Segars, Castellucci, & Ciarmela, 2017; Islam et al., 2013; Protic et al., 2016; Yang, Mas, Diamond, & Al-Hendy, 2016). ECM components, mainly collagen1A1, fibronectin and versican are over-expressed in leiomyoma (Malik & Catherino, 2012), and their upregulation is induced by activin A (Islam et al., 2014). ECM proteins transmit mechanical signals from the outside of the cell to the cell interior through transmembrane integrin proteins. The functional activity of integrins may be modulated by modification of the fluidity of the cell membrane caused by alterations of lipid membrane composition.

Lipid homeostasis is fundamental for cellular function because of the key role of lipids in cell signaling, energy storage, and membrane functions, which are strictly dependent on membrane fluidity (Ibarguren, López, & Escribá, 2014). Lipidomics, or the comprehensive and quantitative analysis of lipid components in a given system, has been used to describe many pathological processes (Han, 2005; Rasmiena, Ng, & Meikle, 2013; Santos & Schulze, 2012). However, to our knowledge it has never been used to investigate uterine leiomyoma. Omega-3 fatty acids (also called ω3 PUFA or $n-3$ PUFA) are polyunsaturated fatty acids (PUFA) that are referred to as 'essential fatty acids' because they cannot be synthesized by mammals and must be obtained from the diet (Rose & Connolly, 1999). ω3 PUFA are commonly found in marine and plant oils and are being increasingly promoted as important dietary components for health and disease prevention. The active components of fish oil are generally considered to be the two ω3 PUFA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Whelan & Rust, 2006). Data from experimental and clinical studies have provided evidence that n-3 fatty acids are anti-inflammatory and immunomodulatory, making n-3 fatty acids potential therapeutic agents for inflammatory and autoimmune diseases (Calder, 2001).

The aim of the present study was to evaluate: (i) if the fatty acid profile of leiomyoma differs from that of healthy myometrium; (ii) if ω3 fatty acids modulate the lipidomic profile of the cell; and (iii) if ω3 fatty acids modulate the expression of ECM and mechanical signaling components as well as intracellular proteins such us sterol regulatory molecules involved in steroid synthesis and transport of cholesterol.

2 | MATERIALS AND METHODS

2.1 | Drugs and chemicals

EPA (cis-5,8,11,14,17-Eicosapentaenoic acid) and DHA (cis-4,7,10,13,16,19-Docosahexaenoic acid) were purchased from Sigma–Aldrich (St. Louis, MO). Both compounds were dissolved in ethanol (absolute) at 30 mM and then further diluted with culture medium to reach 50 μM at the time of cell treatment.Water (H_2O) of Liquid Chromatography-Mass Spectrometry (LC-MS) grade and purchased from Sigma–Aldrich. All others solvents and reagents were of analytical grade. Supelco 37 Component FAME Mix (certified reference material grade) was employed as standard mixture and purchased from Supelco (Bellefonte, PA).

2.2 | Leiomyoma and myometrial tissue collection

The study included premenopausal Caucasian women (41–49 years old) with symptomatic fibroids. Women did not receive hormonal treatment during the 3 months before to surgery. All patients gave their informed consent and the permission of the Human Investigation Committee was granted. Fibroid and myometrial tissues were obtained by hysterectomy or laparotomic myomectomy. The location of fibroids was submucosal and intramural, and their size range was 3–10 cm of diameter. Fibroid tissue was defined based on well-established histopathologic criteria.

2.3 | Primary cell cultures

Myometrial and leiomyoma samples were collected in Hanks'Balanced Salt Solution (HBSS) (Euroclone, Milan, Italy) at the time of surgery. Tissues were washed twice with Dulbecco's PBS (Invitrogen, Life Technologies, Carlsbad, CA) to remove excess blood. Samples were cut into small pieces with 0.1% collagenase type 8 (Serva Electrophoresis GmbH, Heidelberg, Germany) solutions in serum-free Dulbecco's Modified Eagle Medium (DMEM) (Sigma–Aldrich) containing 1% penicillin-streptomycin (Euroclone), 50 μg/L gentamicin (Lonza, Walkersville, MD), and 1% Amphotericin B (Lonza). Tissues were then incubated at 37 °C for about 3–5 hr in a water bath with manual shaken until completely digested. Digested cell suspensions were centrifuged at 1,200 rpm for 10 min, and washed once with fetal bovine serum (FBS) (Sigma–Aldrich). Finally, the cell pellet was dispersed in DMEM containing 10% FBS, 1% penicillin–streptomycin (Euroclone), 50 mg/L gentamicin (Lonza), and 1% Amphotericin B (Lonza), and plated in T25 or T75 plastic dishes, and maintained with same media at 37 °C in 95% air-5% CO_2. The growth medium was changed after 48 hr or 72 hr to remove unattached cells and then subsequently twice a week. The purity of cells was assessed by immunocytochemical staining with

specific smooth muscle cells marker, monoclonal mouse anti-α-smooth muscle actin (α-SMA) (Sigma–Aldrich). The lower passage number (up to 5) of cells was used for experiments to avoid changes in phenotype and gene expression.

2.4 | Transmethylation of uterine myometrium and uterine fibroma tissue fatty acids

The direct transmethylation reaction of the tissue fatty acids (FA) was performed adapting the method of Harris (Harris, Pottala, Vasan, Larson, & Robins, 2012). For each sample, 80 mg of tissue were transferred and freeze dried in a 2 ml screwed cap vial. A total of 250 µl of a solution of BF_3 (12% in MeOH anhydrous from Sigma–Aldrich®) and 250 µl of hexane were added in sequence to the freeze-dried sample. The vial was capped and the suspension was vortexed for 15 s. Then, the vial was put in the oven at 100 °C for 15 min. After cooling, 250 µl of H_2O were added to the sample and, after recapping the vial, the mixture was vortexed for 20 s. Two phases were obtained after centrifugation. A total of 150 µl of the upper hexane phase, containing the fatty acids methyl esters (FAME), were transferred in a 300 µl glass insert in a screwed cap vial. The solution was dried under nitrogen flow, and the FAME were dissolved in 50 µl of hexane. Samples were stored at −20 °C till the gaschromatographic (GC) analysis.

2.5 | Transmethylation of cell pellet fatty acids

Transmethylation reaction was performed modifying the method described by Harris (Harris et al., 2012). Briefly, 30 mg of defrosted cell pellets were suspended in 300 µl of water. The suspension was transferred to a 2 ml screwed cap vial and freeze dried. The transmethylation method to obtain the FAME from the freeze-dried cell pellets was the same employed for the derivatization of the FA from the freeze dried uterine myometrium and fibroma tissues reported above. Samples were stored at −20 °C till the GC analysis.

2.6 | GC-FID analysis of FAME

GC analysis of the FAME in cell pellets and uterine tissues samples was performed with a Varian® 430 GC. The chromatographic system was equipped with a split/splitless injector, a 100 m Varian® capillary column Select™ FAME (0.25 mm, 0.25 µm, #CP7421) and a flame ionization detector (FID). A total of 0.3 µl of sample were injected with a split ratio of 2. The injector and the FID were maintained at 260 °C. Helium (He) was employed as carrier gas with a flow rate of 1.6 ml/min. A gradient of temperature was set, with the oven starting at 160 °C and reaching 240 °C in 20 min and keeping for 15 min before cooling. Peaks were identified by comparison with known standards (Supelco 37 Component FAME Mix). Fatty acids composition (wt %) were calculated by the corrected peak area normalization method. The chromatographic system and the peak integration were managed using Galaxie software (Agilent, Santa Clara, CA).

2.7 | Laurdan fluorescence measurements

Laurdan (6-lauroyl-2-dimethylaminonaphthalene) is a fluorescent probe which locates in the bilayer by its lauric acid tail, with the fluorescent moiety localized at the level of the glycerol backbone (Antollini & Barrantes, 1998). It has a very high partition coefficient from aqueous environments to membranes and it is equally distributed between gel and liquid-crystalline (LC) phases. It is not affected by pH or by the chemical nature of phospholipid polar heads. Its spectral sensitivity to the membrane phase state can be related to its ability to sense polarity and the dynamics of water molecules (dipolar relaxation of water molecules) in the immediate vicinity of the fluorophore (Parasassi, De Stasio, Ravagnan, Rusch, & Gratton, 1991). Laurdan's spectral features are used to calculate the generalized polarization (GP) equation which provides information about the phospholipid phase of the membrane (Bagatolli & Gratton, 1999). Laurdan excitation (Ex) GP spectra were calculated as follows (Parasassi, Loiero, Raimondi, Ravagnan, & Gratton, 1993): Ex GP = $I_{450} - I_{490}/I_{450} + I_{490}$ where I_{450} and I_{490} are the intensities at each excitation wavelength, from 320 to 420 nm, obtained using a fixed emission wavelength of 450 and 490 nm, respectively. In the phospholipid gel phase, Laurdan Ex GP spectra are wavelength independent; in the LC phase, Ex GP values decrease with increasing excitation wavelength, with two coexisting phases, the GP spectrum has an opposite trend.

Low Ex GP values indicate a large water molecular mobility around the probe (thus a fluid membrane), while high Ex GP values indicate a rigid membrane with restricted reorientation of water molecules during the fluorescence lifetime. Laurdan steady-state fluorescence measurements were carried out on a computer-controlled Perkin Elmer (PerkinElmer Ltd., Buckinghamshire, UK) LS55 spectrofluorimeter. The fluorescence background obtained from cells without Laurdan was always subtracted from the data. Laurdan fluorescence spectra were measured at 37 °C and the temperature was measured in the sample by a digital thermometer. Cells were labeled with Laurdan at a final probe concentration of 1 µM. Each Laurdan spectrum corresponds to the average of determinations performed on five different samples ($n = 5$). All spectra were normalized by using Perkin Elmer FL WinLab Software.

2.8 | RNA isolation and real-time PCR

Primary myometrial and leiomyoma cells were treated with EPA or DHA (50 µM), and kept negative control (treated with 0.2% ethanol as we used to dissolve EPA and DHA) for 48 h, and lysed using TRIzol reagent (Ambion, Life Technologies), and stored at −80 °C. Total RNA (colorless upper aqueous phase) was separated using chloroform according to the manufacturer's instructions. After that, RNA was purified and concentrated using ReliaPrep™ RNA Cell Miniprep System (Promega Italia, Milan, Italy). The complementary DNA (cDNA) was generated from 1 µg of RNA using high-capacity cDNA reverse transcriptase (RT) kit (Applied Biosystems, Life Technologies), and newly synthesized cDNA was used for real-time PCR. Real-time PCR was performed on StepOnePlus version

2.2.2 (Applied Biosystems, Life Technologies) in 96-well optical reaction plates with 50 ng cDNA in a final volume of 15 µl, containing 1X TaqMan® fast advanced master mix, with the following TaqMan® gene expression assays (Applied Biosystems, Life Technologies) (Table 1). Controls included RNA subjected to RT-PCR without reverse transcriptase.

2.9 | Statistical analyses

Statistical analyses were performed using GraphPad Prism software (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

TABLE 1 List of primers used in this study

Gene name	Gene symbol	Gene aliases	Reference sequence	Assay ID	Amplicon length
Collagen, type I, alpha 1	COL1A1	OI4	NM_000088.3	Hs00164004_m1	66
Fibronectin 1	FN1	CIG, ED-B, FINC, FN, FNZ, GFND, GFND2, LETS, MSF	NM_002026.2 NM_054034.2 NM_212474.1 NM_212476.1 NM_212478.1 NM_212482.1	Hs00365052_m1	82
Versican	VCAN	CSPG2, ERVR, GHAP, PG-M, WGN, WGN1	NM_001126336.2 NM_001164097.1 NM_001164098.1 NM_004385.4	Hs00171642_m1	72
Inhibin, beta A	INHBA	EDF, FRP	NM_002192.2	Hs00170103_m1	65
Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	ITGB1	CD29, FNRB, GPIIA, MDF2, MSK12, RP11-479G22.2, VLA-BETA, VLAB	NM_002211.3 NM_033668.2 NM_133376.2	Hs00559595_m1	75
Protein tyrosine kinase 2	PTK2	FADK, FAK, FAK1, FRNK, PPP1R71, p125FAK, pp125FAK	NM_001199649.1 NM_005607.4 NM_153831.3	Hs01056457_m1	76
A kinase (PRKA) anchor protein 13	AKAP13	AKAP-13, AKAP-Lbc, ARHGEF13, BRX, HA-3, Ht31, LBC, PRKA13, PROTO-LB, PROTO-LBC, c-lbc, p47	NM_001270546.1 NM_006738.5 NM_007200.4	Hs00180747_m1	105
ATP-binding cassette, sub-family G (WHITE), member 1	ABCG1	ABC8, WHITE1	NM_004915.3 NM_016818.2 NM_207174.1 NM_207627.1 NM_207628.1 NM_207629.1	Hs00245154_m1	58
ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1	ABC-1, ABC1, CERP, HDLDT1, TGD	NM_005502.3	Hs01059118_m1	61
Caveolin 1, caveolae protein, 22 kDa	CAV1	BSCL3, CGL3, MSTP085, PPH3, VIP21	NM_001172895.1 NM_001172896.1 NM_001172897.1 NM_001753.4	Hs00971716_m1	66
Sterol regulatory element binding transcription factor 2	SREBF2	CTA-250D10.14–005, SREBP-2, SREBP2, bHLHd2	NM_004599.3 NR_103834.1	Hs01081784_m1	91
Cytochrome P450, family 11, subfamily A, polypeptide 1	CYP11A1	CYP11A, CYPXIA1, P450SCC	NM_000781.2 NM_001099773.1	Hs00167984_m1	77
Phosphatidylethanolamine binding protein 1	PEBP1	HCNP, HCNPpp, PBP, PEBP, PEBP-1, RKIP	NM_002567.2	Hs00831506_g1	134
Hypoxanthine phosphoribosyltransferase 1	HPRT1	HGPRT, HPRT	NM_000194.2	Hs99999909_m1	100
Actin, beta	ACT B	BRWS1, PS1TP5BP1	NM_001101.3	Hs99999903_m1	171

as mean ± SD. Differences were considered significant when $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The data obtained from the GC-FID analysis of tissues and cells FA were organized and processed by means of Office Excel software (Microsoft). Principal component analysis (PCA) of the results was performed by means the Office Excel add-in Multibase package (Numerical Dynamics, Japan).

3 | RESULTS

3.1 | Total fatty acid composition of the leiomyoma and myometrial tissues

The fatty acid composition of the leiomyoma and myometrial tissues ($n = 8$) is summarized in Table 2. Arachidonic acid was the most abundant fatty acid in both leiomyoma and myometrial tissues, followed by oleic acid, and two saturated species, palmitic and the stearic acids. The fatty acid composition of leiomyoma and myometrial tissues had both a high content of saturated fatty acids (SFA) (> 40%) and a lower content of monounsaturated fatty acids (MUFA) (about 21–22%). The total amount of PUFA was similar in the two tissues, with relative percentages of approximately 35% for each.

Although the fatty acid composition of leiomyoma and myometrial tissues was highly similar, some statistically significant differences were apparent. For example, the myometrial tissue was significantly richer in arachidonic acid (22.4 ± 1.5%) than the myometrial tissue (19.9 ± 1.7%). Also, the leiomyoma tissue had a higher content of linoleic acid, the endogenous precursor of arachidonic acid, than myometrial tissue (8.0 ± 1.4% vs. 5.9 ± 0.7%, respectively).

TABLE 2 Total fatty acid composition (weight % of total fatty acids) of the leiomyoma (FT) and myometrial (MT) tissues, with statistical analysis

Name	Carbon number: double bond number	Weight % of total fatty acids		*p*
		FT	MT	
Myristic acid	14:0	2.2 ± 0.7	2.0 ± 0.2	
Pentadecylic acid	15:0	1.6 ± 0.3	2.0 ± 0.6	
Palmitic acid	16:0	17.6 ± 1.5	17.5 ± 1.4	
Margaric acid	17:0	0.3 ± 0.1	0.3 ± 0.1	
Stearic acid	18:0	16.9 ± 1.4	17.4 ± 0.8	
Lignoceric acid	24:0	2.7 ± 0.5	3.1 ± 0.4	*
	ΣSFA	**41.2 ± 0.8**	**42.3 ± 1.3**	*
Pentadecenoic acid	15:1	0.5 ± 0.2	0.5 ± 0.3	
Palmitoleic acid	16:1 *n*-7	0.8 ± 0.3	0.7 ± 0.2	
Oleic acid	18:1 *n*-9	19.0 ± 1.4	18.1 ± 1.8	
Vaccenic acid	18:1 *n*-7	2.5 ± 0.7	2.2 ± 0.3	
Nervonic acid	24:1	0.2 ± 0.2	0.3 ± 0.1	
	ΣMUFA	**22.3 ± 1.1**	**21.1 ± 1.6**	
Linoleic acid	18:2 *n*-6 cis	8.0 ± 1.4	5.9 ± 0.7	***
γ-Linolenic acid	18:3 *n*-6	0.2 ± 0.1	0.1 ± 0.0	
α-Linolenic acid	18:3 *n*-3	0.1 ± 0.0	0.1 ± 0.0	
Stearidonic acid	18:4 *n*-3	0.5 ± 0.2	0.5 ± 0.1	
Eicosadienoic acid	20:2 *n*-6	0.3 ± 0.1	0.3 ± 0.1	
Dihomo-γ-linolenic acid	20:3 *n*-6	3.2 ± 0.4	3.4 ± 0.8	
Arachidonic acid	20:4 *n*-6	19.9 ± 1.7	22.4 ± 1.5	**
Eicosapentaenoic acid	20:5 *n*-3, EPA	0.2 ± 0.1	0.2 ± 0.1	
Docosapentaenoic acid	22:5 *n*-3, DPA	1.1 ± 0.2	1.0 ± 0.2	
Docosahexaenoic acid	22:6 *n*-3, DHA	1.2 ± 0.2	1.3 ± 0.4	
	ΣPUFA	**34.7 ± 1.0**	**35.1 ± 1.5**	
	ΣPUFA ω3	3.0 ± 0.3	3.0 ± 0.5	
	ΣPUFA ω6	31.6 ± 1.6	32.1 ± 1.3	
	Lipophilic index	20.6 ± 1.0	20.1 ± 1.5	
	Other peaks	0.9 ± 0.3	0.7 ± 0.4	

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Results represent means ± S.D. ($n = 8$);. − $p > 0.05$ (no significant difference); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The total SFA percentage of myometrial tissue was significantly higher than that of leiomyoma tissue (42.3 ± 1.3% vs. 41.2 ± 0.8%, respectively), but no significant differences were found between the levels of palmitic and stearic acids, the two main saturated species. Concerning the SFA profile, the only significant difference was that lignoceric acid (C24:0) was higher in myometrial than in leiomyoma samples (3.1 ± 0.4% vs. 2.7 ± 0.5%, respectively).

For both myometrial and leiomyoma tissues, the ω6 and ω3 fatty acids comprised approximately 30% and 3% of the total fatty acid content, respectively.

3.2 | Effects of DHA and EPA on fatty acid composition of primary myometrial and leiomyoma cells in vitro

The results concerning the fatty acid analysis of primary cells isolated from myometrial tissue and then treated with EPA or DHA in vitro are reported in Table 3. In both treated and untreated myometrial cells ($n = 8$) the SFA were the main constituents, while the PUFA were present in lower abundance. In the untreated control cells, the principal fatty acid was oleic acid (25.6 ± 2.0%) followed by palmitic acid

TABLE 3 Total fatty acid composition (weight % of total fatty acids) of cells isolated from myometrial tissue, untreated (Myo_ctrl) and treated in vitro with DHA (Myo_DHA) and with EPA (Myo_EPA), with statistical analysis

Fatty acids		Myo_ctrl	Myo_DHA	Myo_EPA
Name	Carbon number: double bond number	Weight % of total fatty acids		
Myristic acid	14:0	2.4±0.7	2.5±0.5	2.6±0.5
Pentadecylic acid	15:0	1.3±0.2	1.1±0.2	1.1±0.2
Palmitic acid	16:0	19.9±2.3	21.8±2.1	21.1±1.4
Margaric acid	17:0	0.9±0.1	0.8±0.1	0.8±0.1
Stearic acid	18:0	18.0±2.8	19.7±2.4	18.5±2.4
Lignoceric acid	24:0	1.1±0.3^{b}	0.7±0.1^{a}	0.8±0.1^{a}
	ΣSFA	**43.5±5.3**	**46.6±4.1**	**44.9±2.9**
Pentadecenoic acid	15:1	1.0±0.8	0.8±0.5	0.8±0.4
Palmitoleic acid	16:1 *n*-7	5.7±0.5^{b}	4.3±0.6^{a}	4.2±0.7^{a}
Heptadecenoic acid	17:1	0.6±0.4	0.4±0.3	0.3±0.3
Oleic acid	18:1 *n*-9	25.6±2.0^{b}	18.6±3.0^{a}	17.9±2.7^{a}
Vaccenic acid	18:1 *n*-7	8.9±2.3^{b}	6.7±1.9^{ab}	6.4±1.6^{a}
Nervonic acid	24:1	0.3±0.1^{b}	0.1±0.0^{a}	0.1±0.0^{a}
	ΣMUFA	**42.1±1.1^{b}**	**30.8±4.9^{a}**	**29.7±4.7^{a}**
Linoleic acid	18:2 *n*-6 cis	1.0±0.4^{a}	1.6±0.3^{b}	1.6±0.3^{b}
γ-Linolenic acid	18:3 *n*-6	0.0±0.0	0.1±0.0	0.1±0.1
α-Linolenic acid	18:3 *n*-3	0.1±0.1	0.1±0.0	0.1±0.0
Stearidonic acid	18:4 *n*-3	0.4±0.1^{b}	0.2±0.0^{a}	0.1±0.0^{a}
Eicosadienoic acid	20:2 *n*-6	0.1±0.1	0.1±0.0	0.1±0.0
Dihomo-γ-linolenic acid	20:3 *n*-6	1.1±0.2	1.2±0.3	1.1±0.2
Arachidonic acid	20:4 *n*-6	5.2±1.5^{b}	3.4±0.6^{a}	3.4±0.6^{a}
Eicosapentaenoic acid	20:5 *n*-3, EPA	0.3±0.2^{a}	1.2±0.8^{a}	5.1±2.2^{b}
Docosapentaenoic acid	22:5 *n*-3, DPA	1.7±0.9^{a}	2.2±1.1^{a}	10.5±3.4^{b}
Docosahexaenoic acid	22:6 *n*-3, DHA	2.3±1.2^{a}	11.0±5.6^{b}	1.4±0.8^{a}
	ΣPUFA	**12.3±4.3^{a}**	**21.0±8.4^{ab}**	**23.7±7.0^{b}**
	ΣPUFA ω3	4.8±2.3^{a}	14.7±7.3^{b}	17.4±6.0^{b}
	ΣPUFA ω6	7.5±2.1	6.3±1.1	6.4±1.0
	Lipophilic index	30.8±5.0	27.1±7.0	23.2±5.9
	Other peaks	2.0±0.2	1.6±0.2	1.7±0.3

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Results represent means ± S.D. ($n = 8$); differences were considered significant for $p < 0.05$.

Different superscripts $^{(a,b)}$ denote significant differences ($p < 0.05$), according to the MSD (minimum significant differences) with respect to the other groups. ∑SFA, total weigth % of the saturated fatty acids; ∑MUFA, total weigth % of monounsaturated fatty acids; ∑PUFA, total weigth % of polyunsaturated fatty acids.

(19.9 ± 2.3%) and stearic acid (18.0 ± 2.8%). By contrast, palmitic acid was the most abundant fatty acid in cells treated with either EPA (21.8 ± 2.1%) or DHA (21.1 ± 1.4%), followed by stearic acid and oleic acid. Myometrial cells treated with DHA showed a significant reduction of MUFA content compared to the untreated cells and an enhancement of the PUFA fraction, although this increase did not achieve statistical significance. Upon treatment of myometrial cells with EPA, both the MUFA reduction and the PUFA increase were significant respect to control cells.

With regard to monounsaturated fatty acids, treatment of myometrial cells with DHA caused a reduction in the content of oleic acid (25.6 ± 2.0% to 18.6 ± 3.0%), palmitoleic acid (5.7 ± 0.5% to 4.3 ± 0.6%), and nervonic acid (0.3 ± 0.1% to 0.1 ± 0.0%) relative to control cells. The content of vaccenic acid (18:1 ω7) also appeared to be reduced upon DHA treatment, but this did not reach statistical significance. Treatment with EPA had a similar effect on the polyunsaturated fatty acid composition of myometrial cells and caused the relative content of oleic acid to decrease from 25.6 ± 2.0% in untreated cells to the 17.9 ± 2.7% in treated cells. Also, palmitoleic, nervonic, and vaccenic acids decreased significantly following treatment with EPA.

The PUFA composition of myometrial cells was strongly affected by omega-3 treatments. The arachidonic acid content decreased from a value of 5.2 ± 1.5% in the control samples to values of 3.4 ± 0.6% following treatment with either DHA or EPA. By contrast, DHA and EPA both increased linoleic acid content from 1.0 ± 0.4% to 1.6 ± 0.3%. Treatment of myometrial cells with DHA and EPA caused a small but significant decrease in the level of 18:4 ω3. Considering the amounts of EPA, DHA, and DPA in myometrial cells, the two treatments had different effects. In cells treated with DHA, the EPA, and DPA levels were not different from those of control cells. However, the cells undoubtedly assimilated DHA since the relative DHA content increased from 2.3 ± 1.2% to 11.0 ± 5.6%. On the other hand, myometrial cells treated with EPA showed a significant increase in the levels of EPA (5.1 ± 2.2% to 10.5 ± 3.4%) and DPA (0.3 ± 0.2% to 1.7 ± 0.9%), while the DHA level was not affected by EPA in vitro addition.

Leiomyoma cells (n = 8) responded to treatment with EPA or DHA in manner that was similar to myometrial cells (Table 4). As was observed with myometrial cells, the SFA were the main fatty acids in the treated and untreated leiomyoma cells, representing about 45% of the total fatty acids. Neither EPA nor DHA treatment affected the saturated acidic fraction, except for causing a slight decrease in the level of lignoceric acid.

Treatment of leiomyoma cells with DHA caused a reduction in the total MUFA content from 41.0 ± 3.5% in untreated cells to 32.1 ± 0.4% (p < 0.05) in treated cells. EPA treatment similarly caused the MUFA content to drop to 32.1 ± 1.3% and also caused a significant decrease in the relative content of oleic, palmitoleic, vaccenic, and nervonic acids (Table 4). DHA addition reduced the percentages of the same MUFA species, but did not cause a significant decrease in the level of vaccenic acid.

Treatment with either EPA or DHA increased the level of total PUFA in leiomyoma cells, but this increase was significant only for the cells treated with EPA (p < 0.05). As observed for the myometrial cells, EPA addition caused a significant decrease in the levels of arachidonic acid (6.6 ± 1.9% to 4.0 ± 0.8%) and of C18:4 ω3 (0.3 ± 0.2% to 0.1 ± 0.0%). On the contrary, EPA significantly increased linoleic acid, EPA, and DPA content.

Beyond the significant reduction of the levels of arachidonic and C18:4 ω3, the increase in the content of linoleic acid (p < 0.5) was similar to that caused by the addition of EPA. In contrast to the EPA effect, treatment of leiomyoma cells with DHA caused a significant increase in DHA levels (1.8 ± 1.1% vs. 9.4 ± 2.9%), with no effect on DPA and EPA content. The total PUFA ω3 content of leiomyoma cells was also enhanced from 4.1 ± 2.2% in untreated cells to 12.8 ± 4.2% in DHA treated cells and 13.9 ± 2.0% in EPA treated cells. DHA and EPA treatments had not effect on the PUFA ω6 fraction.

Considering our data (Tables 3 and 4), it is noteworthy that cells isolated from myometrial and leiomyoma tissues had similar responses to treatment with either EPA or DHA. Both treatments provoked a reduction in the MUFA content of the cells and an increase in the PUFA content, and neither affected the fraction of saturated FA. The only differences between the cells treated with EPA and those treated with DHA were found in the ω3 FA composition. The cells treated with DHA were enriched in DHA, with no effects on EPA and DPA. By contrast, treatment with EPA caused an increase in DPA and EPA levels in both myometrial and leiomyoma cells. It is interesting that the concentration of DPA in the cells treated with EPA was mainly increased relative to the level of EPA in the cells.

3.3 | Effect of EPA and DHA on the membrane architecture of myometrial and leiomyoma cells

Laurdan Ex GP spectra indicated that the cell membranes of both untreated myometrial and leiomyoma cells (n = 5) were in the liquid-crystalline phase. In both cell types, EPA and DHA treatment resulted in Laurdan Ex GP values that were higher than those of untreated cells, indicating that the probe was in a more rigid environment with a lower rate of mobility due to a less fluid membrane (Figure 1).

3.4 | Effect of EPA and DHA on mRNA expression of extracellular matrix components and activin A in primary myometrial and leiomyoma cells

EPA or DHA (50 μM for 48 hr) treatment had no significant effect on collagen1A1, fibronectin, versican, or activin A mRNA expression in myometrial and leiomyoma cells (n = 3), compared to the untreated control (Figure 2).

3.5 | Effect of EPA and DHA on mRNA expression of mechanical signaling molecules in primary myometrial and leiomyoma cells

The effects of EPA or DHA treatment on the expression of mechanical signaling molecules, such as integrin β1, FAK, and AKAP13 in myometrial and leiomyoma cells are shown in Figure 3. The real-time PCR showed that AKAP13 was significantly downregulated by EPA and

TABLE 4 Total fatty acid composition (weight % of total fatty acids) of cells isolated from leiomyoma tissue, untreated (Fib_ctrl) and treated in vitro with DHA (Fib_DHA) and with EPA (Fib_EPA), with statistical analysis

		Fib_ctrl	Fib_DHA	Fib_EPA
Name	Carbon number: double bond number	Weight % of total fatty acids		
Myristic acid	14:0	2.1 ± 0.5	2.3 ± 0.6	2.5 ± 0.7
Pentadecylic acid	15:0	1.7 ± 0.5	1.5 ± 0.3	1.5 ± 0.6
Palmitic acid	16:0	20.3 ± 1.9	20.9 ± 2.8	20.8 ± 2.1
Margaric acid	17:0	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Stearic acid	18:0	18.3 ± 3.3	19.9 ± 2.2	18.7 ± 1.6
Lignoceric acid	24:0	1.1 ± 0.3^{b}	0.7 ± 0.2^{a}	0.8 ± 0.2^{a}
	ΣSFA	**44.2 ± 5.5**	**46.1 ± 5.2**	**45.1 ± 3.4**
Pentadecenoic acid	15:1	0.9 ± 0.4	1.3 ± 0.7	1.5 ± 0.8
Palmitoleic acid	16:1 *n*-7	6.5 ± 1.0^{b}	4.9 ± 0.6^{a}	4.9 ± 0.8^{a}
Heptadecenoic acid	17:1	0.2 ± 0.2	0.4 ± 0.3	0.4 ± 0.3
Oleic acid	18:1 *n*-9	24.0 ± 2.3^{b}	19.0 ± 0.6^{a}	18.9 ± 0.4^{a}
Vaccenic acid	18:1 *n*-7	9.2 ± 0.6^{b}	6.5 ± 0.5^{ab}	6.4 ± 0.5^{a}
Nervonic acid	24:1	0.2 ± 0.1^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
	ΣMUFA	**41.0 ± 3.5^{b}**	**32.1 ± 0.4^{a}**	**32.1 ± 1.3^{a}**
Linoleic acid	18:2 *n*-6 cis	1.0 ± 0.2^{a}	1.5 ± 0.1^{b}	1.5 ± 0.2^{b}
γ-Linolenic acid	18:3 *n*-6	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
α-Linolenic acid	18:3 *n*-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Stearidonic acid	18:4 *n*-3	0.3 ± 0.2^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
Eicosadienoic acid	20:2 *n*-6	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.0
Dihomo-γ-linolenic acid	20:3 *n*-6	1.1 ± 0.3	1.2 ± 0.1	1.1 ± 0.1
Arachidonic acid	20:4 *n*-6	6.6 ± 1.9^{b}	4.6 ± 1.8^{a}	4.0 ± 0.8^{a}
Eicosapentaenoic acid	20:5 *n*-3, EPA	0.3 ± 0.1^{a}	1.5 ± 0.7^{a}	5.3 ± 1.8^{b}
Docosapentaenoic acid	22:5 *n*-3, DPA	1.6 ± 0.9^{a}	1.6 ± 0.9^{a}	7.0 ± 0.9^{b}
Docosahexaenoic acid	22:6 *n*-3, DHA	1.8 ± 1.1^{a}	9.4 ± 2.9^{b}	1.3 ± 1.0^{a}
	ΣPUFA	**12.9 ± 2.6^{a}**	**20.3 ± 4.9^{ab}**	**20.7 ± 2.1^{b}**
	ΣPUFA ω3	4.1 ± 2.2^{a}	12.8 ± 4.2^{b}	13.9 ± 2.0^{b}
	ΣPUFA ω6	8.8 ± 1.9	7.5 ± 1.9	6.8 ± 0.8
	Lipophilic index	30.5 ± 4.2	27.2 ± 5.7	25.4 ± 3.1
	Other peaks	1.8 ± 0.4	1.5 ± 0.4	1.7 ± 0.2

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Results represent means ± S.D. ($n = 8$); differences were considered significant for $p < 0.05$.
Different superscripts $^{(a,b)}$ denote significant differences ($p < 0.05$), according to the MSD (minimum significant differences) with respect to the other groups.
∑SFA, total weigth % of the saturated fatty acids; ∑MUFA, total weigth % of monounsaturated fatty acids; ∑PUFA, total weigth % of polyunsaturated fatty acids.

by DHA about 0.8 fold changed over control, in both myometrial and leiomyoma cells (Figure 3). In addition, in myometrial cells, FAK mRNA expression was decreased by EPA and DHA about 0.8 fold changed over control (Figure 3).

3.6 | Effect of EPA and DHA on mRNA expression of sterol regulatory molecules in primary myometrial and leiomyoma cells

Results of real-time PCR, performed on primary myometrial and leiomyoma cells treated with EPA or DHA at 50 μM for 48 hr, showed that ABCG1 and ABCA1 were significantly reduced by EPA and by DHA to about one fifth of the untreated control, in both myometrial and leiomyoma cells (Figure 4). In addition, in myometrial cells, SREBF2 was downregulated 0.72 fold change by DHA treatment compared to untreated control (Figure 4).

3.7 | Effect of EPA and DHA on mRNA expression of mitochondrial enzyme CYP11A1 in myometrial and leiomyoma cells

Considering the role of CYP11A1 in the catabolism of steroid hormones, we aimed to see if treatment with EPA and DHA alters the expression of CYP11A1 in myometrial and leiomyoma cells. We

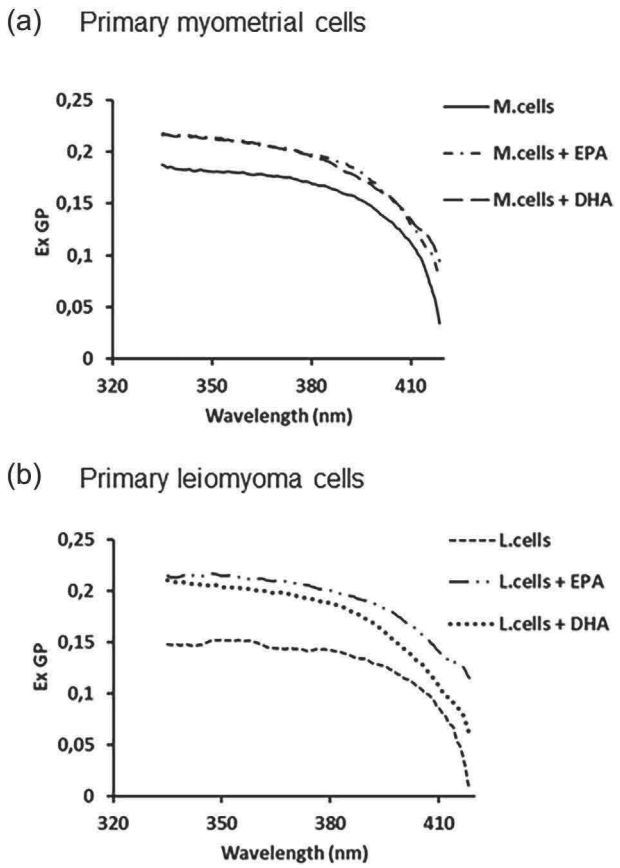


FIGURE 1 (a) Laurdan excitation generalized polarization (Ex GP) spectra in myometrial cells; untreated (—) and treated with EPA (– · –) or DHA (— —). (b) Laurdan excitation generalized polarization (Ex GP) spectra in leiomyomas cells that were untreated (---) or treated with EPA (– · · –) or DHA (· · · ·)*N* = 5

found that CYP11A1 mRNA expression was significantly reduced of about a third by EPA and DHA, in myometrial cells but not in leiomyoma cells (Figure 5).

4 | DISCUSSION

The lipidomic investigation through GC-FID analysis revealed the specific fatty acid composition in leiomyoma and healthy myometrial tissue, for example, our results show that arachidonic acid is present at significantly higher levels in myometrial tissue than in leiomyoma tissue, while linoleic acid was higher in leiomyoma tissue. Although not exhaustive, this study represents the first demonstration that uterine leiomyoma tissue differs from the adjacent normal myometrial tissue with regard to its fatty acid profile .

We also tested if it is possible to modulate the fatty acid composition of primary myometrial and leiomyoma cells cultured in vitro. For this purpose, we tested the effect of treating cells with the ω3 fatty acids, EPA, and DHA. The effects of DHA treatment on leiomyoma cells were also similar to its effects on myometrial cells. Both cell types were enriched in DHA following treatment with DHA, and enriched in EPA following treatment with EPA, demonstrating that the cells are able to incorporate the ω3 fatty acids. The treatment with EPA increased also DPA demonstrating the activation of the elongation process of EPA. We found that both EPA and DHA also reduced the amount of the arachidonic acid. Interestingly, the ability of omega-3 fatty acids to interfere with arachidonic acid metabolism has been reported to be the heart of their anti-inflammatory effects (De Caterina & Libby, 1996). These results encouraged us to explore the possible effects of ω3 fatty acids on the cell membrane, which plays a critical role in cellular communication and function.

4.1 | Our Laurdan Ex

GP results show that myometrial and leiomyoma cell membranes are in the liquid-crystalline phase when untreated but that most of the Laurdan molecules are located in a less fluid environment in the membranes following treatment with EPA or DHA. This was surprising since we expected that treatment of both cell types with long-chain PUFA would be compatible with a much more fluid membrane microenvironment. However, our results are consistent with those of Teague, Ross, Harris, Mitchell, and Shaikh, 2013 who showed that DHA, despite its flexible structure, can increase membrane molecular order in primary B cells, EL4 cells and liposomes of varying composition. Furthermore, Kim, Barhoumi, McMurray, and Chapkin, 2014 used Laurdan fluorescence polarization microscopy to show that membrane enrichment with dietary n-3 PUFA in immunological synapses of CD4+ T cells from DO11.10 T-cell receptor transgenic mice increases membrane molecular order.

Lipid-protein interactions are very important in membranes and proteins may affect lipid properties, such as the acyl chain order, the lateral, and transmembrane distribution and the mobility (Nyholm, 2015). Overall, our data suggest that DHA and EPA remodel membrane architecture with possible effects on several cellular properties including cell signaling. We focused on genes that characterize leiomyomas and that could be modulated in their expression by changes in the structure of the plasma membrane. These include molecules of the ECM, which are anchored externally to the plasma membrane and thus involved in mechanical signals. Considering that we studied reproductive tissues, we also checked molecules of mechanisms involved in steroid synthesis and transport of cholesterol.

While we found no changes in gene expression levels of ECM components and activin A, we detected some modification in the expression of molecules involved in mechanical signal transmission such as integrin β1, FAK, AKAP13, although these changes were mainly restricted to myometrial cells.

Mechanical signals are transmitted from the ECM through integrins, which are heterodimeric transmembrane receptors. Most integrins recruit cytoplasmatic focal adhesion kinase (FAK) and activate downstream pathways, including Rho-dependent signaling (PaszeK et al., 2005). A kinase anchor protein 13 (AKAP13) is a RhoA GTPase-specific guanine exchange factor (Rho-GEF) that converts RhoA from its inactive GDP-bound form to its active GTP-bound form.

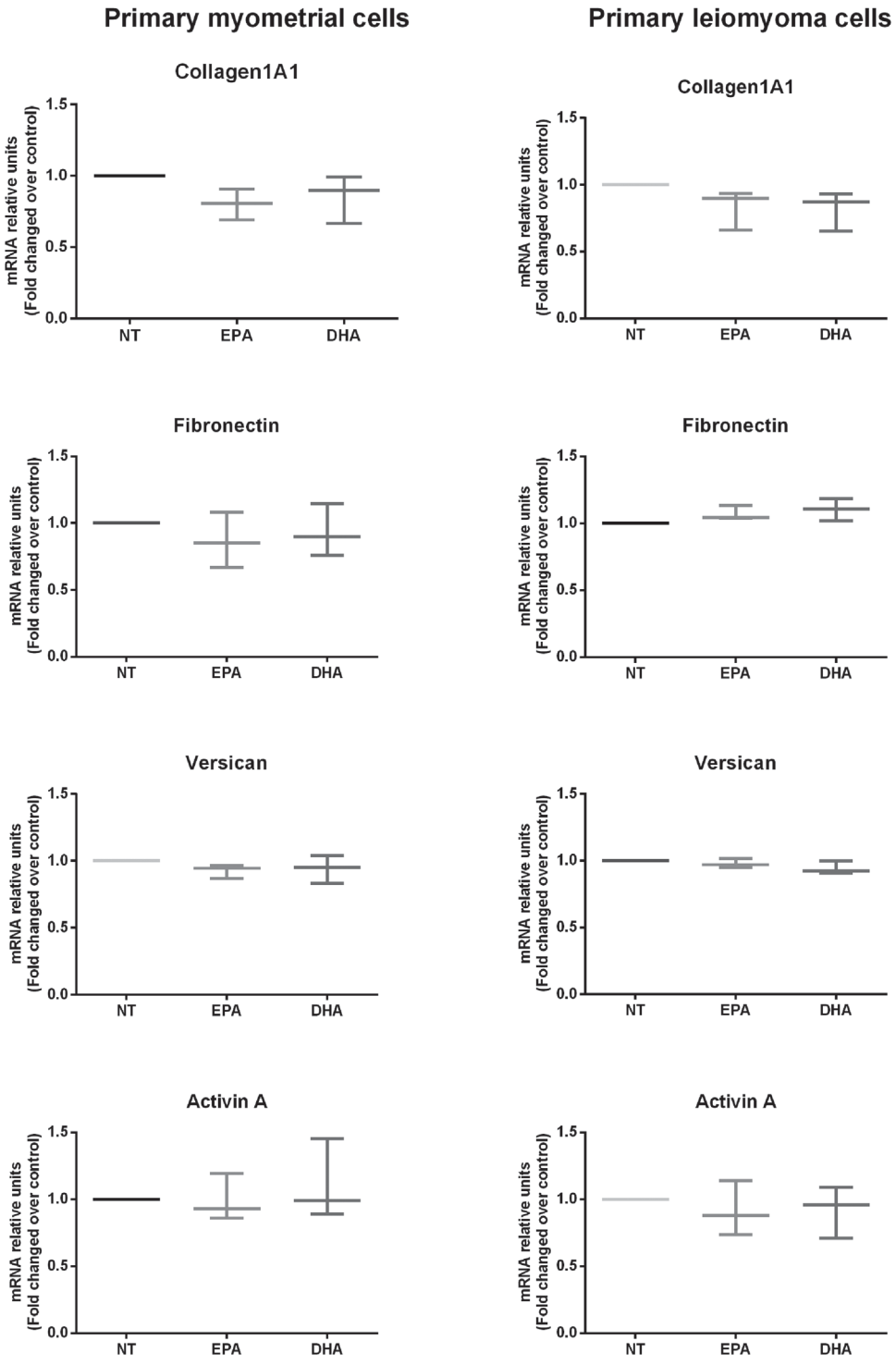


FIGURE 2 Effect of EPA and DHA treatment on mRNA expression of extracellular matrix components and activin A in primary myometrial and leiomyoma cells. Data are expressed as mean ± SD (n = 3). NT, No treatment; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid

The levels of integrin β1 (Chen, Lin, Cheng, & Wing, 2013), AKAP13 (Rogers et al., 2008), and phosphorylated FAK (Chen et al., 2013) were all found to be higher in leiomyoma relative to myometrium.

We found that treatment of cells with ω3 fatty acids reduces the expression of key genes that mediate lipid accumulation, such as ABCG1 and ABCA1. Those proteins are involved in the efflux of cholesterol, the common precursor of steroid hormones and fat-soluble vitamins (A, D, E, K) (Ikonen, 2008), as well as a modulator of human oxytocin receptor membrane activity, stability and affinity to oxytocin. In this regard, altered myometrial cholesterol content has been associated with abnormal oxytocin-induced uterine smooth muscle contraction in rodents. In addition, prevention of accumulation

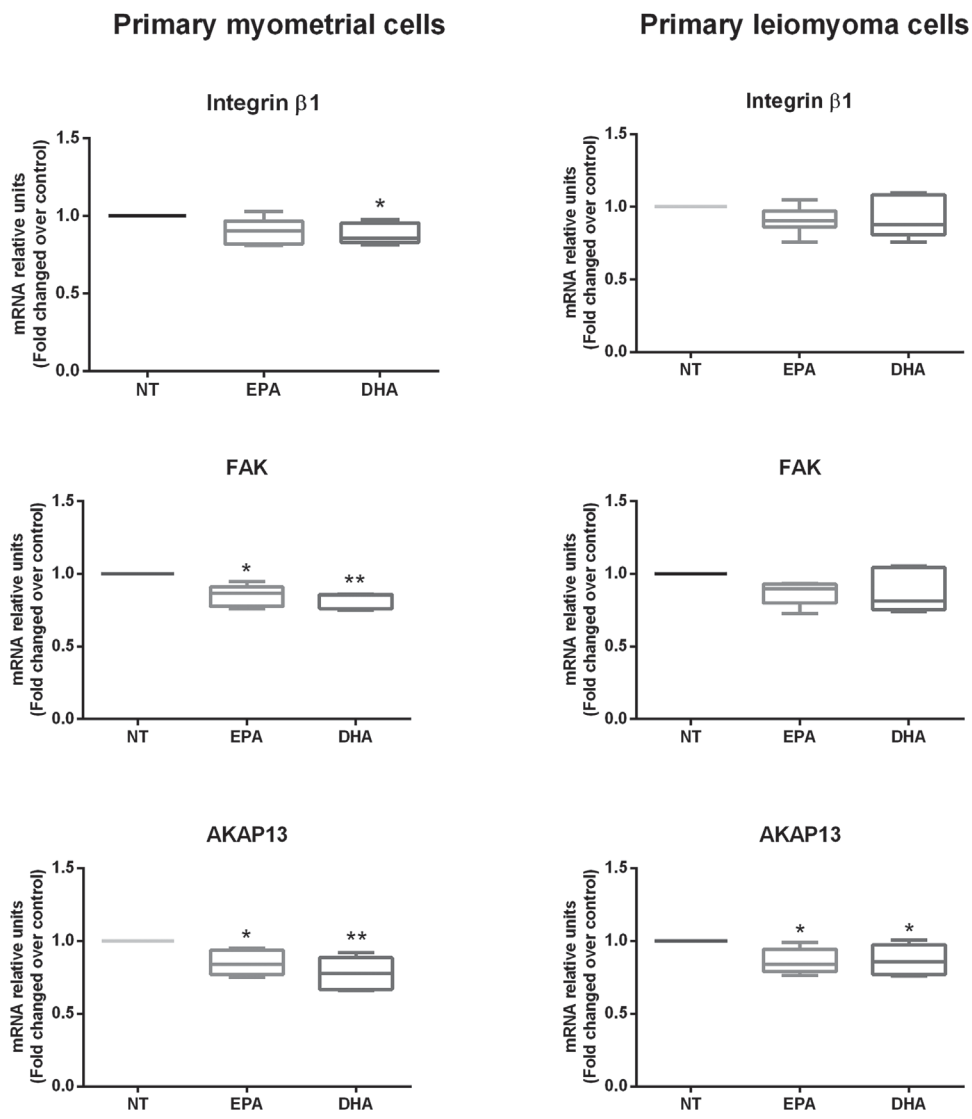


FIGURE 3 Effect of EPA and DHA treatment on mRNA expression of mechanical signaling molecules in primary myometrial and leiomyoma cells. Data are expressed as mean ± SD ($n = 6$). *$p < 0.05$; **$p < 0.01$. NT, No treatment; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid

of cholesteryl esters in mouse myometrium by nuclear liver X receptor-β (LXRβ) occurs via regulation of ABCA1 and ABCG1 (Mouzat et al., 2007).

Elevated levels of serum HDL (high-density lipoprotein)-cholesterol were found in women with fibroids compared with control patients (Sadlonova et al., 2008), suggesting a role of cholesterol regulatory proteins in the pathogenesis of leiomyoma. Recently Borahay and colleagues reported that simvastatin, which is commonly prescribed to lower high cholesterol levels, is able to inhibit the growth of human uterine fibroid tumors (Borahay et al., 2015). ABCG1 and ABCA1 are member of the evolutionarily conserved family of ATP-binding cassette cholesterol transporters. ABCG1 plays a pivotal role in cellular cholesterol efflux to HDLs (Wang, Lan, Chen, Matsuura, & Tall, 2004) but not to lipid-free apolipoprotein A-I (apoA-I) (Vaughan & Oram, 2005). In contrast, the efflux of cellular cholesterol and phospholipids to apoA-I mediated by ABCA1 converts apoA-I into nascent HDL, which can then act as an efficient acceptor for ABCG1-mediated cholesterol efflux (Smith et al., 2004). CAV-1 (Caveolin-1) is a component of caveolae, a "cave-like" invagination of the cell membrane. CAV-1 can interact with ABCG1 and regulate ABCG1-mediated cholesterol efflux (Gu, Wang, & Zhang, 2014). While we found no effect of ϖ3 fatty acids on CAV-1, we found that SREBP-2 (sterol-regulatory-element-binding protein-2) was downregulated in myometrial cells. SREBP-2 is best known for its regulatory role in cholesterol uptake and cholesterol synthesis. SREBP-2 can act as a positive regulator of ABCA1 gene expression (Wong, Quinn, & Brown, 2006). The overexpression of ABCG1 increases the processing of SREBP-2 to the transcriptionally active protein, thus increases in the expression of SREBP-2 target genes and cholesterol synthesis (Tarr & Edwards, 2008).

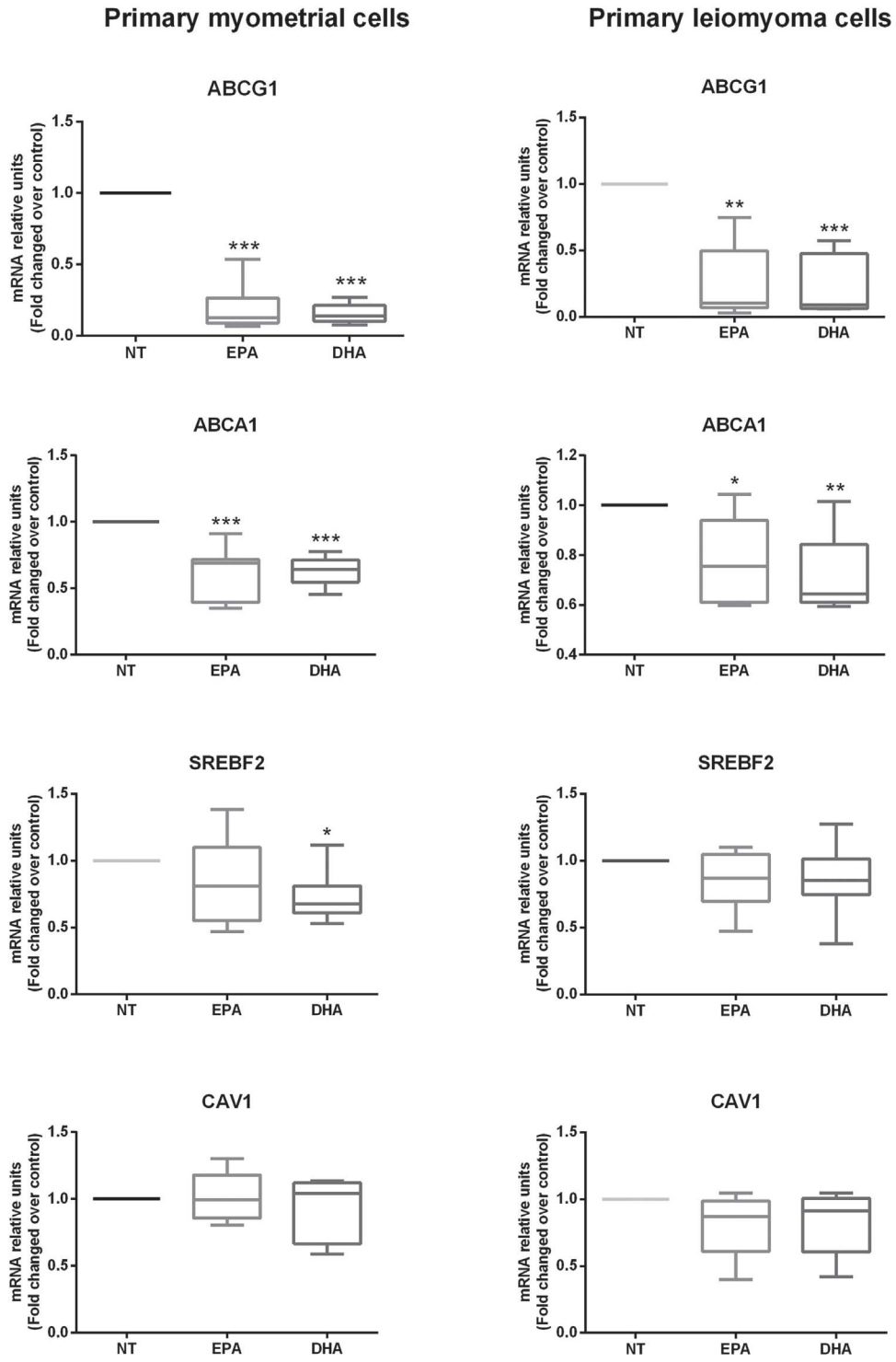


FIGURE 4 Effect of EPA and DHA treatment on mRNA expression of sterol regulatory molecules in primary myometrial and leiomyoma cells. Data are expressed as mean ± SD ($n = 9$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. NT, No treatment; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid

CYP11A1 is a mitochondrial enzyme that catalyzes conversion of cholesterol to pregnenolone. Pregnenolone is the precursor of estrogens, progestogens, mineralocorticoids, glucocorticoids, and androgens, as well as the neuroactive steroids. It is well known that estrogen and progesterone have tremendous effect on uterine leiomyoma growth (Ciarmela et al., 2011; Islam et al., 2013).

Our data showed that treatment with ω3 fatty acids seems to have a greater effect on gene expression levels in myometrial cells, while in

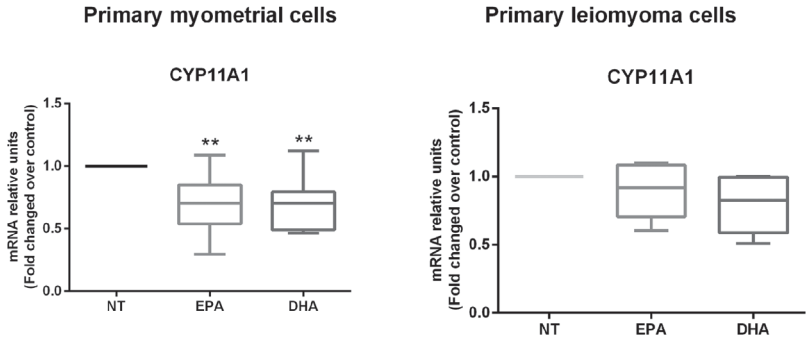


FIGURE 5 Effect of EPA and DHA treatment on expression of CYP11A1 in primary myometrial and leiomyoma cells. Data are expressed as mean ± SD ($N = 9$). $^{*}p < 0.05$; $^{**}p < 0.01$. NT, No treatment; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid

leiomyoma cells this treatment seems to reduce the ability to modulate the gene expression. In myometrial cells, omega-3 fatty acids were also able to reduce CYP11A1, the mitochondrial enzyme that catalyzes the conversion of cholesterol to pregnenolone, the precursor of steroid hormones, suggesting that this cellular type differs in the mechanism of regulation of steroidogenesis and this may contribute to the pathogenesis of uterine leiomyoma.

In conclusion, omega-3 fatty acids modulate lipid profile, remodel membrane architecture, and down regulate expression of genes involved in mechanical signal and cellular lipid accumulation in myometrial cells.

The present study adds the lipidomic approach in leiomyoma characterization in addition to genomic, epigenetic, and proteomic studies, and highlights new insights on molecular mechanisms underlying the pathogenesis of the disease. This new understanding and the demonstration of the possibility to modulate the lipid content will promote clinical applications in drug discovery and therapy.

CONFLICTS OF INTEREST

The authors have nothing to declare.

ORCID

Pasquapina Ciarmela http://orcid.org/0000-0002-4400-3786

REFERENCES

Antollini, S. S., & Barrantes, F. J. (1998). Disclosure of discrete sites for phospholipid and sterols at the protein-lipid interface in native acetylcholine receptor-rich membrane. *Biochemistry (Mosc)*, *37*(47), 16653–16662.

Bagatolli, L. A., & Gratton, E. (1999). Two-photon fluorescence microscopy observation of shape changes at the phase transition in phospholipid giant unilamellar vesicles. *Biophysical Journal*, *77*(4), 2090–2101.

Borahay, M. A., Vincent, K., Motamedi, M., Sbrana, E., Kilic, G. S., Al-Hendy, A., & Boehning, D. (2015). Novel effects of simvastatin on uterine fibroid tumors: In vitro and patient-derived xenograft mouse model study. *American Journal of Obstetrics and Gynecology*, *213*(2), 196.e191–196.e198.

Bulun, S. E. (2013). Uterine fibroids. *The New England Journal of Medicine*, *369*(14), 1344–1355.

Buttram, V. C., Jr, & Reiter, R. C. (1981). Uterine leiomyomata: Etiology, symptomatology, and management. *Fertility and Sterility*, *36*(4), 433–445.

Calder, P. C. (2001). Polyunsaturated fatty acids, inflammation, and immunity. *Lipids*, *36*(9), 1007–1024.

Chen, H. M., Lin, Y. H., Cheng, Y. M., & Wing, L. Y. C. (2013). Overexpression of integrin-beta1 in leiomyoma promotes cell spreading and proliferation. *The Journal of Clinical Endocrinology and Metabolism*, *98*(5), E837–E846.

Ciarmela, P., Islam, M. S., Reis, F. M., Gray, P. C., Bloise, E., Petraglia, F., . . . Castellucci, M. (2011). Growth factors and myometrium: Biological effects in uterine fibroid and possible clinical implications. *Human Reproduction Update*, *17*(6), 772–790.

Cramer, S. F., & Patel, A. (1990). The frequency of uterine leiomyomas. *American Journal of Clinical Pathology*, *94*(4), 435–438.

De Caterina, R., & Libby, P. (1996). Control of endothelial leukocyte adhesion molecules by fatty acids. *Lipids*, *31*(1), 57–63.

Evans, P., & Brunsell, S. (2007). Uterine fibroid tumors: Diagnosis and treatment. *American Family Physician*, *75*(10), 1503–1508.

Flake, G. P., Andersen, J., & Dixon, D. (2003). Etiology and pathogenesis of uterine leiomyomas: A review. *Environmental Health Perspectives*, *111*(8), 1037–1054.

Gallagher, C. S., & Morton, C. C. (2016). Genetic association studies in uterine fibroids: Risk alleles presage the path to personalized therapies. *Seminars in Reproductive Medicine*, *34*, 235–241.

Gu, H. M, Wang, F. Q., & Zhang, D. W. (2014). Caveolin-1 interacts with ATP binding cassette transporter G1 (ABCG1) and regulates ABCG1-mediated cholesterol efflux. *Biochimica et Biophysica Acta*, *1841*(6), 847–858.

Han, X. (2005). Lipid alterations in the earliest clinically recognizable stage of Alzheimer's disease: Implication of the role of lipids in the pathogenesis of Alzheimer's disease. *Current Alzheimer Research*, *2*(1), 65–77.

Harris, W. S., Pottala, J. V., Vasan, R. S., Larson, M. G., & Robins, S. J. (2012). Changes in erythrocyte membrane trans and marine fatty acids between 1999 and 2006 in older Americans. *Journal of Nutrition*, *142*(7), 1297–1303.

Ibarguren, M., López, D. J., & Escribá, P. V. (2014). The effect of natural and synthetic fatty acids on membrane structure, microdomain organization, cellular functions and human health. *Biochimica et Biophysica Acta*, *1838*, 1518–1528.

Ikonen, E. (2008). Cellular cholesterol trafficking and compartmentalization. *Nature Reviews Molecular Cell Biology*, *9*(2), 125–138.

Islam, M. S., Akhtar, M. M., Segars, J. H., Castellucci, M., & Ciarmela, P. (2017). Molecular targets of dietary phytochemicals for possible

prevention and therapy of uterine fi broids: Focus on fi brosis. *Critical Reviews in Food Science and Nutrition*, *57*(17), 3583–3600.

Islam, M. S., Catherino, W. H., Protic, O., Janjusevic, M., Gray, P. C., Giannubilo, S. R., . . . Ciarmela, P. (2014). Role of activin-A and myostatin and their signaling pathway in human myometrial and leiomyoma cell function. *The Journal of Clinical Endocrinology and Metabolism*, *99*(5), E775–E785.

Islam, M. S., Protic, O., Stortoni, P., Grechi, G., Lamanna, P., Petraglia, F., . . . Ciarmela, P. (2013). Complex networks of multiple factors in the pathogenesis of uterine leiomyoma. *Fertility and Sterility*, *100*(1), 178–193.

Kim, W., Barhoumi, R., McMurray, D. N., & Chapkin, R. S. (2014). Dietary fish oil and DHA down-regulate antigen-activated CD4+ T-cells while promoting the formation of liquid-ordered mesodomains. *British Journal of Nutrition*, *111*(2), 254–260.

Malik, M., & Catherino, W. H. (2012). Development and validation of a three-dimensional in vitro model for uterine leiomyoma and patient-matched myometrium. *Fertility and Sterility*, *97*(6), 1287–1293.

Marsh, E. E., & Bulun, S. E. (2006). Steroid hormones and leiomyomas. *Obstetrics and Gynecology Clinics of North America*, *33*(1), 59–67.

Marshall, L. M., Spiegelman, D., Barbieri, R. L., Goldman, M. B., Manson, J. E., Colditz, G. A., . . . Hunter, D. J. (1997). Variation in the incidence of uterine leiomyoma among premenopausal women by age and race. *Obstetrics and Gynecology*, *90*(6), 967–973.

Mouzat, K., Prod'Homme, M., Volle, D. H., Sion, B., Déchelotte, P., Gauthier, K., . . . Lobaccaro, J.-M. A. (2007). Oxysterol nuclear receptor LXRβ regulates cholesterol homeostasis and contractile function in mouse uterus. *Journal of Biological Chemistry*, *282*(7), 4693–4701.

Nyholm, T. K. (2015). Lipid-protein interplay and lateral organization in biomembranes. *Chemistry and Physics of Lipids*, *189*, 48–55.

Okolo, S. (2008). Incidence, aetiology and epidemiology of uterine fibroids. *Best Practice & Research Clinical Obstetrics & Gynaecology*, *22*(4), 571–588.

Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R. M., & Gratton, E. (1991). Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence. *Biophysical Journal*, *60*(1), 179–189.

Parasassi, T., Loiero, M., Raimondi, M., Ravagnan, G., & Gratton, E. (1993). Absence of lipid gel-phase domains in seven mammalian cell lines and in four primary cell types. *Biochimica et Biophysica Acta*, *1153*(2), 143–154.

PaszeK, M. J., Zahir, N., Johnson, K. R., Lakins, J. N., Rozenberg, G. I., Gefen, A., . . . Boettiger, D. (2005). Tensional homeostasis and the malignant phenotype. *Cancer Cell*, *8*(3), 241–254.

Protic, O., Toti, P., Islam, M. S., Occhini, R., Giannubilo, S. R., Catherino, W. H., . . . Ciarmela, P. (2016). Possible involvement of inflammatory/reparative processes in the development of uterine fibroids. *Cell and Tissue Research*, *364*, 415–427.

Rasmiena, A. A., Ng, T. W., & Meikle, P. J. (2013). Metabolomics and ischaemic heart disease. *Clinical Science*, *124*(5), 289–306.

Rogers, R., Norian, J., Malik, M., Christman, G., Abu-Asab, M., Chen, F., . . . Tuan, R. S. (2008). Mechanical homeostasis is altered in uterine leiomyoma. *American Journal of Obstetrics and Gynecology*, *198*(4), 474. e471–474. e411.

Rose, D. P., & Connolly, J. M. (1999). Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacology & Therapeutics*, *83*(3), 217–244.

Sadlonova, J., Kostal, M., Smahelova, A., Hendl, J., Starkova, J., & Nachtigal, P. (2008). Selected metabolic parameters and the risk for uterine fibroids. *International Journal of Gynaecology and Obstetrics*, *102*(1), 50–54.

Santos, C. R., & Schulze, A. (2012). Lipid metabolism in cancer. *FEBS Journal*, *279*(15), 2610–2623.

Smith, J. D., Le Goff, W., Settle, M., Brubaker, G., Waelde, C., Horwitz, A., & Oda, M. N. (2004). ABCA1 mediates concurrent cholesterol and phospholipid efflux to apolipoprotein AI. *Journal of Lipid Research*, *45*(4), 635–644.

Stewart, E. A. (2001). Uterine fibroids. *Lancet*, *357*(9252), 293–298.

Tarr, P. T., & Edwards, P. A. (2008). ABCG1 and ABCG4 are coexpressed in neurons and astrocytes of the CNS and regulate cholesterol homeostasis through SREBP-2. *Journal of Lipid Research*, *49*(1), 169–182.

Teague, H., Ross, R., Harris, M., Mitchell, D. C., & Shaikh, S. R. (2013). DHA-fluorescent probe is sensitive to membrane order and reveals molecular adaptation of DHA in ordered lipid microdomains. *Journal of Nutritional Biochemistry*, *24*(1), 188–195.

Vaughan, A. M., & Oram, J. F. (2005). ABCG1 redistributes cell cholesterol to domains removable by high density lipoprotein but not by lipid-depleted apolipoproteins. *Journal of Biological Chemistry*, *280*(34), 30150–30157.

Walker, C. L., & Stewart, E. A. (2005). Uterine fibroids: The elephant in the room. *Science*, *308*(5728), 1589–1592.

Wang, N., Lan, D., Chen, W., Matsuura, F., & Tall, A. R. (2004). ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(26), 9774–9779.

Whelan, J., & Rust, C. (2006). Innovative dietary sources of n-3 fatty acids. *Annual Review of Nutrition*, *26*, 75–103.

Wong, J., Quinn, C., & Brown, A. (2006). SREBP-2 positively regulates transcription of the cholesterol efflux gene, ABCA1, by generating oxysterol ligands for LXR. *Biochemical Journal*, *400*, 485–491.

Yang, Q., Mas, A., Diamond, M. P., & Al-Hendy, A. (2016). The mechanism and function of epigenetics in uterine leiomyoma development. *Reproductive Sciences*, *23*(2), 163–175.

How to cite this article: Islam MS, Castellucci C, Fiorini R, et al. Omega-3 fatty acids modulate the lipid profile, membrane architecture, and gene expression of leiomyoma cells. *J Cell Physiol*. 2018;233:7143–7156. https://doi.org/10.1002/jcp.26537