Obstetrics and Gynecology International

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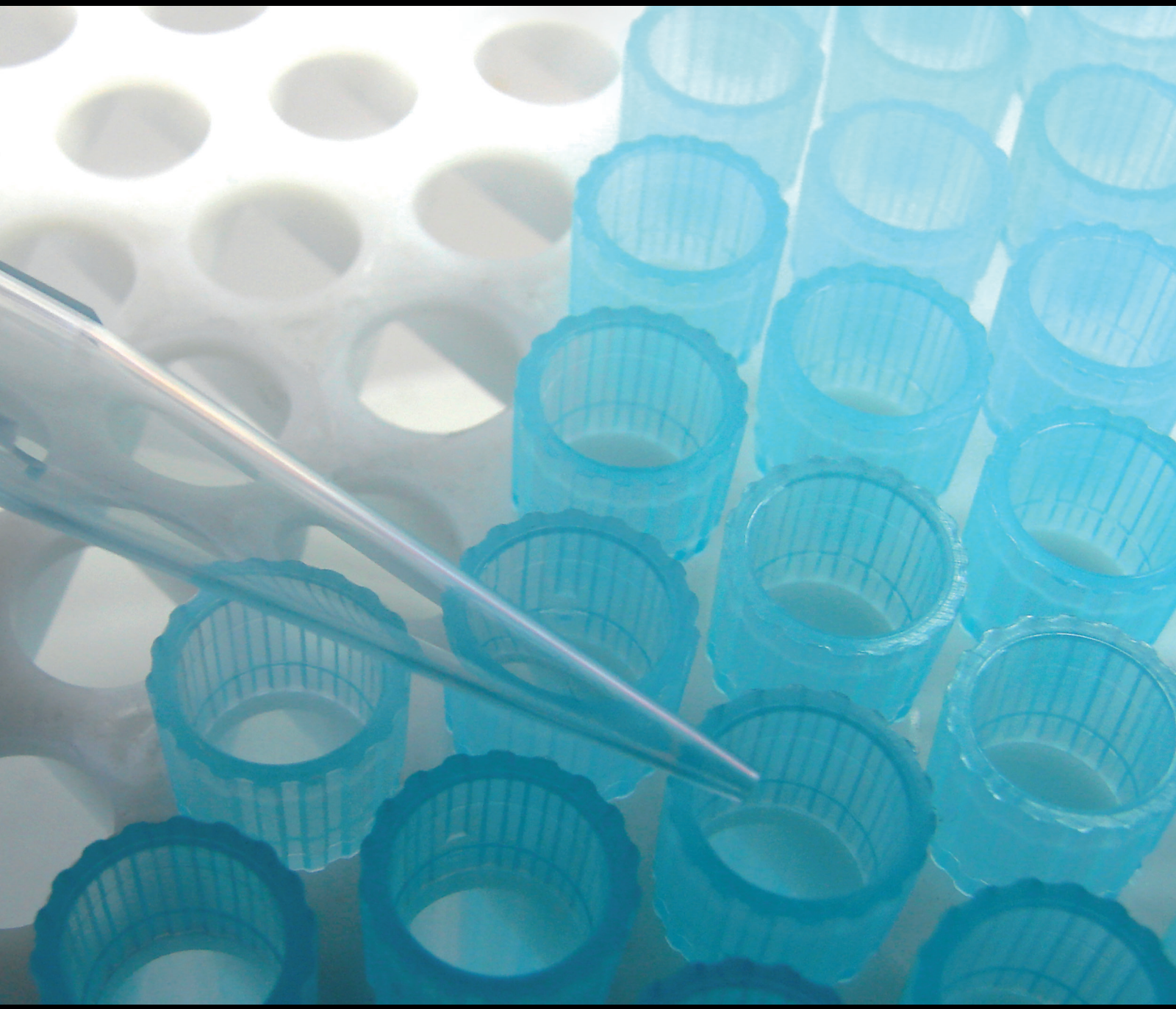
Guest Editors: Pasquapina Ciarmela, Hilary Critchley, Gregory M. Christman, and Fernando M. Reis



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Editorial Board

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Editorial

Pathogenesis of Endometriosis and Uterine Fibroids

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Endometriosis and uterine fibroids are important, common pathological conditions that impose a major healthcare burden.

Endometriosis is defined as the presence of endometrial tissue outside the uterine cavity and represents one of the most frequent gynaecological disorders, affecting 10–15% of all women of reproductive age and >30% of the infertile women.

Uterine fibroids (leiomyomas or myomas) are benign tumors of the myometrium. Uterine leiomyomas affect as many as 77% of women in reproductive age, of whom 20–50% are symptomatic.

Although they are nonneoplastic conditions, they heavily impact women's health and fertility and are a common indication for surgery, and the socioeconomic cost is huge. The mechanisms of formation remain unclear.

The knowledge and the understanding of the pathogenesis of these conditions are essential to develop successful medical therapies and to understand the mechanisms of action of the currently available therapies and are of interest to clinicians and basic and clinical researchers.

The focus of this special issue is to highlight novel aspects pertaining to endometriosis and uterine fibroids focusing on the pathogenetic mechanisms and new avenues of enquiry (novel hypotheses).

The special issue presents reviews, research articles, and a clinical study and is the effort of a truly international group of researchers giving varied experiences from across the world.

The paper entitled "*The natural history of uterine leiomyomas: light and electron microscopic studies of fibroid phases, interstitial ischemia, inanosis, and reclamation*" and the companion paper entitled "*The natural history of uterine leiomyomas: morphometric concordance with concepts of interstitial ischemia, inanosis*" by G. Flake et al. describe a fascinating natural history of uterine leiomyoma hypothesizing progressive developmental changes occurring in many uterine fibroids.

The paper entitled "*Uterine fibroids: pathogenesis and interactions with endometrium and endomyometrial junction*" by A. Ciavattini et al., summarizes the available literature concerning current knowledge on pathogenesis of uterine fibroids considering risk factors, genetic, epigenetic, hormonal, and growth and differentiation contributors. This review also describes how endomyometrial junction disruption may play a crucial role in fibroid-related infertility, uterine bleeding, and growth of submucosal and intramural myomas.

In the paper entitled "*Angiogenesis and endometriosis*" A. L. L. Rocha et al. review the evidence for the important role of angiogenesis in the pathogenesis of endometriosis and discuss the rationale for the search of antiangiogenic agents as a new therapeutic option in the treatment of endometriosis.

In the paper entitled "*Interplay between misplaced Müllerian-derived stem cells and peritoneal immune dysregulation in the pathogenesis of endometriosis*" A. Simone Laganà et al. hypothesize that during postpubertal age, under

the influence of different stimuli, misplaced and quiescent endometriotic cells derived from Müllerian structures of the embryonic female reproductive tract may acquire new phenotype, biological functions, and immunogenicity. These cells may differentiate, specializing in epithelium, glands, and stroma to form a functional ectopic endometrial tissue.

The paper entitled "*Gene expression of leptin and long leptin receptor isoform in endometriosis: a case-control study*" is an original clinical study suggesting a putative role of leptin in the development of endometrial implants. In this study, A. P. Nácul et al. report a significantly higher serum leptin/BMI ratio in women with endometriosis as well as a significantly higher expression of leptin and long form leptin receptor transcripts in the ectopic endometrium compared to the eutopic endometrium of patients with endometriosis and those with normal pelvis (controls).

Overall, this special issue is an excellent resource for researchers and physicians and provides "state of the art" information on these very common benign uterine/pelvic conditions, which have a major impact on women's quality of life. Although this special issue does not focus directly on therapies, this should be a valuable compendium for researchers, students, and physicians to stimulate continuing efforts to further the understanding of the pathogenesis of endometriosis and uterine fibroids and to help to develop new therapies.

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Research Article

The Natural History of Uterine Leiomyomas: Light and Electron Microscopic Studies of Fibroid Phases, Interstitial Ischemia, Inanosis, and Reclamation

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We propose, and offer evidence to support, the concept that many uterine leiomyomas pursue a self-limited life cycle. This cycle can be arbitrarily divided on the basis of morphologic assessment of the collagen content into 4 phases: (1) proliferation, (2) proliferation and synthesis of collagen, (3) proliferation, synthesis of collagen, and early senescence, and (4) involution. Involution occurs as a result of both vascular and interstitial ischemia. Interstitial ischemia is the consequence of the excessive elaboration of collagen, resulting in reduced microvascular density, increased distance between myocytes and capillaries, nutritional deprivation, and myocyte atrophy. The end stage of this process is an involuted tumor with a predominance of collagen, little to no proliferative activity, myocyte atrophy, and myocyte cell death. Since many of the dying cells exhibit light microscopic and ultrastructural features that appear distinct from either necrosis or apoptosis, we refer to this process as inanosis, because it appears that nutritional deprivation, or inanition, is the underlying cause of cell death. The disposal of myocytes dying by inanosis also differs in that there is no phagocytic reaction, but rather an apparent dissolution of the cell, which might be viewed as a process of reclamation as the molecular contents are reclaimed and recycled.

1. Introduction

The etiology of uterine leiomyomas (or fibroids) is unknown, and their pathogenesis has been incompletely determined. Because they are so common (80% in African-American women and 70% in Caucasian women in one study [1]), it would be reasonable to assume that women share a common risk factor for the development of fibroids. One such factor is menstruation, and perhaps more importantly is the occurrence of dysmenorrhea with associated abnormal uterine contractions [2, 3], which is estimated to occur in up to 70% of women by the fifth year after menarche [4]. Patients with primary dysmenorrhea experience varied patterns of myometrial hyperactivity, including contractions of increased amplitude, very frequent contractions, and/or a high basal tone, and this increased contractile activity is associated with a reduction in uterine blood flow [5]. If focal injury (ischemic or otherwise) to the myometrium occurs during menstruation, the reparative response could be similar to that which occurs following injury to blood vessels. In response to vascular intimal injury, smooth muscle cells of the media migrate into the intima, proliferate, and synthesize

extracellular matrix [6]. During the healing response, these smooth muscle cells are thus transformed into cells that have the capacity to divide and to synthesize extracellular proteins, while losing the capacity to contract. These changes are mirrored by the electron microscopic changes in which the smooth muscle cells exhibit a decrease in contractile filaments and an increase in protein synthesizing organelles such as the rough endoplasmic reticulum, free ribosomes, and Golgi apparatus [7].

The histologic changes that characterize the smooth muscle cell response to vascular injury are similar to those that occur in uterine fibroids. Uterine fibroids are characterized by two primary histologic features: the proliferation of smooth muscle cells and the production of a collagenous matrix. While the mitotic activity of fibroids is variable and generally modest, the proliferative rate of many fibroids is greater than that of the adjacent myometrium [8]. The collagenous component of fibroids is also variable in quantity. For example, one subtype of leiomyoma, the cellular leiomyoma, usually displays little extracellular matrix, consisting primarily of closely packed fascicles of smooth muscle cells, while many fibroids contain abundant fibrous matrix, which may even exceed the smooth muscle component itself. Likewise, the size of fibroids is also quite variable, from those that are barely visible (1-2 mm) to those as large as 10 cm or more. While recognizing this heterogeneity of size, proliferative activity, cellularity, and fibrotic stroma among fibroids, it is our impression that the majority of fibroids fall between the extremes of the hypercellular tumors and the hypocellular, predominantly fibrotic tumors. And thus, it would seem that the size and growth of fibroids are probably dependent upon both the proliferation of the smooth muscle cells and the synthesis and deposition of extracellular matrix.

It has been our observation that fibroids with extensive accumulation of collagen generally exhibit less mitotic activity. Based upon this observation and the conjectural analogy of fibroid development to the reparative response of smooth muscle cells in vascular injury, we hypothesized that the growth of uterine fibroids begins with a predominantly proliferative phase that either precedes or occurs concomitantly with the production of extracellular matrix. At some stage in the life of a fibroid, however, this progressive elaboration of matrix then seems to predominate over the proliferative response, resulting in the ultimate appearance of a hyalinized, involuted tumor. With this concept in mind, we have arbitrarily divided the development of fibroids into 4 hypothetical phases based upon the quantity of collagen present in the tumor. We have further hypothesized that the proliferative rate of the tumors would be the greatest in the early phases and would then diminish with progression to the final phase of involution.

2. Materials and Methods

2.1. Study Participants and Specimens

2.1.1. Study Participants. Fibroid tumor specimens were obtained by consent from women undergoing hysterectomy or myomectomy at the George Washington University Medical Center between June, 1996 and April, 1999, as part of the NIEHS Uterine Fibroid Study. Details of patient recruitment, demographics, and collection of gross pathology data have been previously reported [9].

2.1.2. Specimens. Following fixation in 10% buffered formalin, representative sections of at least one and as many as six fibroid tumors were submitted from each patient. Up to six sections were taken from tumors that were of sufficient size, with four of these sections taken from the periphery of the tumor and two from the central portion of the tumor. Sections of myometrium and endometrium were also submitted from many of the patients. The fixed tissues were routinely processed, embedded in paraffin, sectioned at 4-5 μm, and stained with hematoxylin and eosin. Representative sections were stained with Masson's trichrome stain to accentuate the collagen.

2.2. Light Microscopy. A total of 2151 sections from 460 fibroid tumors were available for microscopic examination. The slides were examined with an Olympus BX50 microscope, using objectives ranging from 2x to 100x. An Olympus ocular micrometer WHN10x-H/22 was used for nuclear measurements. The magnifications cited in the figure legends represent the original objective magnification multiplied by the tube length magnification of 3.3.

2.3. Developmental Phases of Fibroids. On the basis of microscopic estimation of the area of the tumor occupied by extracellular matrix in H&E stained slides, we arbitrarily categorized the fibroid tumors into four phases as follows:

Phase 1 = no, or insignificant, collagen matrix

Phase 2 = <10% collagen

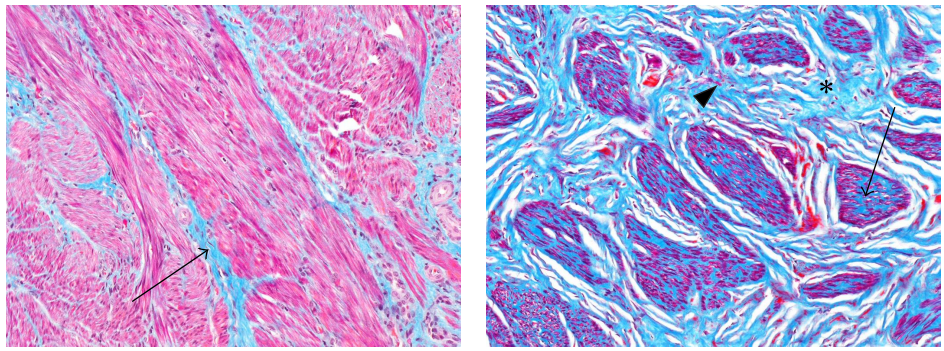
Phase 3 = 10–50% collagen

Phase 4 = >50% collagen.

When more than 1 section of the tumor was available, all sections were included in the estimation of percent collagen (Table 1).

2.4. Mitotic Counts. The H&E stained sections of all tumors were scanned with the 20x objective of an Olympus BX50 microscope until a mitosis was identified, and then the number of mitoses in 10 high power (40x objective) fields was counted. Only structures consistent with the prometaphase, metaphase, anaphase, or telophase stages of mitosis were counted. When more than one section of a tumor was available, the mitotic counts from each section were added and the sum was divided by the number of sections to give an average mitotic count for each tumor.

2.5. Gross Tumor Size. Gross tumor size data (<2 cm or ≥2 cm) were available for most (86%) of the tumors examined microscopically. Following assignment of Phase (1–4) for each tumor, the gross tumor sizes were obtained from



(a) (b)

Figure 1: Intrafascicular and interfascicular fibrosis. (a) Masson's trichrome stain of normal myometrium for comparison with (b) Phase 3 fibroid, also stained with Masson's trichrome, which stains muscle red and collagen blue. Note that in the myometrium (a) most of the blue staining collagen is between the fascicles (interfascicular) (arrow), whereas blue collagenous stroma in the fibroid is also noted between the individual myocytes of the muscle fascicles (intrafascicular fibrosis) (arrow), without the interposition of fibroblasts, indicating that the collagenous stroma is being produced by the myocytes themselves. In addition, the interfascicular blue collagenous stroma (asterisk) is more abundant in the fibroid (b) than in the myometrium (a). Red staining cells within the interfascicular stroma of the fibroid (arrowhead) probably represent transformed myocytes, or fibroblasts. Original objective magnification of (a) and (b): 10x.

Table 1: Fibroid Phases.

Phase	Estimated collagen content	Functional status
Phase 1	No, or insignificant, collagen matrix	Proliferation of myocytes
Phase 2	<10% collagen	Proliferation of myocytes, and synthesis of collagen
Phase 3	10–50% collagen	Proliferation, synthesis of collagen, and early senescence in late Phase 3
Phase 4	>50% collagen	Involution

the surgical pathology records for comparison of tumor size with tumor phase.

2.6. Transmission Electron Microscopy (TEM). Specimens for TEM were collected, with no identifiers or links to patient identification in accordance with guidelines by the National Institutes of Health Office of Human Subjects Research, at the time of hysterectomy or myomectomy at Duke University Medical Center in Durham, North Carolina. This on-site collection allowed for the immediate sectioning of tissue into 1 mm cubes and fixation in 2.5% glutaraldehyde. Following fixation, the tissues were processed routinely for TEM in a Lynx Automatic Tissue Processor (Electron Microscopy Sciences, Hatfield, PA) by buffer rinsing, postfixing in 1% osmium tetroxide, dehydrating in an ascending graded series of ethanols and acetone, and infiltrating and embedding in epoxy resin. Semithin sections (or "thick sections", a 500 to 800 nm section) were cut and stained with toluidine blue, examined by a pathologist, and areas within the stained section chosen for thin sectioning (a 90 nm or "gold section"). Areas selected for thin sectioning were trimmed and cut and then placed on copper grids. The grids were examined on a Tecnai G2 12 BioTwin 120 KV TEM (FEI, Hillsboro, Oregon). Digital photomicrographs were taken from selected areas of interest.

2.7. Statistical Analysis of Mitotic Counts. Descriptive statistics included means, standard errors of the mean, and minimum and maximum mitotic counts per tumor. Because mitotic counts were not normally distributed, counts were compared across phases using one-sided Mann-Whitney tests.

3. Results

3.1. Developmental Evolution of Fibroids: Phasing by Collagen Content. Phasing of fibroids was based upon the microscopically estimated percent of total tumor area occupied by extracellular fibrous matrix in H&E stained slides. Small amounts of collagenous matrix around blood vessels and between muscle fascicles, unaccompanied by obvious spindle cell or fibroblastic proliferation, were considered to be part of the normal structural support and were not included in the total percentage. This perivascular and interfascicular matrix appeared to be a relatively minor component in most fibroids except for those tumors assigned to the Phase 1 category in which fibrous matrix was otherwise minimal. Collagen deposition within the tumors was variably interfascicular, intrafascicular, or a combination of the two (Figure 1). After applying the criteria discussed in the methods section in which tumors were categorized on the basis of percent collagen content (Phase 1 = no, or insignificant, collagen matrix; Phase 2 = <10% collagen; Phase 3 = 10–50% collagen; Phase 4 = >50% collagen), it became clear that most fibroids would fall into the Phase 3 category, lesser numbers into the Phase 2 category, and the least number into the Phase 1 and 4 categories (Table 2). Although most fibroid tumors exhibited collagen production, there were a few tumors that showed no obvious fibrosis; these were generally small

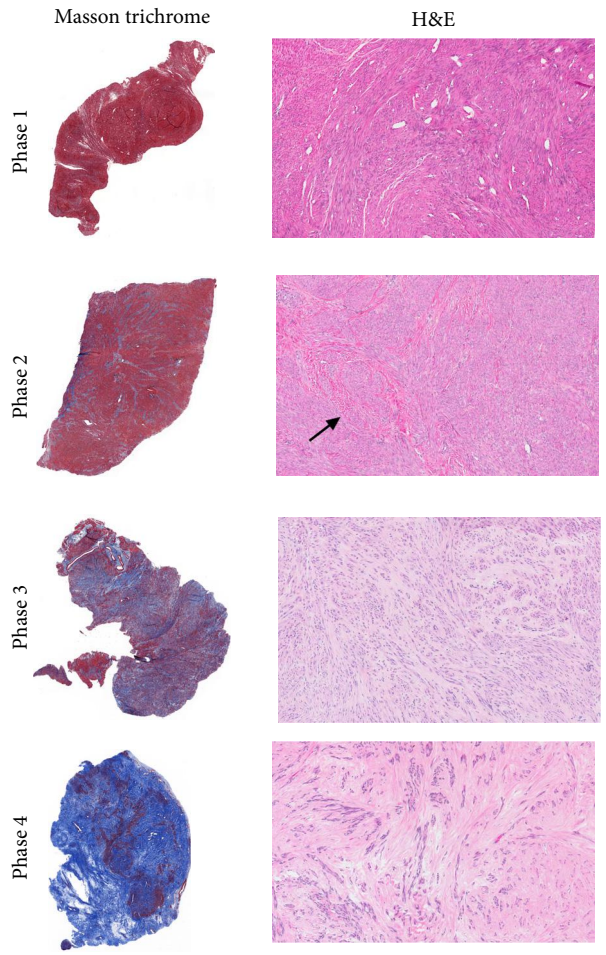


Figure 2: Fibroid Phases 1–4. Representative examples of the four phases of fibroid development are shown, with the Masson trichrome stain (1x image) on the left and the H&E stain of the same tumor (10x image) on the right. The progressive increase in blue staining collagen from Phase 1 to Phase 4 is well shown in the Masson trichrome stained sections. The corresponding H&E images on the right also demonstrate the virtual absence of collagen in the Phase 1 tumor, the appearance of interspersed pink collagenous fibers (arrow) in Phase 2, the more abundant pale pink collagenous stroma of Phase 3, and the predominance of pink, hyalinized stroma in Phase 4. Note also the abundance of microvessels (small ovoid spaces) in Phase 1 and the paucity of vessels in Phases 3 and 4.

and may significantly represent an early stage of development, and for this reason, the category of Phase 1 was created. Among patients with more than one fibroid, the majority (69%) were found to have tumors in more than one phase, suggesting that the tumors in these patients either arose at different times or were genetically different.

Phase 1 and Phase 2 tumors consist predominately of myocytes, whereas in Phase 3 and Phase 4 tumors, the balance is progressively tilted towards a predominance of dense extracellular matrix (Table 1). As the quantity of collagenous tissue increases, the microvessel density often appears to decrease; this is particularly noticeable in comparing Phase 1 and Phase 4 tumors. Representative examples of H&E and Masson trichrome stained fibroids from each of the 4 Phases are shown in Figure 2.

Table 2: Mitotic counts of tumors in Phases 1–4.

Phase	No. of tumors	Mitotic counts/10 HPF			
		Min	Max	Mean	S.E.
1	20	0	2.0	0.4085	0.1531
2	116	0	3.5	0.4281	0.0556
3	270	0	2.4	0.2769	0.0279
4	26	0	0.0	0.0000	0.0000

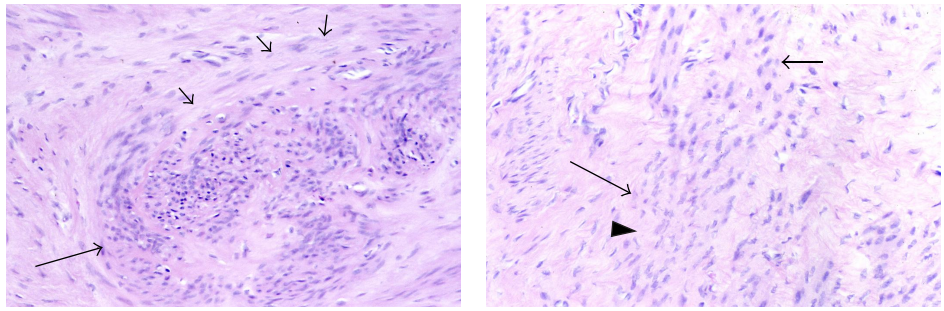
Table 3: Gross size of tumors in phases 1–4.

Phase	No. of tumors	No. of tumors ≥2 cm	% of tumors ≥2 cm
1	17	10	58.8
2	111	59	53.2
3	244	183	75.0
4	22	17	77.3

Mitotic figures were infrequent in most tumors and were not identified in the Phase 4 tumors (Table 2). Phase 3 tumors also showed a decline in average mitotic count, compared to Phases 1 and 2, consistent with the hypothesis of decreased proliferation as the collagenous matrix accumulates in the later developmental phases. The differences in average mitotic count between Phases 1 and 4 ($P = 0.0003$), Phases 2 and 3 ($P = 0.0105$), Phases 2 and 4 ($P < 0.0001$), and Phases 3 and 4 ($P < 0.0001$) were all statistically significant. Myometrial samples were also available from 67 of the patients. Out of the total of 287 myometrial samples, mitotic activity (1/10 HPF) was found in only one sample from each of two patients.

Analysis of gross tumor size data revealed an increase in average size of tumors in Phases 3 and 4, as compared to Phases 1 and 2 (Table 3). The trend towards increased tumor size with progression in phase is statistically significant, with a one-sided Cochran-Armitage trend value of $P = 0.0001$. This finding correlates with our hypothesis that the growth of the tumors is the result of both the proliferation of myocytes and the accumulation of extracellular collagen. In addition, this result, in combination with the reduction in mitotic count in Phases 3 and 4, further supports our hypothesis that as the tumors grow and accumulate collagen, the proliferative rate of the myocytes eventually declines.

3.2. Phases 1 and 2: Proliferation and Synthesis of Collagen (Phenotypic Transformation). At the interface between fascicles of myocytes and zones of collagenous matrix in leiomyomas, the myocytes often exhibit cytologic changes that are evident with the light microscope. Chief among these are the reduction of eosinophilic cytoplasm and the tendency of nuclei to become thinner and more pointed at the poles. The cytoplasmic pallor of these transformed myocytes is suggestive of a diminution in the number of myofilaments, which occupy most of the cytoplasm of normal smooth muscle cells (Figure 3). In addition, the nuclei of



(a) (b)

Figure 3: Phenotypic transformation from smooth muscle cells to fibroblast-like cells. In (a), the cells in the center and to the right are in close fascicular apposition and exhibit the typical pink cytoplasm of smooth muscle cells, while the cells above and to the left are more widely separated and have a paler cytoplasm resembling that of fibroblasts. Note that as the smooth muscle cells at the bottom (long arrow) appear to stream upwards and then to the right, there is a morphologic transformation to fibroblast-like cells with paler cytoplasm (short arrows). A similar transformation is noted in (b) as the organized, closely packed smooth muscle cells with pinker cytoplasm at the top (short arrow) transform into more widely spaced, paler fibroblast-like cells in the bottom half (long arrow). Note that the transformed cells have already produced a collagenous stroma (arrowhead), and that the cytoplasm of the transformed cells is less distinct and not clearly contiguous with that of their neighbors. Original magnification of (a) and (b): 66x.

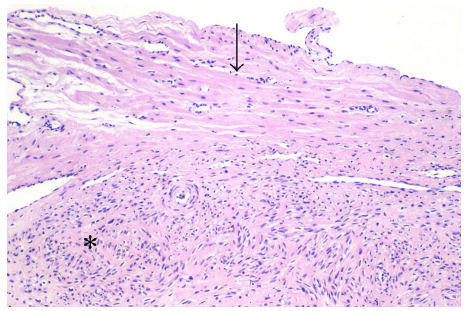


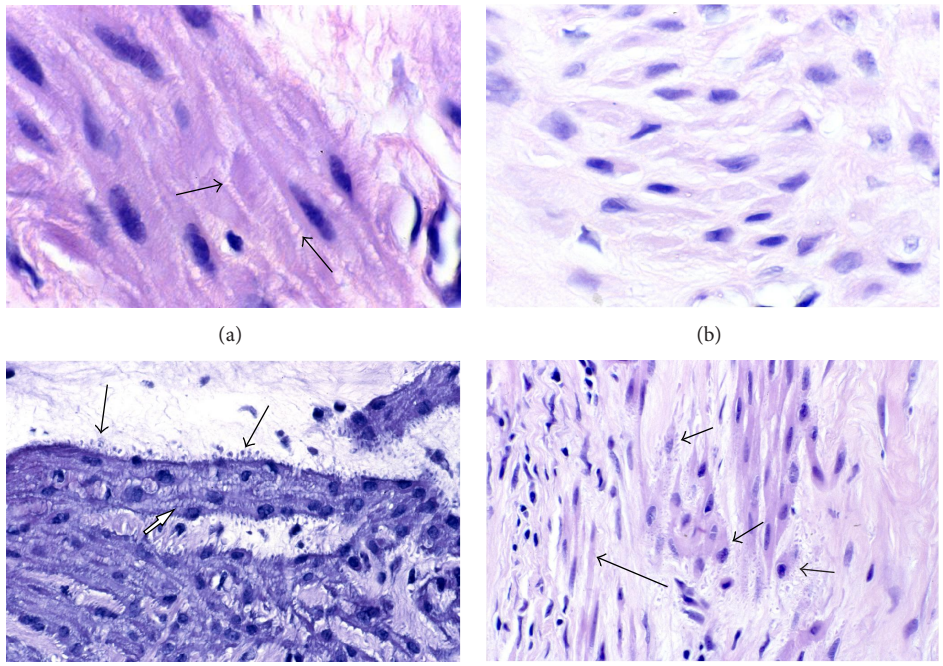
Figure 4: Phenotypic transformation: loss of parallel orientation of fibers. The parallel, linear orientation of the myometrial smooth muscle cells (arrow) contrasts with the haphazard, disorganized arrangement of the fibroid tumor cells (asterisk). Original magnification: 33x.

the transformed cells sometimes appear to be more widely spaced because of the intervening stroma between the cells.

If the cytoarchitectural features of leiomyomas are compared to the myometrium itself, several differences are apparent. First, it is evident that the fascicular pattern of the myometrium becomes less well defined in leiomyomas, sometimes appearing microfascicular, sometimes disorganized and haphazardly fascicular, and sometimes relatively patternless. In addition, the parallel linear arrangement of normal myocytes usually noted in the myometrium is also replaced to varying degrees by a less orderly and sometimes completely disorganized pattern of cells in fibroid tumors (Figure 4). Since the muscle fascicle is important to coordinated contraction and might be considered the functional unit of the myometrium, the loss of this pattern signals an important deviation from the normal contractile phenotype.

In some sections of myometrium, close examination of myometrial myocytes with the 40x or 100x objectives reveals lateral intercellular attachments between adjacent myocytes (Figure 5(a)). We refer to these as lateral bars, and it is significant that these often disappear as myocytes undergo transformation in leiomyomas. We believe that these lateral bars are the result of 2 phenomena: (1) disproportionate shrinkage of myocytes, in comparison to the interstitial (intercellular) tissue during the alcohol dehydration step of tissue processing and (2) the tenacity of gap junctions connecting adjacent myocytes. Myocytes shrink disproportionately during the dehydration phase of tissue processing because of the high water content of muscle cells. Despite the retraction of muscle cells from each other, however, the remarkable adherence of the gap junctions remains unbroken, resulting in lateral linear extensions of the myocyte cytoplasm. Since the gap junctions are critical conduits for the influx of calcium during coordinated contraction, the loss of these lateral bars in leiomyomas is further indication of transformation to a phenotype in which contraction is no longer important (Figure 5(b)). The lateral bars also attest to the plasticity of the myocytes, a property which would be expected in a contractile cell and which is probably lost as the cells undergo transformation and reduce their content of actin filaments. Myocytes with short, stubby lateral buds are sometimes seen and are thought to represent a transitional, intermediate stage in the transformation process, as the myocytes retract from their neighbors en route to assuming their proliferative, synthesizing phenotype (Figures 5(c) and 5(d)). Interestingly, these lateral bars are not seen in electron micrographs of myometrium, which we believe is related to the postfixation step with osmium tetroxide that stabilizes proteins and causes cell swelling that tends to offset the shrinking effect of the dehydration solvents [10].

By transmission electron microscopy, it is apparent, in side-by-side comparisons, that the cytoplasm of myometrial myocytes is more uniformly electron dense than the cytoplasm of leiomyoma tumor cells due to the greater concentration of thin myofilaments (Figures 6(a) and 6(b)). In contrast, the cytoplasm of leiomyoma cells has a more heterogeneous appearance due to the presence of more



(a) (b) (c) (d)

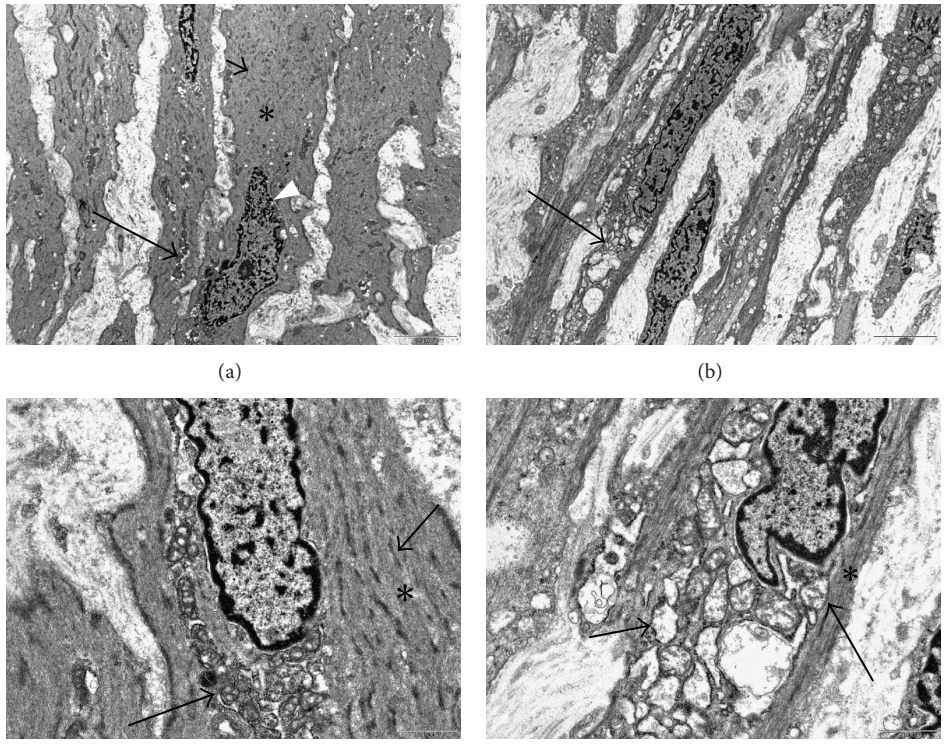
Figure 5: Phenotypic transformation: loss of lateral bars. Lateral bars (arrows) connecting myometrial smooth muscle cells in (a) are believed to represent sites of gap junction attachments between adjacent cells for purposes of coordinated contraction. These lateral bars, or intercellular attachments, are not seen between the fibroid tumor cells in (b). We believe that this is another indication of the phenotypic transformation to non-contractile cells. In (c), many of the fibroid myocytes exhibit lateral buds (long arrows), imparting a knobby appearance to the free borders of the cells. It is thought that these buds may represent remnants of the lateral bars. Note that the cytoplasmic borders of contiguous myocytes within the fascicle, however, do not show these lateral buds, but rather show retention of lateral bars, or attachments (short arrow), as noted in the myometrium. In (d), there are a few myocytes displaying lateral buds, which have a more frayed or feathered appearance (short arrows). Note that in the fully transformed cells on the left (long arrow), there are neither lateral bars or lateral buds. Original magnification of (a) and (b): 330x, (c) and (d): 132x.

abundant endoplasmic reticulum, which is often swollen, and the frequent presence of vacuoles, lysosomes, and swollen mitochondria. There is a clear reduction in the thin filaments, and the associated dense bodies, in the tumor cells, when compared to myometrial smooth muscle cells. Concomitantly, a prominent increase in the endoplasmic reticulum is seen in the cytoplasm of the tumor cells, often to the extent that this organelle occupies most of the central portion of the cell, with the fine filaments relegated to the periphery of the cell (Figure 6(d)). It is also our impression that the tumor cell mitochondria, although frequently swollen, are reduced in number in comparison with the myometrial cells. For example, the large clusters of mitochondria which are sometimes seen in myometrial smooth muscle cells have not been observed in the leiomyoma cells in our samples. And it is these three changes, that is, the reduction in myofilaments, the increase in endoplasmic reticulum, and the possible reduction in mitochondria that are ultrastructural features of the phenotypic transformation from a myometrial contractile cell to a fibroid synthesizing cell.

An associated morphologic consequence of this transformation, seen with both the light and electron microscope, is that the transformed cells have less cytoplasm because of this reduction in myofilaments, and thus have a more slender, streamlined appearance. The nuclei also appear to be thinner and often have pointed ends, in contrast to the more frequently rounded ends of the myometrial cells (Figures 6(c) and 6(d)).

3.3. Phases 3 and 4: Excessive Synthesis of Collagen Leads to Atrophy, Injury, and Inanosis

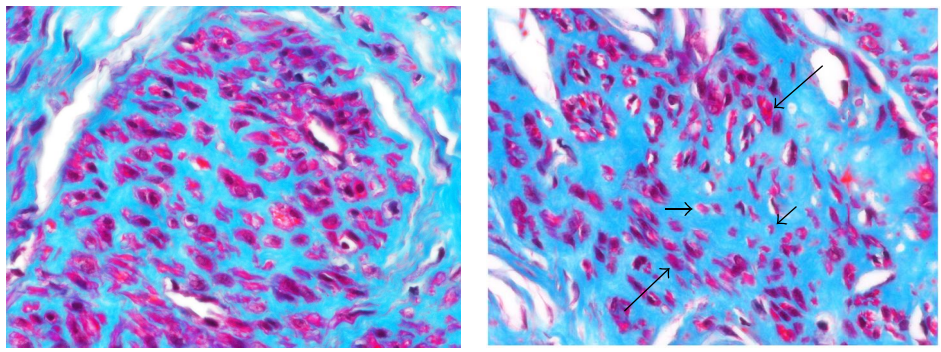
3.3.1. Atrophy. By the examination of Masson's trichrome stained sections, it is apparent that the collagen in fibroids is produced by the myocytes themselves since there are no interspersed fibroblasts between the myocytes (Figure 7(a)). In association with the increasing collagen deposition in the later phases (Phases 3 and 4) of fibroid development, it is also evident that myocyte atrophy is occurring (Figure 7(b)). This is sometimes evident by comparing one fascicle of myocytes with another. The atrophic process involves a reduction in size of both the cytoplasm and the nucleus of myocytes. In the late stages of atrophy, the cells are often reduced to slender, elongate, wispy structures (Figure 8(a)). Some of the atrophic myocytes will be noted to exhibit changes in shape, such as the presence of lateral pseudopodal projections or buds (pinnate atrophy) (Figure 8(b)). Whether the latter represent a form of decapitation membrane budding (similar to apocrine secretion) associated with the atrophic process,



(a) (b)

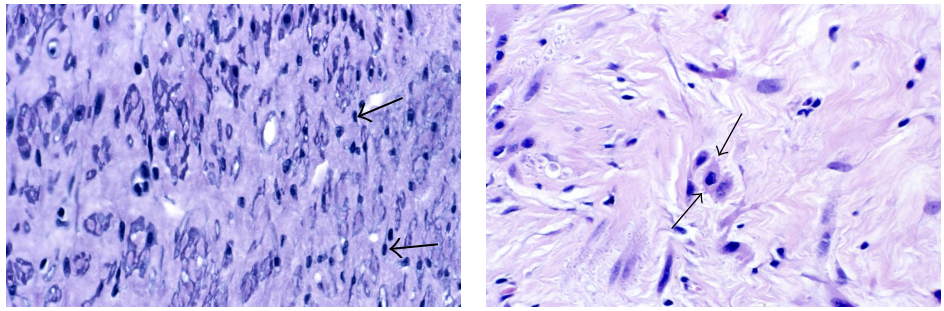
(c) (d)

Figure 6: Ultrastructural features of phenotypic transformation. This side-by-side comparison of myometrial smooth muscle cells in (a) with the transformed fibroid myocytes in (b) illustrates some of the electron microscopic features of the phenotypic transformation. The myometrial myocytes in (a) display abundant, relatively homogeneous cytoplasm that is filled with fine actin filaments (asterisk), oriented in parallel array in the long axis of the cells. Also evident are interspersed dense bodies (short arrow), focal clusters of mitochondria (long arrow), and a nucleus with a crenulated nuclear border (arrowhead) that is suggestive of the contractile state. In contrast, the fibroid in (b) exhibits more intercellular stroma and slender, streamlined cells with less cytoplasm, marked reduction in the fine actin filaments, increased swollen endoplasmic reticulum (arrow), and elongated, pointed nuclei. In (c), the myometrial myocyte displays a cytoplasm filled with actin fine filaments (asterisk), interspersed dense bodies (short arrow), clusters of mitochondria (long arrow), and a nucleus with a rounded end. In contrast, the fibroid myocyte in (d) exhibits extensive, dilated endoplasmic reticulum (short arrow), swollen mitochondria (long arrow), greatly reduced myofilaments limited to the periphery of the cell (asterisk), and a nucleus with a pointed end. Original magnification of both (a) and (b): 2550x, (c) and (d): 11,500x.



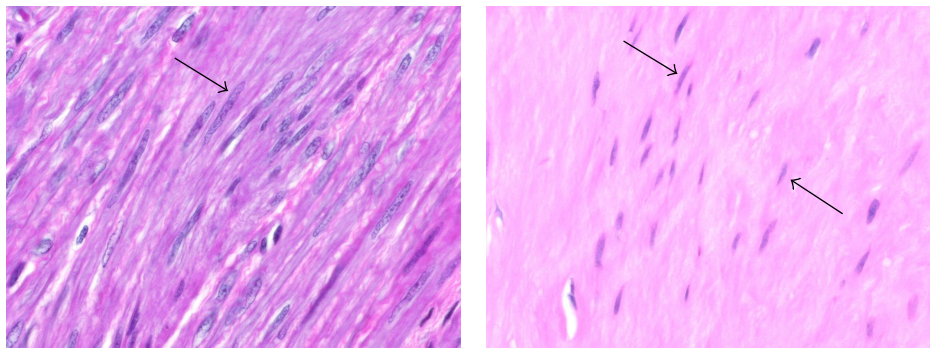
(a) (b)

Figure 7: Collagen production by tumor myocytes. (a) In this Masson trichrome stained section, the interstitium of the tumor fascicle is expanded by abundant blue collagen that separates the red staining fibroid myocytes. No interspersed fibroblasts are noted in the blue collagenous stroma, indicating that the stromal matrix has been produced by the tumor myocytes themselves. (b) Many of the myocytes in the central portion of this Masson trichrome stained section are reduced in size (short arrows) in comparison with those above and below (long arrows). These appear to be atrophic cells, although the possibility of artifactual tangential sectioning cannot be excluded. Original objective magnification of (a) and (b): 40x.



(a) (b)

Figure 8: (a) Wispy atrophy of tumor myocytes. As collagen production and deposition continues, the tumor myocytes become further encased in matrix, progressively more atrophic, and often reduced to slender, wispy structures. Note that not only the cytoplasm but also the nuclei of some cells appear to be reduced in size (arrows). (b) Lateral buds versus pinnate atrophy. Sometimes the atrophic cells exhibit lateral projections (arrows), which are similar to the previously described lateral buds associated with phenotypic transformation (Figure 7), but are occurring late in the atrophic process. Whether these lateral projections are the residua of lateral buds, or whether they might represent a form of decapitation budding associated with the atrophic process (pinnate atrophy) is uncertain. Original magnification of (a) and (b): 132x.



(a) (b)

Figure 9: Cytoplasmic and nuclear atrophy. The eosinophilic cytoplasm of the fibroid myocytes in (b) is indistinct but appears diminished and less abundant than that of the myometrial myocytes in (a), and there is an obvious increase in the stroma that is separating the fibroid cells of this Phase 4 tumor. In addition, the nuclei of the myometrial cells in (a) are uniformly long and straight (arrow), while the fibroid nuclei in (b) exhibit variation in length and contour (arrows) and often appear to be shortened, suggestive of early nuclear shrinkage. Original objective magnification of both (a) and (b): 40x.

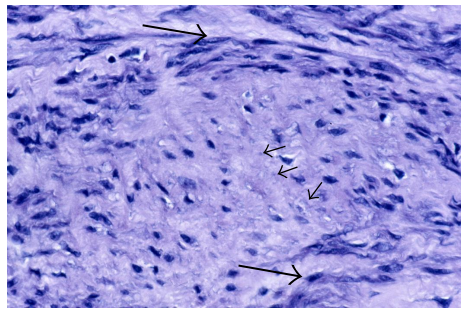
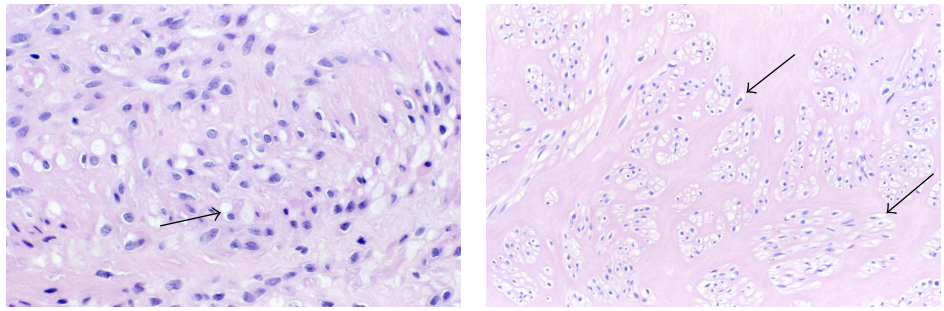


Figure 10: Nuclear atrophy. In this image from a late phase fibroid, the myocytes at the top and lower right (long arrows) retain some visible cytoplasm and nuclear length, while the cells in the center of the fibrotic matrix show little remaining cytoplasm and nuclei that are small, variably shaped, and appear to be atrophic. In addition, near the most central portion of this image there are a few pale staining, barely visible nuclei of cells that are assumed to be nonviable (short arrows). Original magnification: 132x.

or alternatively the remnants or the shedding of intercellular nexus attachments (gap junctions) as the cells transform from myocytes to fibroblast-like cells is not clear.

Although the nuclei of fibroid myocytes seem to retain their size and shape during much of the atrophic process, the nuclei often appear to shrink in size during the late stages of atrophy. To make this evaluation, it is necessary to examine cells cut in the longitudinal axis, but even with this in mind it is hard to exclude the possibility of partial tangential sectioning. However, if we compare side-by-side images, taken at the same magnification, of nonatrophic myometrial myocytes (Figure 9(a)) with fibroid myocytes from an atrophic area in a Phase 4 fibroid (Figure 9(b)), there does appear to be reduction in the length and width of many of the fibroid myocyte nuclei. Using an ocular micrometer, most of the measurable nuclei in the myometrial image (Figure 9(a)) ranged from 22.5 to 27.5 μm in length, while the nuclear lengths of the fibroid nuclei in this image (Figure 9(b)) measured from 10.0 μm to 20.0 μm, with most



(a) (b)

Figure 11: Vacuolization of myocytes. In Phase 3 and 4 fibroids, cytoplasmic vacuolization (arrows) is common and may reflect the loss of myofilaments and other changes related to injury or involution. As noted in both (a) and (b), the myocytes are often surrounded by an amorphous (hyaline) matrix. Original magnification of (a): 132x. Original magnification of (b): 66x.

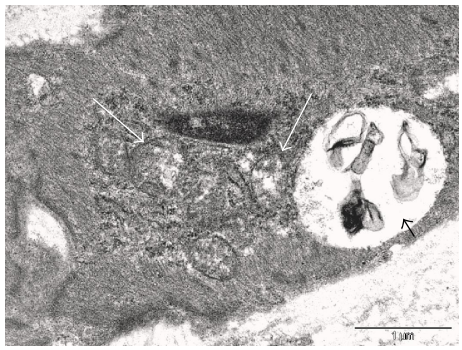


Figure 12: Injury and autophagy. A cluster of degenerating mitochondria (long arrows) near the center of the image are swollen and show fragmentation and loss of cristae. The myofilaments of the surrounding cytoplasm also appear granular and degenerate. To the right of the mitochondria, there is vacuole, probably autophagic, containing membranous debris (short arrow). The image suggests that the degenerate mitochondria and myofilaments are being drawn into an autophagic vacuole. Original magnification: 20,500x.

of the latter measuring between 12.5 and 15.0 μm. In later stages of atrophy, smaller nuclei may be noted (Figure 10). The apparent nuclear atrophy would imply that some loss of nucleoplasm is consistent with viability, although there is obviously a limit to nuclear atrophy, just as there is a limit to the loss of the metabolic and synthetic organelles of the cytoplasm.

3.3.2. Injury (Degenerative Change). In addition to shrinking in size, the myocytes often exhibit cytoplasmic vacuolization, particularly in the Phase 3 and 4 tumors (Figure 11). The vacuolization may take the form of diffuse cytoplasmic pallor surrounding a central or eccentric nucleus, or there may be one large cytoplasmic vacuole that appears to displace the nucleus to one side of the cell. In some tumors, several large foci or clusters of multiple vacuolated cells may be seen (Figure 11(b)). In view of the association of this change with the later phase tumors that are also exhibiting atrophic features, it seems likely that the vacuolization is related to the involutional changes that are occurring in these tumors. That is, the vacuoles could represent either swollen endoplasmic reticulum due to injury or possibly active synthesis [11], or phagolysosomes or autophagic vacuoles as the atrophic cells dispose of nonessential organelles and myofilaments [12].

Examination of fibroids by electron microscopy offers evidence that both injury and autophagocytosis are occurring. In addition to the swelling of endoplasmic reticulum and mitochondria in leiomyoma cells, the mitochondrial cristae are often decreased and fragmented (Figure 12). Lysosomes and autophagic vacuoles also appear to be increased (Figures 13(a) and 13(b)), sometimes containing myelin figures and sometimes lying adjacent to mitochondria or degenerating myofilaments, suggesting that these structures are being engulfed by the autophagic vacuoles. These changes support the concept of atrophy with autophagic disposition of myofilaments and nonessential organelles, as well as injury, in later stage fibroids (Figures 13(c) and 13(d)).

3.3.3. Vascular Changes in Fibroids. The myocytes within the blood vessels of fibroids frequently exhibit changes that mirror those of the tumor myocytes. Medial hypertrophy of vessels due to smooth muscle cell proliferation is commonly seen (Figure 14(a)). Similarly, in tumors displaying fibrosis, there is often a corresponding fibrosis of the blood vessel walls, either medial or intimal (Figure 14(b)). Other changes noted in both the vessels and the tumor itself include vacuolization (Figure 14(c)), hyalinization (Figure 14(d)), mucinosis or myxoid change, and myocyte atypia or symplastic change, characterized by nuclear enlargement and hyperchromasia. The consequence of these vascular changes, such as the smooth muscle hyperplasia, fibrosis, or hyalinization, is progressive stenosis of blood vessel lumens, which contributes to the ischemic environment that results in eventual atrophy of tumor myocytes. In addition to the reduced blood flow from stenosis of the lumens, hyalinized arterioles are probably also unable to dilate and thus unable to increase the blood flow in response to the low oxygen tension.

3.3.4. Inanosis. Within fibroid tumors that have accumulated extracellular collagenous matrix—that is, Phase 3 and 4 tumors—there are almost invariably found a few small,

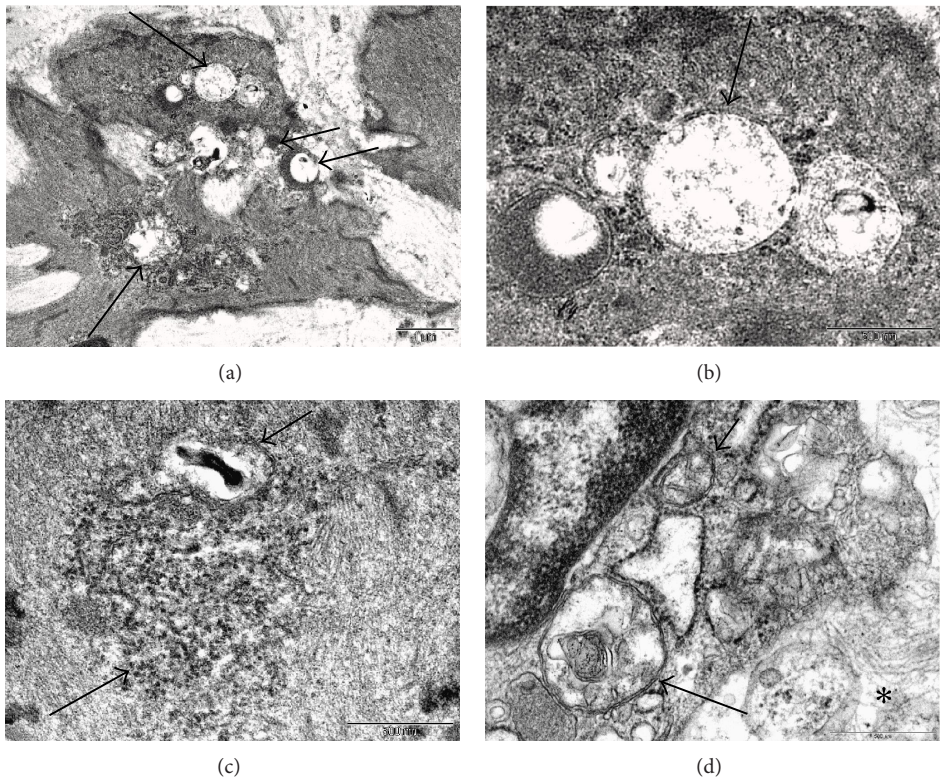


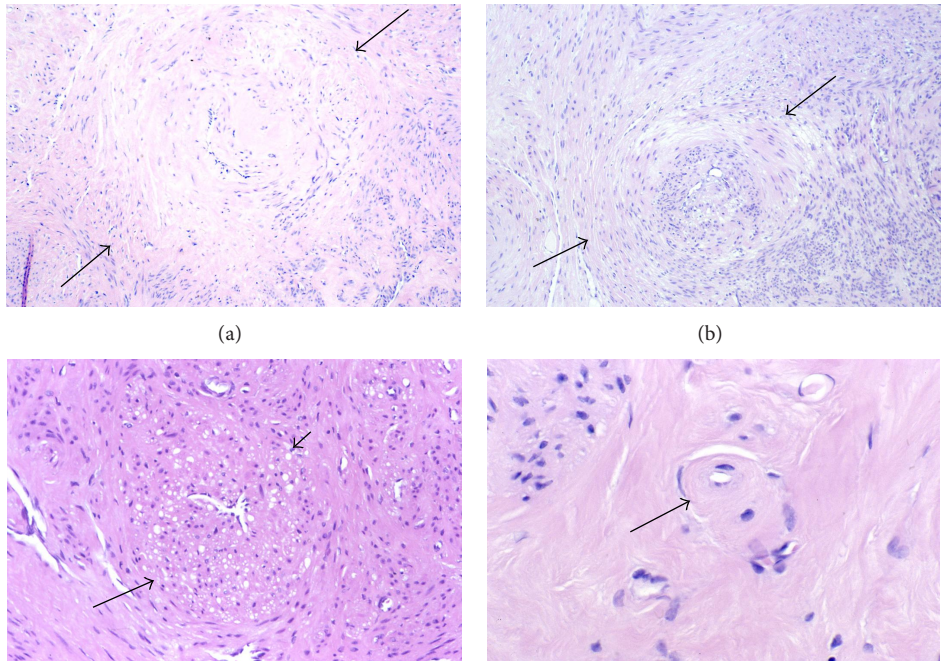
Figure 13: Lysosomes and Autophagy. The myocyte in (a) contains several vacuoles (long arrow at top and left lower) and lysosomes (shorter, horizontal arrows in middle). Higher magnification of the 4 structures at the top of the cell in (b) shows partial fusion of vacuoles to form a double membraned autophagosome in the middle (arrow). Granular debris in the vacuolar lumens may represent degenerate myofilament particles. The image in (c) shows degenerate myofilaments in the center (long arrow) and an autophagic vacuole (short arrow) which appears to be engulfing the degenerate filaments. The myofilaments on either side of this central area appear to be intact. The cell in (d) exhibits more advanced changes with loosely expanded cytoplasm separating the myofilaments on the right (asterisk), degenerating and swollen mitochondria (short arrow), and an autophagosome with membranous and particulate debris (long arrow). Original magnification of (a): 11,500x. Original magnification of (b), (c), and (d): 43,000x.

pale shrunken myocytes (Figure 15). Because of the small size and pale staining nucleus and cytoplasm of these cells, they are inconspicuous. They are most commonly found in small clusters of two to four cells entrapped within the areas of most abundant collagenous matrix, which is usually of the amorphous or hyaline type. These cells are only a fraction (1/3 to 1/5) of the size of normal myocytes. Their nuclei are tiny, rounded to ovoid, pale staining, and never fragmented. Nucleoli are not seen. Their cytoplasm retains an ovoid to fusiform shape, is very pale to lightly eosinophilic, and is in contact with the extracellular matrix. Since each of these cells is surrounded and insulated by the interstitial matrix, they may lie in proximity to each other, but they are usually not contiguous.

Within a given field, these shrunken cells are generally similar in size and tinctorial qualities, although size and tinctorial preservation of these cells will vary from one focus to another. That is, some cells may be markedly reduced in size but retain slight basophilia of the nucleus, whereas the shrunken cells in other areas will exhibit nuclei which are even more diminutive and so pale as to be barely visible. When measured with an ocular micrometer, the smallest nuclei will measure from 1 to 2 μm in diameter. The shrunken cells will usually lie at considerable distance from the nearest visible capillaries, separated by more than 30 μm and sometimes by as much as 50 μm from the closest vessels (Figure 15(a)). Thus, it appears in many tumors that as the quantity of collagenous tissue increases, the microvessel density correspondingly decreases. It is also evident that there is never any observed phagocytosis of these cells nor any adjacent inflammatory response.

Although the argument could be made that these small, pale cells represent tangential cuts of larger cells, it is clear that atrophy of myocytes is occurring since other myocytes in these hyalinized areas are also reduced in size—but to a lesser degree and with retention of normal nuclear basophilia. In addition, it would seem unlikely to have so many small, pale nuclei in one field as a result of tangential sectioning.

The mildly to moderately atrophic myocytes with retention of normal nuclear staining are probably viable. The viability of severely shrunken cells with retention of some nuclear staining is questionable. On the other hand, those cells exhibiting the most extreme degrees of nuclear and cytoplasmic atrophy, with such loss of nuclear staining that the nuclei are barely visible, are assumed to be nonviable. These latter cells are regarded as myocyte tombstones,



(a) (b) (c) (d)

Figure 14: Vascular changes within fibroids mirror those of the fibroid tumor cells. Atrophy of the tumor myocytes occurs as a result of both vascular and interstitial changes. The vascular smooth muscle cells within fibroid tumors often exhibit changes similar to those occurring in the tumor myocytes, including hypertrophy and hyperplasia of the medial myocytes (a), medial hyperplasia and intimal fibrosis (b), vacuolization (c), and hyalinization (d). Arrows mark the outer perimeters of the thickened vessels. Note that each of these vessels also shows marked stenosis of the lumen, which contributes to the ischemic, atrophic process. Original magnification of (a): 33x, (b): 33x, (c): 66x, and (d): 132x.

and they are the histologic hallmark and end stage of the atrophic process that we have designated as inanosis.

The term inanosis denotes a condition of cellular inanition resulting from gradual nutritional deprivation. This catabolic process of severe atrophy, eventuating in cell death by inanosis, is the consequence of the elaboration of extracellular matrix within the tumor as well as within the vessel walls of the tumor. Blood flow is diminished by progressive stenosis of vascular lumens resulting from the intratumoral vascular smooth muscle proliferation and fibrosis that mirrors the pathology of the fibroid tumor itself. Of equal if not greater import, however, is the interstitial fibrosis occurring within and between the fascicles of tumor myocytes themselves. The consequence of this process is the progressive separation of myocytes from capillaries, thus increasing the diffusion distance for vital nutrients to reach the myocytes from the capillaries, eventually resulting in atrophy and ultimately in cell death. This condition of interstitial ischemia is probably exacerbated by the dense collagenous character of the fibroid extracellular matrix, which most likely contains less ground substance for the diffusion of oxygen and nutrients.

3.3.5. Postinanotic Changes (Reclamation). A variety of changes are noted in both the nonviable cells and the surrounding stroma of involuting fibroids that are suggestive of resorption of the nonviable cells, a process that we refer to as reclamation. Although most inanotic nuclei are small, rounded structures, some exhibit sharply angulated shapes, one of the more frequent of these being the triangulated nucleus (Figure 16(a)). Such a nuclear shape would be unusual for a normal, viable cell. In addition, some inanotic cells are seen with nuclei exhibiting two or more pointed projections resembling burrs (Figure 16(b)). It is possible that these abnormal nuclear shapes could be artifacts of processing or sectioning, although that seems less likely for the burr-shaped nuclei. We refer to the burr-shaped nuclei as showing acanthanuclear alteration by way of analogy to the acanthocytic red blood cells seen in certain pathologic states such as severe hepatocellular injury and the genetic disorder abetalipoproteinemia [13]. Since the abnormal red blood cell shapes in these latter disorders are related to aberrations in the relative amounts of cholesterol and specific phospholipids in the lipid bilayers of the plasma membrane, we have considered the possibility that a similar phenomenon could be responsible for the irregular shapes occurring in some inanotic nuclei. Such distortions in the nuclear membranes of presumed nonviable inanotic cells could be related to a loss of nuclear membrane integrity, secondary to extraction of membrane components for recycling purposes.

More advanced degrees of reclamation are suggested by fields in which inanotic cells exhibit hollow or empty nuclei, with only shells of cytoplasm remaining (Figure 16(c)). Fragments of apparent cytoplasmic debris, resembling the previously described lateral buds, but possibly representing decapitated segments of cytoplasm associated with atrophy, are occasionally noted in the stroma (Figure 16(d)).

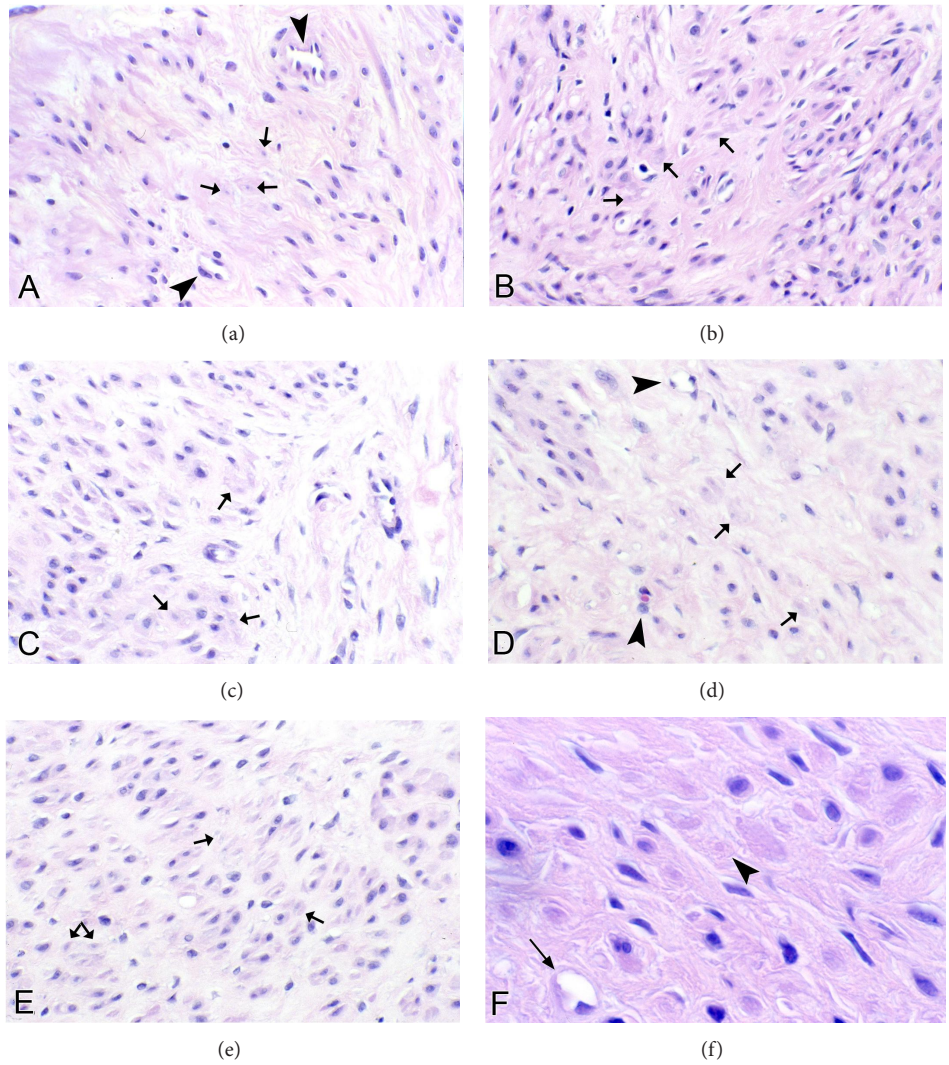


Figure 15: Inanosis of tumor myocytes. Interstitial and vascular ischemia lead to myocyte atrophy and eventual cell death, a process that we refer to as inanosis. In this panel, each image shows cytoplasmic and nuclear atrophy of tumor myocytes, as well as scattered, shrunken cells with marked nuclear pallor that are probably nonviable (arrows in (a)–(e)). In addition to those marked by arrows, there are other similar pale, presumably nonviable cells scattered among the viable cells with blue nuclei, resulting in a mottled pattern. Note the absence of inflammatory reaction. (a) The pale, inanotic cells are widely separated from the capillary at the top and the capillary at the bottom (arrowheads). (b) The pale, presumably dead cells are scattered among atrophic, but viable, cells with dark blue nuclei. (c) The pale, inanotic cells are shrunken but usually maintain elongate shapes. (d) Note that the pale, inanotic cells in the center are the most distant cells from the capillary with the open lumen at the top and the apparent capillary with a red cell in the lumen at the bottom (arrowheads). (e) Pale cells are noted here and there among the atrophic, viable cells in a field without any obvious capillaries. No inflammation is present. Original magnification of photos ((a)–(e)): 132x. (f) Oil immersion image of inanotic cells (myocyte tombstones) with pale nuclei and cytoplasm, in a fibrotic, atrophic field of a Phase 3 tumor. The cell in the center of the field (arrowhead) is located 76 μm from the capillary in the left lower corner (arrow). Original magnification of (f): 330x.

Both the hollow nuclei and the cytoplasmic fragments may lie within clear spaces in the stroma that are believed to be resorption pits. Finally, in the hyalinized areas of some Phase 4 fibroids, the tissue exhibits occasional foci with a spongiform or motheaten appearance due to the presence of multiple empty spaces. These spaces are believed to be the sites of complete resorption of inanotic cells and cell debris and thus are thought to represent the end stage of reclamation (Figures 16(e) and 16(f)).

An interesting feature of reclamation is the lack of an inflammatory reaction or recruitment of macrophages. We have considered the possibility that it might be difficult for inflammatory cells to pass through the dense collagen of these fibrotic areas; however, the finding of leukocytes with filopodial extensions traversing through the stroma of some fibroid tumors (Figure 17) indicates that at least some fibroids are accessible to the influx of inflammatory cells. Since there is no accompanying inflammatory reaction or evidence of

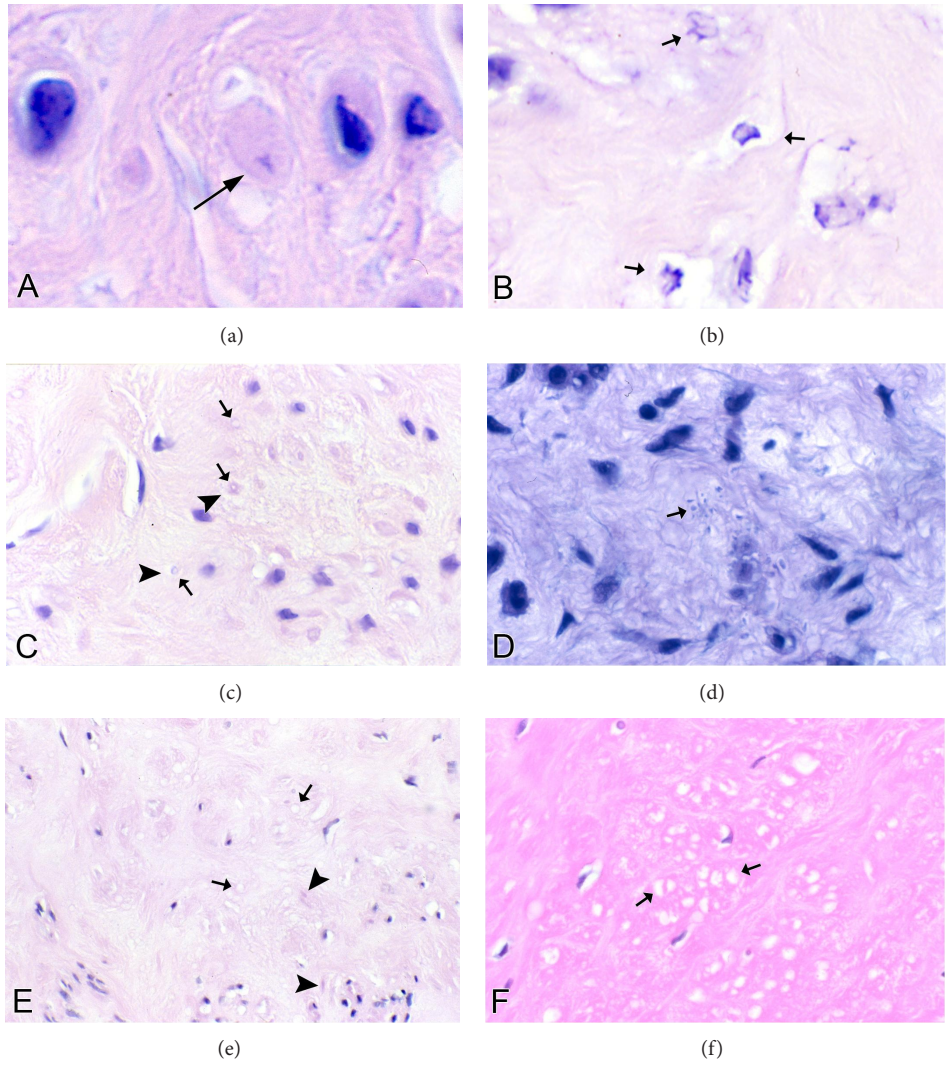


Figure 16: Reclamation. Cytologic and histologic changes are sometimes noted in involuting fibroids that are suggestive of cell resorption, a process that we refer to as reclamation. Angulated nuclear shapes, such as the triangular shaped nucleus in (a) (arrow) and the burr-shaped nuclei (acanthanuclear alteration) in (b) (arrows), are aberrant shapes that would not be expected in viable cells and may be related to loss of nuclear membrane integrity. Structures that appear to be hollow or empty nuclei, with only a shell of surrounding cytoplasm, are noted in (c) (arrows), and these structures often lie within clear spaces in the stroma that may represent resorption pits (arrowheads). Small fragments of probable cytoplasmic material are sometimes noted within clearings in the stroma, as seen in (d) (arrow); whether these are discarded lateral buds or decapitated particles of cell cytoplasm is uncertain. Finally, clear, circular spaces are sometimes noted within hyalinized areas of Phase 4 tumors, as seen in (e) and (f) (arrows) that are believed to be the end stage of the reclamation process. In (e), inanotic dead cells are also noted (arrowheads). In (f), the clear spaces are numerous, resulting in a spongiform appearance. Original magnification of (a): 330x, (b): 330x, (c): 330x, (d): 198x, (e): 132x, and (f): 40x.

phagocytosis, we feel that this is a process distinct from that normally associated with reaction to necrotic or apoptotic cells. Thus we refer to this process of non-phagocytic, presumably enzymatic, resorption and recycling of cellular components as reclamation since the body is reclaiming the molecular contents for use by other cells.

3.4. Transmission Electron Microscopy (TEM) of Fibrotic Areas. Ultrastructural analysis of fibrotic areas reveals much more than can be appreciated by light microscopy. Thin, wispy atrophic cells, degenerating cells, and remnants of dead cells can be found entrapped in the dense collagenous matrix (Figures 18(a) and 18(b)). Many of these cells or cell fragments are too small to be visualized with the light microscope. Occasional cells show membrane budding with some of the buds loosely attached (Figure 18(b)), suggesting that this may be a form of decapitation membrane budding associated with atrophic downsizing. Some degenerating cells contain swollen endoplasmic reticulum and degenerating mitochondria that largely fill the cytoplasm. Fine myofilaments are prominently reduced (Figures 18(c) and 18(d)). Lysosomes often appear increased. Irregular vacuolar structures containing cytoplasmic debris and probably

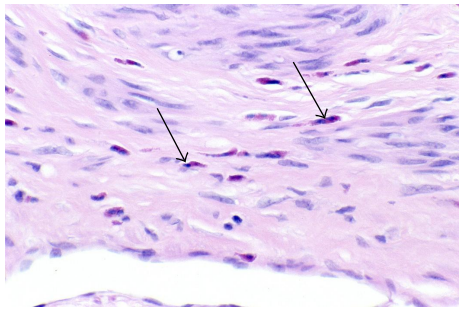


Figure 17: Leukocytes traversing fibrotic stroma of a fibroid tumor. Multiple ameboid eosinophils (arrows) with filopodial extensions are traversing the fibrotic stroma of this fibroid, seemingly unimpeded. Foci such as this indicate that the fibrotic stroma of fibroids can be penetrated by granulocytes and weakens the argument that the lack of inflammatory response to the dead and fragmented inanotic cells is due to the physical resistance of the dense fibrotic stroma in fibroids. Original magnification: 132x.

representing phagolysosomes are sometimes seen within the cytoplasm. Double membraned autophagic vacuoles are occasionally encountered (Figures 18(e) and 18(f)), frequently containing membranous debris and sometimes containing ribosomes or the stacked membranes of the Golgi apparatus. These changes are consistent with the atrophic and vacuolar degenerative changes noted with the light microscope and presumably reflect both atrophic downsizing as well as ongoing degeneration related to ischemia and nutritional deprivation.

Frequently, nuclei are not seen in these degenerating cells, although this could be related to tangential sectioning. Atrophic, but otherwise intact appearing, nuclei may be seen in markedly atrophic cells with greatly reduced cytoplasm that is largely degenerated, indicating that the nucleus is capable of downsizing and also that it is probably the last organelle to degenerate. Isolated, or naked, nuclei may be seen without surrounding cytoplasm, indicating that both the nuclear and cytoplasmic fragments may be remnants of dead cells (Figure 19(a)). Occasional degenerating cells with small nuclei exhibiting irregular, undulating or serrated borders are noted; this is an appearance similar to that of the acanthanuclear alteration noted with the light microscope and described above (Figure 19(b)).

Another feature often noted with TEM of fibrotic areas in fibroids is that fine filaments and cytoplasmic organelles such as mitochondria and ribosomes may be seen outside of any enclosing cytoplasmic membrane, lying free within the surrounding collagen fibers (Figure 19(c)). Although we have questioned whether this appearance is due to tangential sectioning, the myofilaments and other structures are sometimes seen adjacent to collagen fibers, thus offering support for an extracellular location, and they are often associated with adjacent cells that appear viable (Figure 19(d)). It seems more likely that this release of cellular structures is indicative of leaky cell membranes (degradative porosity) associated with dead or dying cells during the postinanotic state of reclamation, but the possibility cannot be excluded that this could be a mechanism of inanotic expulsion that might occur in the atrophic, inanotic phase. Nevertheless it is notable that despite the presence of cell organelles and cytoplasmic particles lying within the extracellular matrix, no inflammatory cells are present, and no macrophages with apoptotic bodies are noted. This suggests that cytokines have not been released during the slow, progressive process of inanosis, which now may be complete and giving way to an orderly disposal process of reclamation.

In addition, occasional cells exhibit entire segments of cytoplasm in which both the organelles and the cytoplasm itself are degenerated and are lacking a well-defined surrounding plasma membrane. This latter appearance, as well as the presence of islands of degenerated cytoplasmic fragments in the collagenous stroma, suggests that atrophic, degenerating cells may be capable of autoamputation of segments of cytoplasm (Figure 19(e)). Thus, atrophy of cells may involve multiple processes, including lysosomal and autophagosomal digestion of organelles and other constituents, decapitation membrane budding, and possibly expulsion of cell organelles and filaments and/or segmental autoamputation.

Reclamation, or the non-phagocytic resorption of dead cells and cell fragments, can be readily appreciated in the fibrotic areas of leiomyomas. In fact, in almost any fibrotic field, there will be fragments of cells or cytoplasmic particles dispersed within the stroma that are indicative of cell breakdown or expulsion, in the absence of inflammatory reaction (Figures 18 and 19). In addition to the dispersed fragments of cell cytoplasm and organelles lying free in the collagenous matrix, naked nuclei are sometimes seen (Figure 19(a)), with the latter often showing swollen or incomplete nuclear membranes and degenerated, clumped chromatin. Disintegrating cells with absence of plasma membrane in focal areas will be seen (Figure 19(f)). Characteristically, both the naked nuclei and the disintegrating cells are surrounded by clear zones with reduced collagenous matrix (Figures 19(a)–19(f)). These clear zones are believed to be resorption sites, similar to the resorption pits produced by osteoclasts or the digestion chambers of degenerating nerve fibers, in which organic material is digested by proteases. Both matrix metalloproteinases and lysosomal enzymes released from the disintegrating cells could be sources of this toxic milieu, which will ultimately result in the degradation of cellular material to the molecular level, so that it can be absorbed into the circulation and recycled. Thus, the images speak for themselves: degradation of cells and cellular contents is clearly occurring in these fibrotic areas without the assistance of phagocytic cells.

In summary, based upon our observations and our interpretation of these findings, as reported above, we have attempted to illustrate the concepts of phenotypic transformation, interstitial ischemia, inanosis, and reclamation in a graphic representation (Figure 20).

4. Discussion

Our observations suggest that many and probably most fibroids pursue a self-limited life cycle, which may be arbitrarily divided into 4 Phases (Table 1). Phase 1, in which

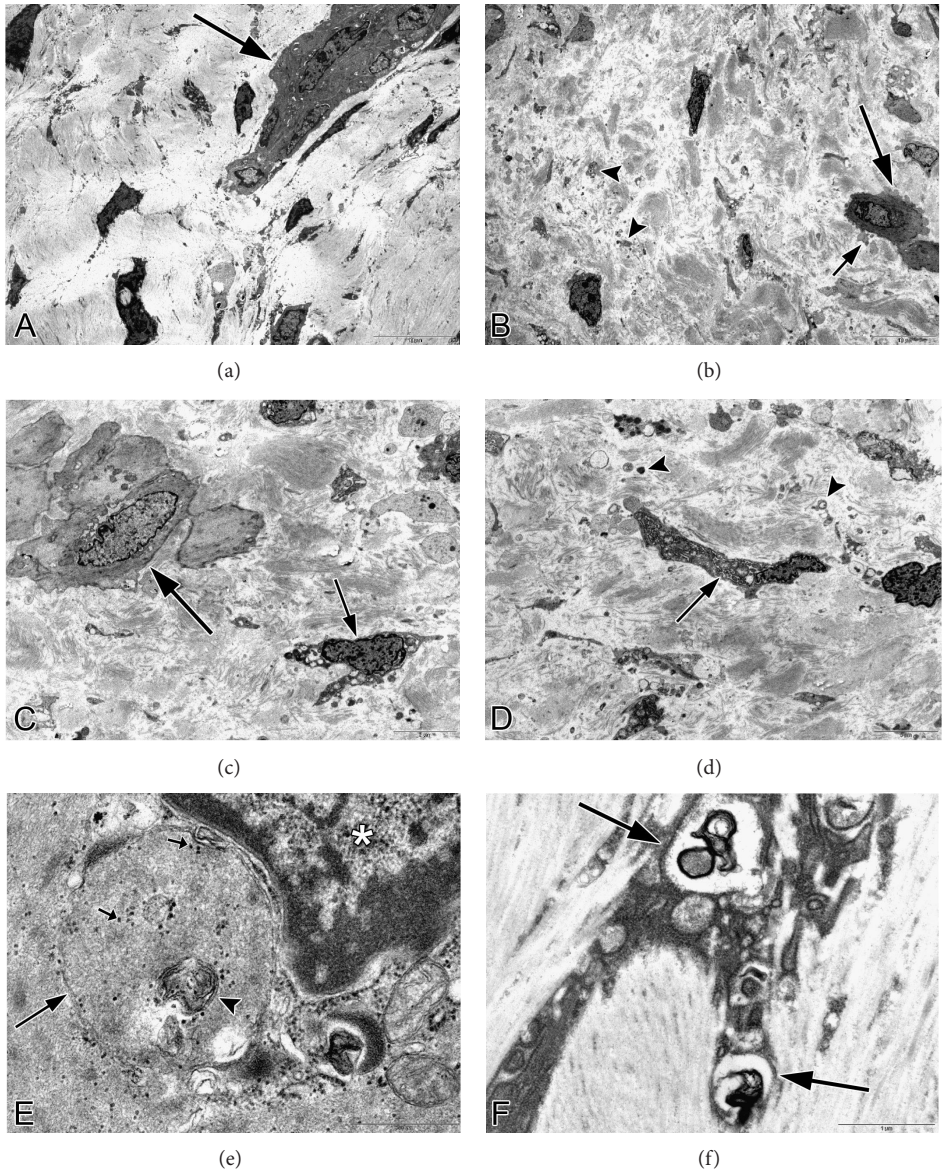
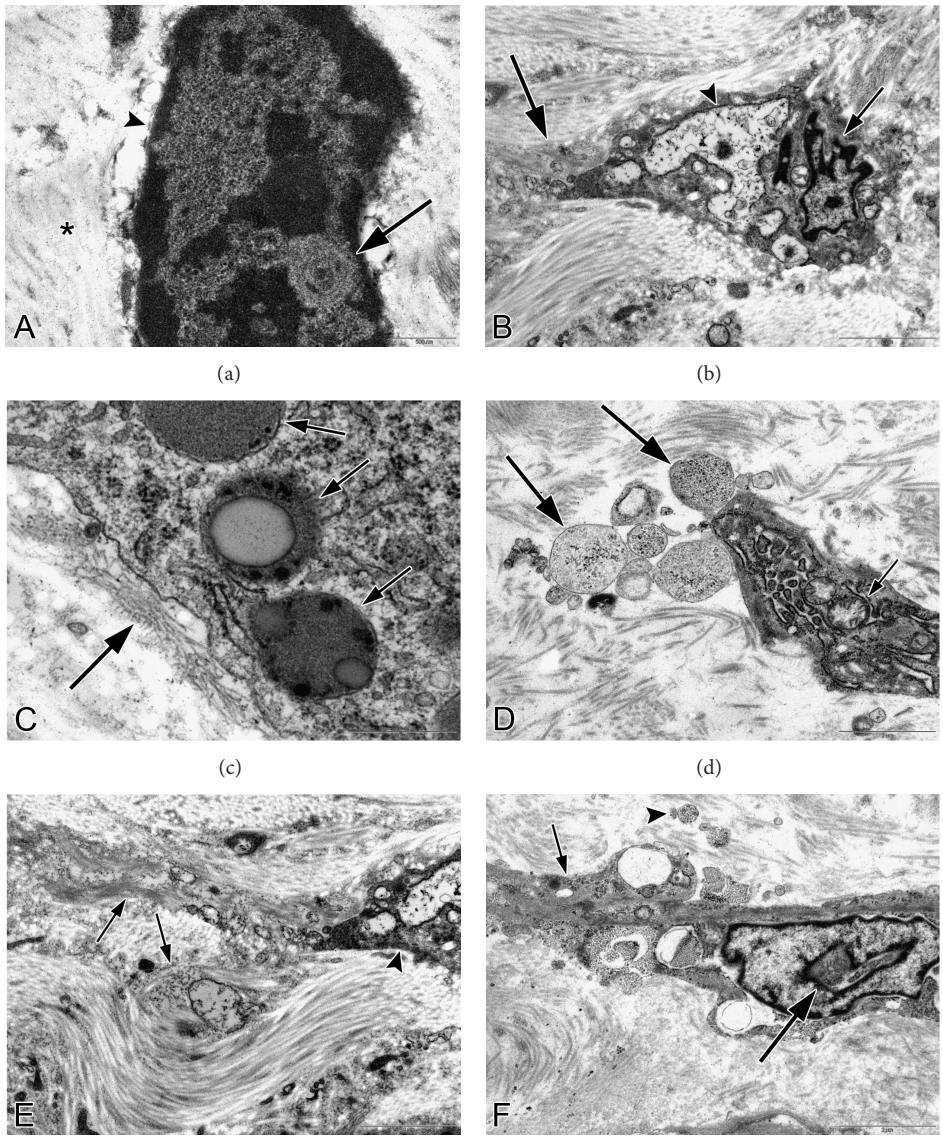


Figure 18: Ultrastructure of inanosis and reclamation. Electron microscopic examination of fibrotic areas in fibroids provides even more dramatic evidence of shrunken myocytes, widely spaced within the fibrotic stroma. In (a), there is a cluster of cells which have retained some cytoplasm (arrow) and provide a frame of reference for the small, shrunken cells. In (b), a cell in the lower right (long arrow) exhibits cytoplasmic budding, with one or two of these knob-like fragments apparently pinching off from the cell membrane (small arrow); we refer to this as decapitation membrane budding. Note that the loose, watery stroma contains numerous particles of cellular debris (arrowheads), without any evidence of inflammatory cell infiltrate or phagocytosis. In (c), the shrunken cell in the lower right (short arrow) contrasts with the less atrophic cell in the upper left (long arrow). The cytoplasm of the shrunken cell contains vacuoles, lysosomes, and degenerating organelles with few remaining myofilaments. The slender, atrophic cell in the center of the fibrotic field in (d) has retained its shape and endoplasmic reticulum (arrow), but has lost most of its myofilaments. The surrounding matrix contains abundant cellular debris (arrowheads), imparting a junkyard appearance. In (e), there is an autophagosome (long arrow) containing ribosomes (short arrows) and membranous debris which could be of endoplasmic reticulum origin (arrowhead). The remainder of the contents of the autophagosome resembles the adjacent cytosol and probably consists of degenerated myofilaments. The nucleus (asterisk) is in the upper right of the image. The cell in (f) exhibits two double membraned autophagosomes (arrows) containing electron-dense membranous debris. Original magnification of (a): 1700x, (b): 1250x, (c): 2550x, (d): 2550x, (e): 43,000x, and (f): 20,500x.

no, or insignificant, collagen matrix is present, is characterized predominantly by proliferation of myocytes. Similar to the experimentally-induced injury response of vascular smooth muscle, the proliferation of uterine myocytes may represent an injury response to the hypoxia of vasoconstriction occurring during menstruation, especially in women with the myometrial hypercontractility associated with dysmenorrhea. Under the continual stimulus of estrogen



(a) (b) (c) (d) (e) (f)

Figure 19: Ultrastructure of inanosis and reclamation. The nucleus in (a) is lying free within the stroma and shows a swollen, discontinuous nuclear envelope (arrowhead) that is being resorbed. The nucleus itself appears relatively intact except for the whorl in the lower right (arrow). Note the loosely expanded, watery stroma (asterisk) surrounding the nucleus. The nucleus of the shrunken cell in (b) displays a rippled or serrated border (short arrow) resembling the acanthanuclear alteration sometimes seen with the light microscope. The large vacuolar structure in the cytoplasm (arrowhead) could be dilated endoplasmic reticulum or possibly a phagolysosome and may correspond to the cytoplasmic vacuoles seen with the light microscope. The cytoplasmic tail at the left (long arrow) is degenerated and could be a precursor to autoamputation. In (c), myofilaments (long arrow) appear to lie outside of the cell membrane within a loosely expanded, watery stroma, suggesting that they have either leaked out or have been extruded. Three phagolysosomes (short arrows) are present in the cytoplasm of the cell. The cell in (d) shows budding of cytoplasmic debris (long arrows) from the elongate end of the cell. Some of the rounded particles could be autophagolysosomes with free ribosomes and degenerated cytoplasm. The cytoplasm of the cell consists almost entirely of dilated ER and swollen mitochondria (short arrow), with few remaining filaments. The tail of the cell in (e) exhibits complete degeneration of cytoplasm and organelles (arrows), with apparent loss of the cell membrane, while the remaining portion of the cell on the right (arrowhead) appears to be viable; the appearance suggests that cells might be capable of segmental autoamputation. The cell in (f) illustrates both the end stage of inanosis and the resorptive process of reclamation. The cytoplasmic contents are degenerated, granular, and vacuolated (short arrow), and the cell membrane has been breached in some areas, resulting in extrusion of degenerated cytoplasm into the adjacent watery stroma with disrupted collagenous matrix (arrowhead). The nuclear chromatin is condensed, and there is a degenerative structure in the middle (long arrow). Note that in each photo ((a)–(f)), the inanotic cells or cell particles are surrounded by loosely expanded, watery stroma, which is believed to correspond to the clear spaces sometimes seen around inanotic cells with light microscopy and thought to represent resorption pits associated with the reclamation process. Original magnification of (a): 26,500x, (b): 9,900x, (c): 43,000x, (d): 9,900x, (e): 9,900x, and (f): 9,900x.

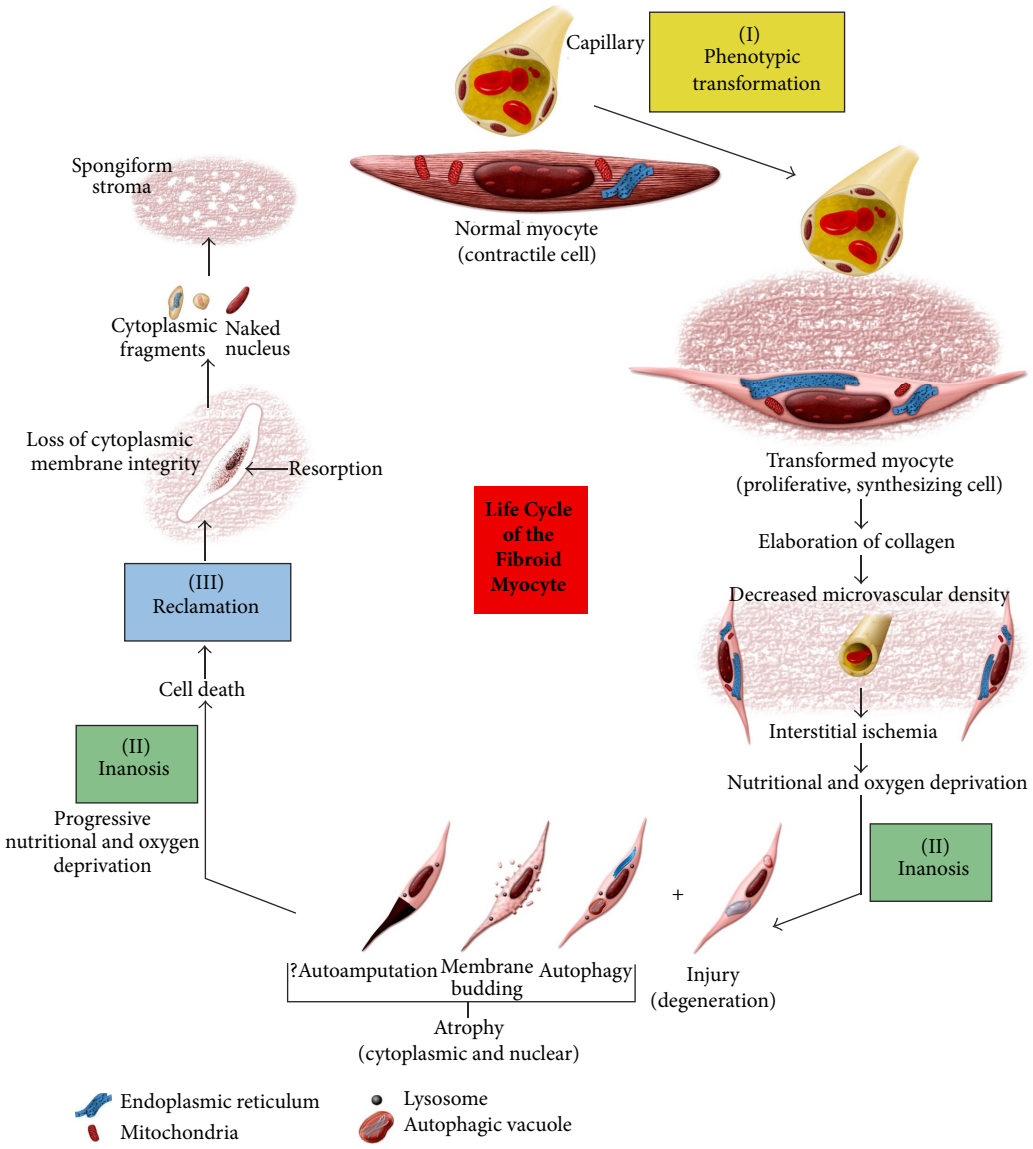


Figure 20: Life cycle of the fibroid myocyte. In this graphic representation, we have attempted to summarize our impression of the fibroid myocyte life cycle, from (I) phenotypic transformation to a proliferating, synthesizing cell, to (II) inanotic injury, atrophy, and ultimately cell death resulting from interstitial and vascular ischemia, to (III) reclamation in which dead cells and cell contents are resorbed by enzymatic degradation without the involvement of phagocytic cells. Illustration by David Sabio, Experimental Pathology Laboratories.

and progesterone, the smooth muscle cells continue to proliferate, eventually becoming biclonal or monoclonal as individual cell types with growth advantages emerge. Eventually and perhaps even at the onset in some tumors, the proliferating myocytes begin to elaborate collagen (Phase 2 = <10% collagen), completing the injury response in which smooth muscle cells are transformed from a contractile phenotype to a proliferative/synthetic phenotype. In Phase 3, proliferation continues, but the balance now tilts progressively in favor of collagen synthesis (10–50% collagen), and the extracellular matrix begins to accumulate and even predominate. Not only are the myocytes now further removed from the nearest capillaries because of the excess matrix, but also the angiogenesis seems to lag behind the growth in size of the tumor. Perhaps the production of fibrogenic growth factors, such as FGF [14] and TGF-β, exceeds the production of angiogenic growth factors such as VEGF, because the microvessel density appears to decline progressively as the tumors grow and age. Since all cells must lie within a reasonable distance of capillaries to receive oxygen and nutrients, eventually a state of interstitial ischemia will supervene. The histologic consequence of this interstitial ischemia is cellular atrophy, which is characteristic of late Phase 3 tumors and is one of the defining features of Phase 4 tumors.

During the final phase (Phase 4) in the life of a fibroid, the excessive production of extracellular matrix reaches a maximum (>50% collagen), angiogenesis is reduced or at least fails to keep pace with the growth of the tumor,

myocyte proliferation is greatly diminished or absent, and cellular atrophy occurs. This is the phase of involution, characterized by large areas of hyaline matrix with scattered islands of atrophic myocytes. Necrosis due to infarction is often seen in Phase 4, but infarction may occur in any phase. Likewise, apoptotic cells are occasionally noted. However, a third form of cell death, inanosis, the ultimate endpoint of atrophy and the consequence of nutritional deprivation, is a characteristic and defining feature of this final phase.

In some respects, the process of inanosis occurring within fibroids can be considered ischemic in nature and in this respect likened to both necrosis and apoptosis which may occur as a result of ischemia. A fundamental difference between these processes, however, is the rapidity with which they occur. Necrosis and apoptosis are both relatively rapid consequences of severe to moderately severe ischemic injury, while the histologic features of inanosis suggest that this is a very slow atrophic process which probably progresses over days to months until cellular proteins have been depleted to the point that vital cellular functions can no longer be maintained. In fact, zones of necrosis are not infrequently found in fibroids, presumably resulting from more sudden and profound ischemia, and rare cells which appear apoptotic are noted among the myocytes of Phase 3 and 4 tumors. From a quantitative standpoint, however, the atrophic cells of inanosis are more commonly found in late phase fibroids than are apoptotic cells, and they occur more consistently than do zones of necrosis.

In addition to pathogenetic differences, the morphologic features of inanosis, particularly the nuclear changes, are distinct from those of apoptosis. Apoptosis is a well-described and morphologically defined pattern of cell death. Other than cell shrinkage, there is very little similarity between apoptosis and inanosis by light microscopy. When the apoptotic cell shrinks, it also tends to retract from the surrounding tissue, rounds up and condenses the cytoplasm, whereas the atrophic cells of inanosis shrink to a more marked degree but usually maintain their shape and their connection with the surrounding stroma. While the apoptotic cell often exhibits hypereosinophilic cytoplasm, inanotic cells typically show cytoplasmic pallor, probably because of progressive depletion of cytoplasmic protein and organelles. Moreover, the nuclear changes are particularly different in that apoptotic cells characteristically condense their chromatin in peripheral aggregates against the nuclear membrane and then subsequently often fragment, whereas the myocyte tombstone nucleus is markedly reduced in size, pale staining, and nonfragmented. When the nuclear pallor of inanotic cells is marked and the nuclear size is diminished to 2 μm or less, it is assumed that the cell is nonviable. This is based upon the fact that if all of the DNA of a human nucleus was condensed, it would occupy a volume equivalent to a cube measuring 1.9 μm on a side [15]. If this volume is then converted to that of a sphere to more nearly approximate the shape of a cell nucleus, it would be equivalent to a sphere with a diameter of 2.356 μm. Thus, it must be assumed that cells having nuclei less than 2.356 μm in diameter, as well as nuclear pallor, have lost nucleic acid material, and that such loss would be incompatible with cell survival. As we have noted in our description of inanosis in Section 3, some of the smaller inanotic nuclei measured between 1 and 2 μm, although admittedly it is always difficult to exclude tangential sectioning.

Morphologic comparisons of inanosis with necrosis are more problematic than comparisons with apoptosis because necrosis is less well defined than apoptosis and also more variable in appearance. The histologic hallmarks of necrosis are usually considered to be the regional involvement of multiple contiguous cells, hypereosinophilia and swelling of the cytoplasm, variable nuclear changes including pyknosis, karyolysis, or karyorrhexis, and an accompanying inflammatory reaction because of the rupture of cell membranes with the release of cell contents. However, if the definition of necrosis is extended to include individual cell death with cytologic changes of cell shrinkage, nuclear and cytoplasmic pallor, and lack of inflammatory reaction, then inanosis would not be distinguishable from necrosis and would have to be considered as a variant of necrosis. At the least, however, there are a few features of inanosis that are not typically associated with necrosis. The degree of cell and nuclear shrinkage and pallor in inanosis is marked, the pattern of cell involvement is often mottled with viable cells between the dead cells, and there is no inflammatory reaction despite ultimate membrane degradation and release of cell contents. Necrotic cells probably do not achieve the marked degree of shrinkage seen in inanosis because there is often no preceding atrophy and because they are more rapidly eliminated by the enzymatic and phagocytic action of inflammatory cells attracted to the site by cytokines and the leakage of their cytoplasmic contents. Finally, it is of interest that autophagy, which we have shown by EM is clearly occurring in the late phase fibroid tumors, has been shown to be cytoprotective when the availability of oxygen and nutrients is poor, and in such environments is effective in inhibiting the induction of both apoptosis and necrosis [16].

In this regard, the argument might also be made that inanosis is in fact autophagic cell death. While recognizing that there is no clear answer to this question, we favor the concept that autophagy is cytoprotective to cells deprived of nutrients, and that cell death in this circumstance is more likely related to a critical lack of oxygen and essential nutrients available to the cell in its environment, rather than being secondary to a self-destructive overzealous autophagic process. If nutritionally deprived cells, struggling to survive, possess the regulatory controls to suppress apoptosis, necrosis, and apparently the release of cytokines as suggested by the lack of inflammation, then it seems likely that autophagy would also be a finely tuned and regulated survival mechanism that would not promote the demise of the cell which it is designed to protect [17, 18].

Comparative morphologic features of necrosis, apoptosis, and inanosis are summarized in Table 4.

In summary, the distinguishing features of inanosis in fibroids are the slowness of the process, the combined vascular and interstitial ischemic pathogenesis resulting from the elaboration of an extensive extracellular matrix, and the consequent cellular atrophy presumably due to nutritional and oxygen deprivation, which culminates in the formation of the hallmark cell, the myocyte tombstone. An alternative

Table 4: Comparison of necrosis, apoptosis, and inanosis.

	Necrosis (ischemic)	Apoptosis	Inanosis
Basic mechanism	Energy independent	Energy-dependent	Energy independent
Speed of Process	Rapid	Rapid	Slow
Individual cell versus groups of cells	Usually groups	Individual	Individual
Typical pattern	Regional groups of cells	Individual cells	Mottled (interspersed viable cells)
Histologic hallmark	Zonal (multicellular) coagulative necrosis	Apoptotic nuclear changes; tingible body macrophages	Myocyte tombstone
Nuclear changes	Pyknosis, karyorrhexis, or karyolysis	Pyknosis and karyorrhexis	Shrinkage and pallor
Cytoplasmic volume	Cell swelling	Cell shrinkage	Cell shrinkage
Cytoplasmic shape	Variable	Rounded	Normal
Cytoplasmic tinctorial features	Hypereosinophilic	Hypereosinophilic	Pallor
Relation of cell to adjacent cells or stroma	Variable	Detaches from neighboring cells	Maintains connection initially
Inflammation	Usually	No; macrophages	No
Disposition of dead cells	Phagocytosis	Phagocytosis	Gradual dissolution (Reclamation)
Release of cytoplasmic contents	Yes	No	Yes

term for inanosis, combining these distinctive characteristics, is slow vasculointerstitial ischemic and atrophic cell death.

Clearly there is no evidence of phagocytosis of the inanotic cells, nor is there any reason to hypothesize enzymatic digestion by leukocytes since there is no inflammatory reaction associated with inanosis. Perhaps there is an orderly, internal as well as extracellular, disposition process for the recycling of amino acids and other important molecules from the myocyte tombstones of inanosis. This process may be viewed as a type of reclamation in which the molecular contents of the cell are reclaimed by the body and recycled. Reclamation may be mediated by both lysosomal degradation and matrix metalloproteinases. The lack of an inflammatory reaction or recruitment of macrophages seems contrary to the notion that the extrusion of cytoplasmic contents will evoke an inflammatory reaction as occurs with necrosis. It could be argued that cytokines that might be released into the dense stroma do not reach the capillaries because of the dense fibrosis; however, there seems no reason for a starving cell to release cytokines. Since it has been reported that nutritional deprivation inhibits apoptosis in cells [19], it seems plausible that starving cells might also restrict the production and release of cytokines. Evidence has also been presented that autophagy, initiated by starvation or metabolic stress, may prevent the death of cells by necrosis [20]. Finally, the electron microscopic image of shrunken, fragmented, dead myocytes, with dissolution of cell membranes and cytoplasmic contents, and extruded cell material in the adjacent stroma, without phagocytic involvement, is evidence in itself for the existence of a noninflammatory, nonphagocytic, presumably enzymatic, degradative process, which we refer to as reclamation.

From a broader standpoint, might such a mechanism as reclamation be relevant to the routine removal of effete cells from our viscera as well, since we know that most of the cells in our body have a limited lifespan and are periodically replaced? Apoptotic cells are removed by phagocytosis, and yet phagocytes with apoptotic bodies are only infrequently encountered in histologic sections of viscera. And from the standpoint of energy efficiency, why would nature develop a system that would require the energy input of a second cell (such as a phagocyte) to remove senescent cells on a regular basis?

5. Conclusions

We have presented morphologic studies to support our hypothesis of the contribution of collagen synthesis to both the enlargement and the eventual involution of uterine fibroids. The excessive elaboration of extracellular matrix ultimately results in hyalinization of both the smaller vessels and the interstitium, leading to reduced microvascular density, interstitial and vascular ischemia, and nutritional deprivation of the tumor myocytes. Atrophy through autophagy then develops as a survival mechanism for individual cells, until a critical point is reached when the nutritional deficits are incompatible with survival and inanotic cell death occurs. Inanosis differs from both apoptosis and necrosis in several respects, including the initiating factors, the slowness of the process, and the morphologic features. Future studies of immunohistochemical and molecular markers are needed to complement these morphologic observations and to provide additional evidence for the differentiation of inanosis from apoptosis and necrosis. Further elucidation of the process of inanosis may offer insights into the pathogenesis of cachexia in neoplastic diseases since nutritional deprivation may play a key role in each. Finally, disposition of inanotic cells seems to occur by a reclamation process of nonphagocytic, presumably enzymatic dissolution in which cellular molecules are reclaimed and recycled for utilization elsewhere.

Our proposals and our concepts are based upon our observations and our attempt to understand the biology of these tumors. Although we believe that many fibroids undergo involutional changes later in their development, we neither propose that these changes occur in all fibroids, nor that the involutional changes occurring in some fibroids result in shrinkage or disappearance of those tumors. In fact, we have shown that the later phase tumors tend to be larger on average because of the accumulated collagenous matrix, and it is the gross size of these tumors that is recognized as one of the major determinants of their morbidity.

Conflict of Interests

All authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

The Natural History of Uterine Leiomyomas: Morphometric Concordance with Concepts of Interstitial Ischemia and Inanosis

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Based upon our morphologic observations, we hypothesize and also provide morphometric evidence for the occurrence of progressive developmental changes in many uterine fibroids, which can be arbitrarily divided into 4 phases. These developmental phases are related to the ongoing production of extracellular collagenous matrix, which eventually exceeds the degree of angiogenesis, resulting in the progressive separation of myocytes from their blood supply and a condition of interstitial ischemia. The consequence of this process of slow ischemia with nutritional and oxygen deprivation is a progressive myocyte atrophy (or inanition), culminating in cell death, a process that we refer to as inanosis. The studies presented here provide quantitative and semiquantitative evidence to support the concept of the declining proliferative activity as the collagenous matrix increases and the microvascular density decreases.

1. Introduction

We hypothesize that many uterine leiomyomas (fibroids) undergo progressive obsolescence and eventual involution, largely as a result of the excessive elaboration of collagen into the interstitial matrix, thereby increasing the distance between tumor myocytes and their blood supply. Since the smooth muscle cells of fibroid blood vessels mirror the phenotypic transformational changes of the tumor myocytes, the blood vessels of fibroids also become progressively more fibrotic and hyalinized. Thus, tumor myocytes are subjected to a reduced supply of essential nutrients and oxygen as a consequence of both vascular and interstitial ischemia.

If the growth of fibroid tumors was solely dictated by those genetic and epigenetic changes that promote an increased proliferative rate, then the tumor myocytes should continue to proliferate and the tumor would continue to grow. On the other hand, if vascular and interstitial ischemia do develop within these tumors as the deposition of collagen continues, the proliferative capacity of myocytes would probably be diminished as the diffusion of nutrients and oxygen is impeded in both the fibrotic, thickened vessels and the fibrotic interstitium. In addition, if the rate of angiogenesis is not equivalent to or greater than the rate of fibrogenesis, the tumor myocytes would be subjected to the additional stress of an increased distance between myocytes and capillaries (reduced microvascular density).

With these concepts of excessive production and accumulation of collagen, reduced microvascular density, and combined vascular and interstitial ischemia in mind, we

hypothesized that, in general, tumors composed of the highest percentage of collagenous matrix would have the lowest microvascular density and the lowest proliferative rate. The morphometric studies that we report herein offer support for this hypothesis.

2. Materials and Methods

2.1. Study Participants. Subjects were recruited from the George Washington University (GWU) Medical Center Obstetrics and Gynecology Department surgical rosters as part of the National Institute of Environmental Health Sciences (NIEHS) Uterine Fibroid Study. Informed consent was obtained, and the study was approved by the institutional review boards at the NIEHS and GWU. Details of patient recruitment, demographics, and collection of gross pathology data have been previously reported [1].

2.2. Developmental Phases of Fibroids. We arbitrarily categorized the fibroid tumors in a large fibroid study (NIEHS-Uterine Fibroid Study) into four phases on the basis of a microscopic estimation of the percentage of the tumor occupied by extracellular matrix in H&E-stained slides. All tumors in this study were therefore placed in 1 of the 4 phases on the basis of the following estimation:

phase 1 = no, or insignificant, collagen matrix,

phase 2 = <10% collagen,

phase 3 = 10–50% collagen,

phase 4 = >50% collagen.

2.3. Selection of Cases for Morphometric Study. Cases were selected at random from the previously phased fibroid tumors in the NIEHS Uterine Fibroid Study. Five fibroid tumors were selected from each of the 4 phases, for a total of 20 tumors. The only restrictions placed upon the selection of cases were as follows.

(a) Gross tumor size data must be available.

(b) There must be sufficient tissue in the paraffin block to be able to recut for special stains.

(c) The fibroid fragment must be large enough (>5 mm) to accurately evaluate the various parameters under study.

(d) There should be no cautery edge artifact or other significant histologic artifacts in the section.

2.4. Size of Tumors. Gross dimensions of the fibroid tumors were obtained from the fibroid worksheets used in the NIEHS Uterine Fibroid Study. Some tumors were only recorded as <2 cm or ≥2 cm.

2.5. Image Analysis of Collagen Content. Sections of fibroid tumors were cut at 5 μm, placed on microscopic slides, and stained with Masson's trichrome stain. This stain provided an ideal contrast for our imaging purposes because the collagen is stained blue and the muscle is stained red. The Masson's trichrome-stained slides were then cleaned with an isopropanol solution and scanned with the Aperio Scanscope XT Scanner (Aperio Technologies, Inc., Vista, CA), a machine which uses line-scanning technology to capture high-resolution, seamless digital images of glass slides. After scanning, the slides were viewed with the Aperio Imagescope v. 11.1.2.752, a digital slide viewing and analysis program. Image analysis was performed using the Aperio colocalization algorithm. This algorithm calculates the contribution of multiple stains at every pixel location in the image. For the analysis of the Masson trichrome stains, the algorithm's parameters were set for the identification of all blue collagen staining present. The data output from the Aperio colocalization algorithm was expressed as the percentage of collagen present in each sample. This data was exported to an excel table and graphed.

2.6. Mitosis Counting. For the mitosis counts, as well as the PCNA and vascular counts, an Olympus BX50 microscope was used. The H&E-stained slides were scanned with the 20x objective until a mitosis was identified, and then the number of mitoses in 10 high-power (40x) fields was counted. Only structures consistent with the prometaphase, metaphase, anaphase, or telophase stages of mitosis in which hairlike chromatin fibers could be seen were counted.

2.7. Immunohistochemistry

PCNA. Formalin-fixed, paraffin-embedded tissue sections (5 μm thickness) were deparaffinized in xylene, rehydrated in ethanol and submerged in 3% hydrogen peroxide for 15 minutes. Antigen retrieval was achieved by microwave method with distilled water (power level 5 for two cycles of five minutes each). Tissue sections were incubated with primary antibody (Monoclonal mouse Anti-PCNA, Chemicon International, Inc., Temecula, CA) at a 1 : 1200 dilution (diluted equally in 1% nonfat dry milk and 1% bovine serum albumin (BSA)) for 30 minutes, followed by incubation with Goat anti-mouse IgM secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1 : 400 dilution (diluted in 1% BSA) for 30 minutes. Next, Streptavidin Peroxidase SS Label (BioGenex, San Ramon, CA) was applied to the sections for 30 minutes. All incubations were performed at room temperature in a moist chamber, and each step was followed by two rinses in 1X Wash Buffer (Dako, Carpinteria, CA). Visualization was performed with DAB chromagen for 6 minutes and counterstained in hematoxylin for 30 seconds. The area (or areas when necessary to count more than one) of the greatest staining with the PCNA antibody was chosen for the counts in each fibroid. All cells with nuclear staining were counted, regardless of the intensity. A total of 1000 cells, including both stained and unstained, were counted, using a 25 square (5 × 5) grid.

Factor 8 (von Willebrand Factor). Formalin-fixed, paraffin-embedded tissue sections (5 μm thickness) were deparaffinized in xylene and hydrated through a graded series of ethanol. The slides were then quenched in 3% hydrogen

peroxide for 15 minutes at room temperature. Ready-to-use Carezyme Pepsin antigen retrieval solution (Biocare Medical) was added to the tissue slides and allowed to incubate for 5 minutes at 37°C. Afterwards, the tissues were blocked with normal serum from the Vectastain Rabbit Elite Kit (Vector Laboratories) for 20 minutes at room temperature. Rabbit anti-human Factor VIII Antibody (Biocare Medical) was applied to the tissues at 1 : 800 for one hour at room temperature. Normal rabbit serum (Jackson ImmunoResearch) was used instead of the primary antibody (at the same dilution as the primary antibody) for negative control staining. The secondary antibody and label complex reagents from the Vectastain Rabbit Elite Kit were both incubated on the tissue for 30 minutes at room temperature. The stain was visualized by using DAB chromagen (Dako) and hematoxylin counterstain. Lastly, the slides were dehydrated, cleared in xylene and coverslipped.

2.8. Microvascular Density (Factor 8 Counts). Each tumor was scanned at low power (4x or 10x objective) to find the areas of most prominent vascular density with the Factor 8 stain. Vessels were counted with a 20x objective, and an eyepiece containing a 5 × 5 grid (each side = 750 μm) to simplify the counting. Only structures that had the linear shape of vessels, whether with open or compressed lumina, or cross sectional profiles of vessels, were counted. Individual cells stained with the Factor 8 antibody were not counted. All vessels within the 5 × 5 grid, using the 20x objective, were counted in each of five different microscopic fields, and the five scores were then averaged to give an average microvessel density score for each fibroid.

The distance between vessels was also measured in some tumors, using the 20x objective and an ocular micrometer. This could only be accomplished with any accuracy in areas where several vessels had been cut in cross-section.

2.9. Statistical Analysis of Collagen Content, PCNA Proliferation Index, and Microvessel Density. Based upon the image analysis software evaluations of the mean percentage of collagen in the Masson trichrome-stained slides from each of the four phases, analysis of variance (ANOVA) and Fisher's least significant differences (LSD) multiple comparisons procedure were used to compare the percent of collagen among the four phases. Similar statistical procedures were used for the analysis of the PCNA proliferation indices and the Factor 8 microvessel density determinations.

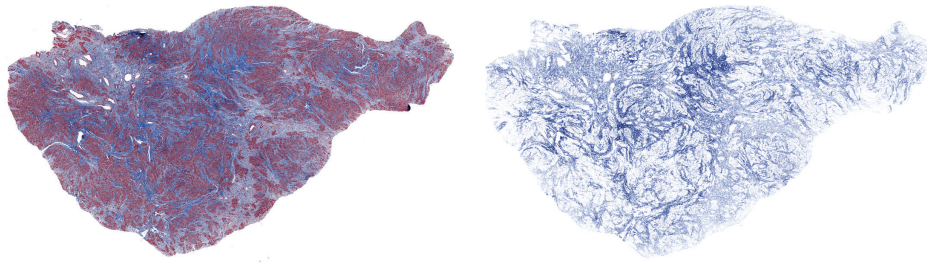
2.10. Simulation Study of Myocyte to Vessel Distances. To supplement the analyses of the Factor 8 microvessel density studies, we used the Monte Carlo simulations to estimate the average and maximum distances between myocytes and blood vessels in phase 1 and phase 4 tumors. These simulations generated hypothetical 750 μm × 750 μm microscopic fields (the size of the eyepiece grid) in which blood vessels and myocytes were assumed to be uniformly distributed throughout areas not occupied by collagen. Although smooth muscle cells can vary considerably in size, for the purposes of these calculations, myocytes were assumed to be aligned in parallel and to be 100 μm long and 10 μm wide [2, 3]. It was further assumed that, in the absence of collagen, myocytes would fill the entire field such that there would be 750 μm/100 μm × 750 μm/10 μm = 7.5 × 75 = 562.5 myocytes per field. Blood vessels were assumed to be round with a diameter of 7 μm. Mean numbers of blood vessels and percentages of collagen present in a 750 μm × 750 μm field were determined from the actual phase 1 and phase 4 tumors. Using these means, 500 hypothetical phase 1 fields and phase 4 fields were simulated, and for each field, the average distance and maximum distance were determined between the myocytes and their closest blood vessel. The likely ranges of average and maximum distances were estimated as the 2.5th percentile to the 97.5th percentile of the 500 simulated average or maximum distances, for each phase. Average distance and maximum distance were compared between phase 1 and phase 4 using z-statistics.

3. Results

3.1. Size of Tumors. Although exact measurements were not available for some tumors, all tumors were at least placed into a <2 cm or ≥2 cm category. On this basis, only 2 of the phase 1 tumors were >2 cm, while 3 of the phase 2 tumors were >2 cm, and all five of the tumors in both phase 3 and phase 4 were ≥2 cm (Table 1). Although the numbers are small, the data tend to support the concept that as fibroid tumors grow, most of them will accumulate extracellular matrix and that this continued deposition of collagen probably contributes, along with tumor cell proliferation, to the increasing size of the tumors.

3.2. Phasing of Fibroids by Collagen Content. In order to more accurately quantitate the percentage of collagen present and to evaluate the validity of the visual microscopic estimation, the microscopic sections of Masson's trichrome-stained slides of all 20 fibroids were scanned in the Aperio Scanscope and analyzed with Aperio Imagescope software (Figure 1). The individual tumor results within each phase are listed in Table 1, and the average percent of collagen for each phase is shown in Table 2. These data support the validity of visual estimation of the collagen content of fibroid tumors in H&E-stained slides. Almost all tumors within phases 2, 3, and 4 were found to have collagen components that fell within the estimated ranges of <10%, 10–50%, and >50%, respectively, and the collagen content of phase 1 tumors was minimal in each case (Table 1). In addition, the average values revealed broad differences between the phases, and there was no overlap in the collagen content of individual tumors between phases 2, 3, and 4. Finally, the computerized results demonstrate a progressive rise in the percentage of collagen from phase 1 to phase 4 (Figure 2), verifying the categorization of fibroids by visual estimation on H&E-stained slides.

Myometrial tissue was also available from 8 of the 20 patients in the study. The average collagen content of these 8 specimens was 19.4% (Figure 2), which exceeds the average collagen content of phase 1 and 2 tumors, and is lower than that of the phase 3 and 4 tumors. This intermediate collagen



(a) (b)

Figure 1: Image analysis of fibroid collagen content. By light microscopic examination of the H&E-stained section of this tumor, the collagen content was estimated to be more than 10% and less than 50% and thus to fall into the phase 3 category. The image on the left (a) shows the Masson's trichrome-stained section of this tumor, while that on the right (b) depicts the markup image of the same section in which only the blue-stained collagen has been colocalized, allowing for quantitation of the percent of collagen, which was found to be 38.4%.

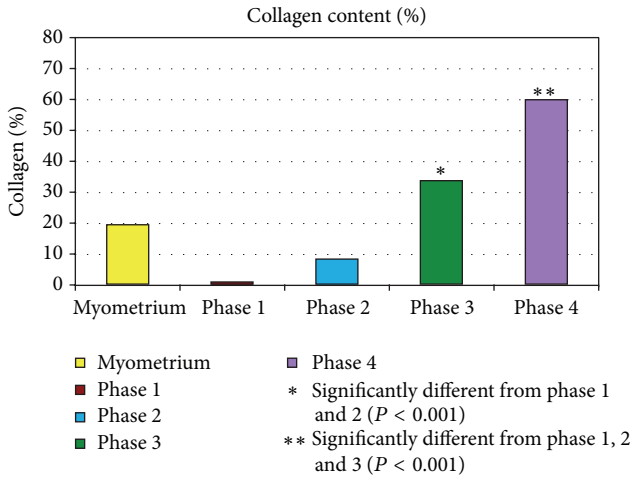


Figure 2: Mean percent collagen content of fibroid phases 1–4 by image analysis. The bar graph depicts the progressive accumulation of collagen in the transition from phase 1 to 4. There were 5 samples per phase group and the values represent the means. The myometrial bar is the mean value of 8 samples. *Significantly different from phases 1 and 2 ($P < 0.001$). **Significantly different from phases 1, 2 or 3 ($P < 0.001$).

content of the myometrium is consistent with the notion that the early development of fibroids is marked primarily by myocyte proliferation, in contrast to the later phases (phases 3 and 4) in which there is excessive elaboration and accumulation of collagenous matrix. It is likely, however, that the collagen content of the myometrium is also variable and may be affected by a number of factors, such as the age of the patient, the reproductive history, and the location within the uterine wall.

Statistically, the percent of collagen differed significantly among the four phases (ANOVA P value < 0.0001, meaning that at least one phase was significantly different from the others). By Fisher's LSD, there was no significant difference in the percent of collagen between phases 1 and 2. Phases 1 and 2 were significantly lower than phase 3 and phase 4 ($P < 0.001$). Phase 3 was significantly lower than phase 4 ($P < 0.001$) (Figure 2).

3.3. Proliferative Activity. Proliferative activity of fibroids has previously been shown to be increased in comparison to the myometrium [4]. As noted in Table 1, the tumors chosen for this study exhibited few mitotic figures, with only 1 tumor exhibiting more than 1 mitosis in 10 high-power fields. However, it is notable that none of the phase 4 tumors exhibited any mitotic activity. Of the 28 myometrial samples available from 8 of the 20 patients, no mitotic figures were identified.

On the other hand, immunohistochemical nuclear staining for the proliferation marker, PCNA, revealed positive staining of some nuclei in all tumors. The percentage of positive staining cells (PCNA proliferation index) and the intensity of staining, varied from one area to another in each tumor. As noted in Table 1, the average percentage of nuclei staining with the PCNA antibody declined progressively from phase 1 to 4. Within the tumors of each phase, the number of PCNA positive nuclei was variable from one tumor to another, but notably the highest value in any of the phase 4 tumors (20.3%) was less than the lowest of the phase 1 tumors (24.3%), and the average PCNA value for the phase 1 tumors was more than 3 times that of the average value for the phase 4 tumors (48.6% versus 15.0%). Representative examples of PCNA staining are shown in Figure 3(a), and the average scores for each phase are depicted in the bar graph in Figure 3(b).

The percent of PCNA labeling for phase 1 samples was significantly higher than that for phase 4 samples ($P < 0.01$). The percent of PCNA labeling for phase 2 and phase 3 samples was intermediate between phase 1 and phase 4 and did not differ significantly from either phase 1 or phase 4.

3.4. Microvessel Density (MVD). This parameter was assessed by immunohistochemical staining for Factor 8. The values listed in Table 1 represent the number of vessels counted with the 20x objective in a 0.5625 mm^2 field (750 μm × 750 μm). The average values decline from a high of 92 in the phase 1 tumors to a low of 33 in the phase 4 tumors, a roughly threefold decline in MVD. Representative images from each phase are shown in Figure 4(a), and average microvessel counts for each phase are depicted in the bar graph in

Table 1: Summary of individual tumor data.

Fibroid	Size (cm)	Collagen[b] (%)	Mitoses	PCNA (%)	Factor 8 (microvessel density)
Phase[a] 1					
1a	<2	1.14	1	24.3	92
2a	>2	0.17	0	54.7	79.2
3a	<2	1.86	1	69.9	113.0
4a	>2	0.61	0	35.2	56.6
5a	<2	0.37	0	59.1	119
Average	**2 > 2 cm**	**0.83%**	**0.4**	**48.6**	**92**
Phase 2					
1b	1.5 × 1.0 × 1.0	5.11	0	18.1	45.0
2b	>2	6.04	0	13.4	99.6
3b	>2	11.89	0	56.9	40.2
4b	4.0 × 3.0 × 2.5	3.33	1	25.3	25.4
5b	1.8 × 1.9 × 1.0	14.64	1	48.8	73.6
Average	**3 > 2 cm**	**8.20%**	**0.4**	**32.5**	**57**
Phase 3					
1c	>2	30.81	0	3.1	27.0
2c	4.0 × 5.0 × 3.0	36.55	0	8.2	8.8
3c	>2	33.36	0	19.1	27.6
4c	7.3 × 6.5	29.99	2	50.2	66.8
5c	3.5 × 3.0 × 2.0	38.41	0	47.6	56.4
Average	**5 > 2 cm**	**33.82%**	**0.4**	**25.6**	**40**
Phase 4					
1d	3.0 × 3.0	50.02	0	13.8	29.0
2d	6.0 × 5.0 × 4.0	72.65	0	19.1	43.6
3d	≥2.0	51.76	0	20.3	18.8
4d	14 × 7.5	67.42	0	6.3	40.8
5d	5 × 4.5	58.11	0	15.5	31.6
Average	**5 > 2 cm**	**59.99%**	**0.0**	**15.0**	**33**

[a]Phase was based upon light microscopic estimation of the percentage of collagen in the tumor in H&E-stained slides.
[b]Collagen (%) was determined by image analysis of Masson's trichrome-stained slides.

Table 2: Mean collagen content of phases 1–4.

Phase	H&E-stained slides collagen content (%)	Aperio colocalization % collagen (mean ± s.e.)
1[a]	No, or insignificant, collagen matrix	0.83 ± 0.30
2	<10%	8.20 ± 2.16
3	10–50%	33.82 ± 1.62
4	>50%	59.99 ± 4.39

[a]$n = 5$ slide samples per phase group.

Table 3: Monte Carlo simulations of mean and maximum distances of myocytes from vessels.

	Mean distance, μm	Maximum distance, μm
Phase 1[a]	34.9 (32.5–38.5)	141.4 (114.1–186.3)
Phase 2	43.5 (39.8–49.1)	168.6 (132.2–235.1)
Phase 3	52.3 (46.1–60.0)	190.3 (143.6–263.2)
Phase 4	58.0 (50.5–68.4)*	195.6 (144.2–283.4)*

[a]$n = 5$ slide samples per phase group.
*Statistically significant versus phase 1 ($P < 0.001$).

Figure 4(b). As noted in Table 1, there is a variation in the microvessel counts of individual fibroids within each phase, but the overall trend is one of decreasing MVD from phase 1 to 4. Variation in MVD was also apparent from one field to another of the same fibroid, particularly in phases 2, 3, and 4. For example, one of the phase 3 fibroids (2c) with a microvessel count of 8.8 displayed a higher level of vascularity (microvessel count of 19.2) in a section from another area of the same tumor (data not included in Table 1), and one of the phase 4 tumors (4d) with a microvessel count of 40.8 displayed other areas of reduced vascularity with counts of 23 and 24 (data not included in Table 1). In the Phase 4 tumors, the vascularity of hypocellular, hyalinized areas was sometimes equivalent to that of the residual cellular areas, while in other areas of hyalinization the MVD was considerably reduced as shown in Figures 4(a) (4), and 5. Since hyalinized areas with reduced vascularity were not included in the microvessel counts, it is likely that the overall MVD of the more fibrotic tumors, such as the phase 3 and 4 tumors, is even lower than measured; and further, that the difference in MVD between phase 1 and phase 4 tumors is probably greater than calculated since the hyalinized areas were not included.

The distances between the vessels in a hyalinized area of a phase 4 tumor in Figure 5 ranged from 110 μm to 360 μm, with only a few surviving atrophic cells in the intervening hyalinized stroma, suggesting that the greater distances exceeded the physiologic limits of cell survival. In Figure 6, most of the cells lie within 50–75 μm of the two vessels in the field. At the arrowhead, there is a lone surviving cell that is 50 μm from the central capillary; at the short arrow, a single cell is noted that is approximately 100 μm from the central vessel; and at the long arrow, there appears to be a nuclear ghost located 70 μm from the central vessel. No visible cells are noted in the stroma beyond these points. Fields such as this suggest that the maximum cell to vessel distance compatible with cell survival in a hyalinized fibroid stroma is between 70 and 100 μm. If a distance of 100 μm between a cell and the nearest vessel is taken as the physical limit for cell survival [5], then the absence of cells, or the presence of nuclear ghosts, in some areas between 50 and 70 μm lends credence to the concept that the regional density of the collagenous stroma in fibroid tumors probably offers an additional impediment to the free diffusion of oxygen and nutrients necessary for cell survival.

The MVD, by Factor 8 staining, was significantly higher in phase 1 samples than in phases 2, 3 or 4 ($P < 0.05$). The MVD did not differ significantly among phases 2, 3 and 4 samples.

3.5. Simulation of Average and Maximum Myocyte to Vessel Distances. Simulation of the mean and maximum distances

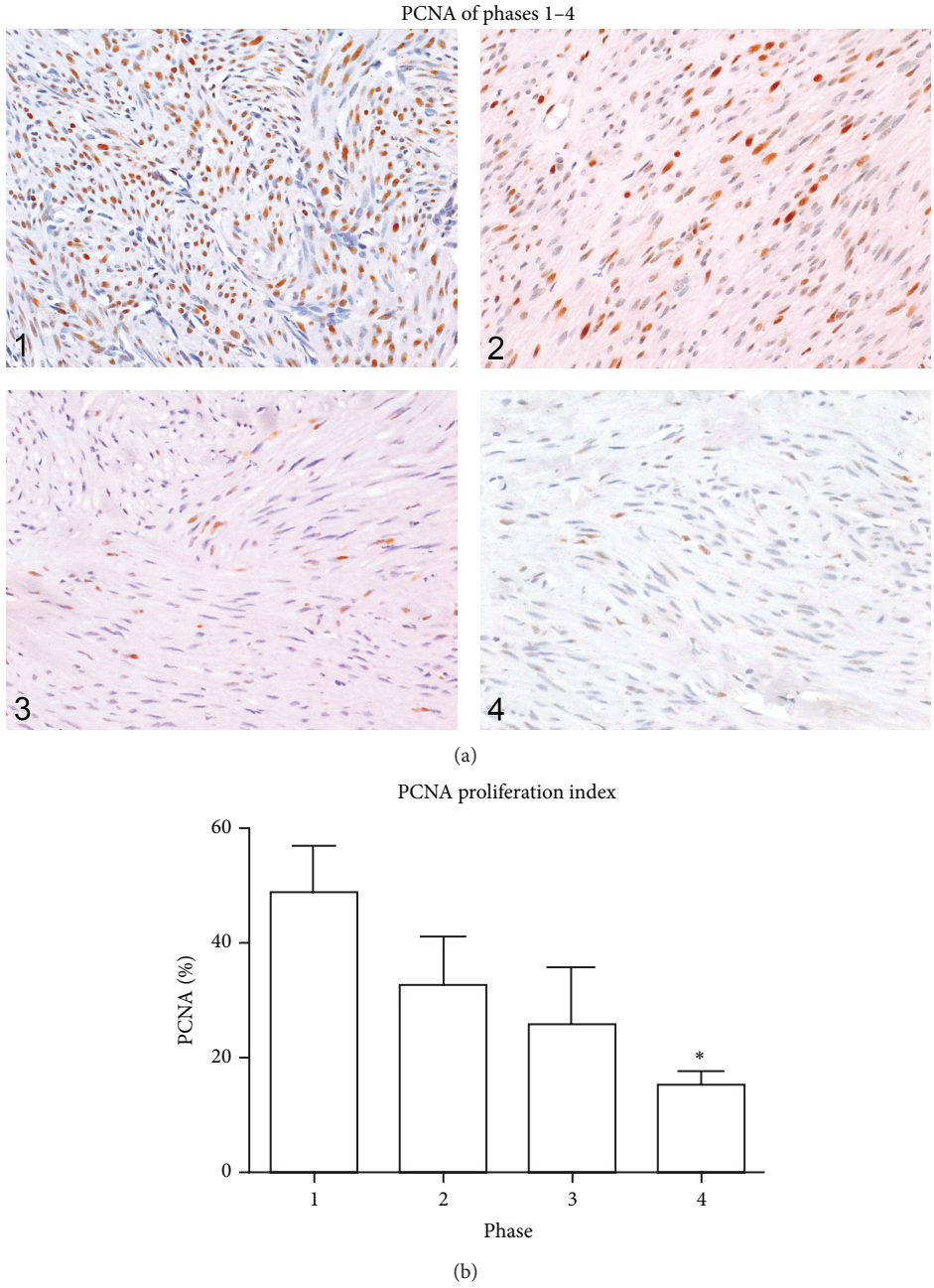


Figure 3: Fibroid phases: comparison of PCNA staining. (a) In this panel, a representative image of PCNA immunostaining from a fibroid in each of the four phases is shown. The percentage of PCNA positive nuclei in areas of maximum staining within the 4 fibroids in this panel was 59.1, 25.3, 19.1, and 15.5 for phases 1, 2, 3, and 4, respectively. A few PCNA positive nuclei are present in the phase 4 photo, but these are less intensely stained and thus less obvious than those in the other phases. All images were taken with the 20x objective. (b) Bar graph of Mean PCNAs from each phase. Although there was significant variation of PCNA scores within each phase of tumors in this study, as noted in Table 1, the mean scores of each phase declined progressively from phase 1 to phase 4. There were 5 samples per phase group and the values represent the mean ± SEM. *Significantly different from phase 1 ($P < 0.01$).

of myocytes from blood vessels in each phase is summarized in Table 3. Phase 1 tumors contained a mean of 92 blood vessels per 750 μm × 750 μm area and a mean collagen content of 0.83% (Table 1). Using this empirical data, the average distance of myocytes from blood vessels in Phase 1 tumors was estimated to be 34.9 μm by Monte Carlo simulations (Table 3). The "likely range," or the range within which one would find 95% of the averages, was 32.5 to 38.5 μm. The maximum distance of myocytes from blood vessels in phase 1 tumors was estimated to be 141.4 μm, with a likely range of 114.1 to 186.3 μm.

Phase 4 tumors contained a mean of 33 blood vessels per 750 μm × 750 μm area and a mean collagen content of 59.99% (Table 1). Based on this empirical data, the average distance of

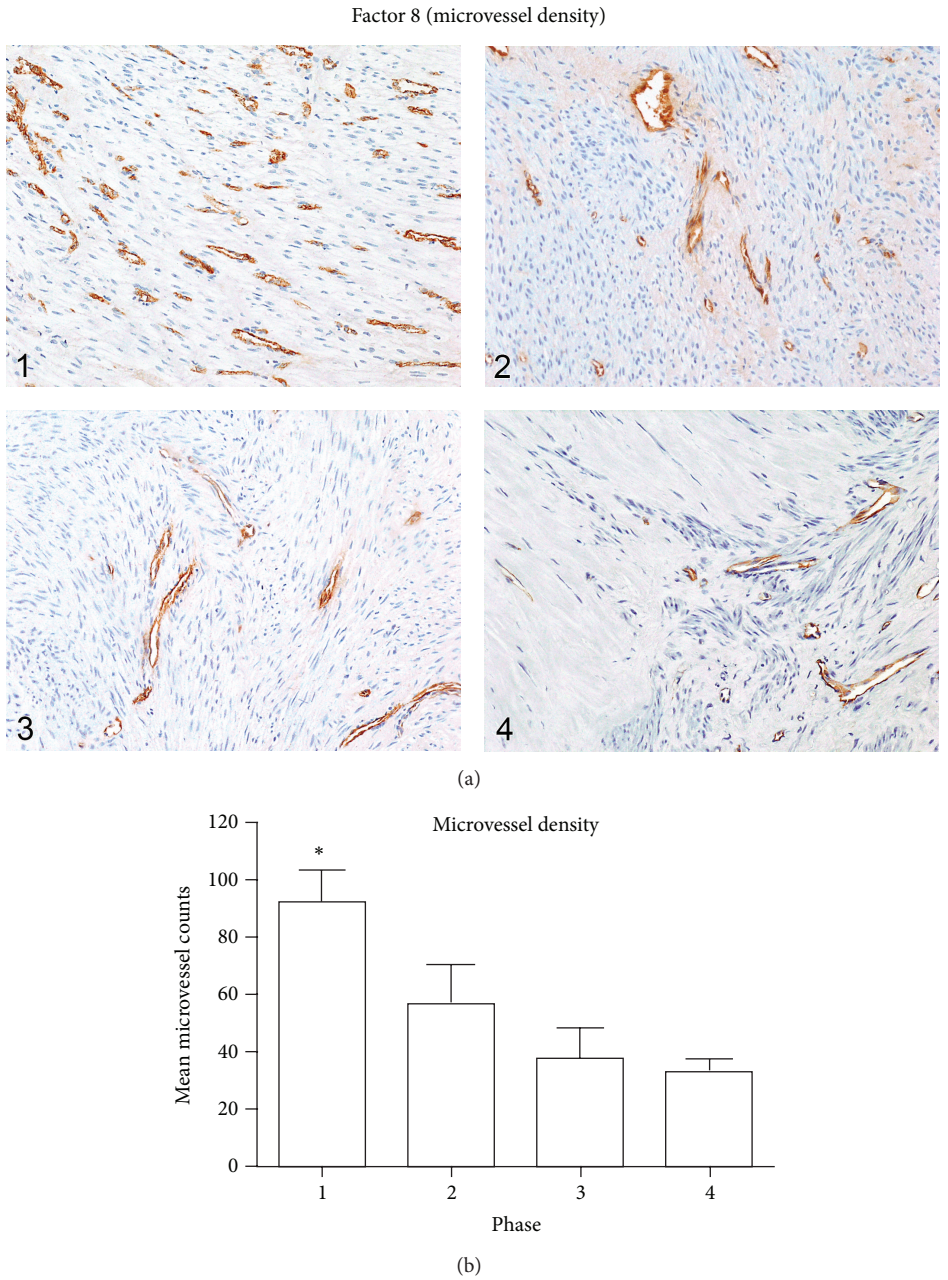


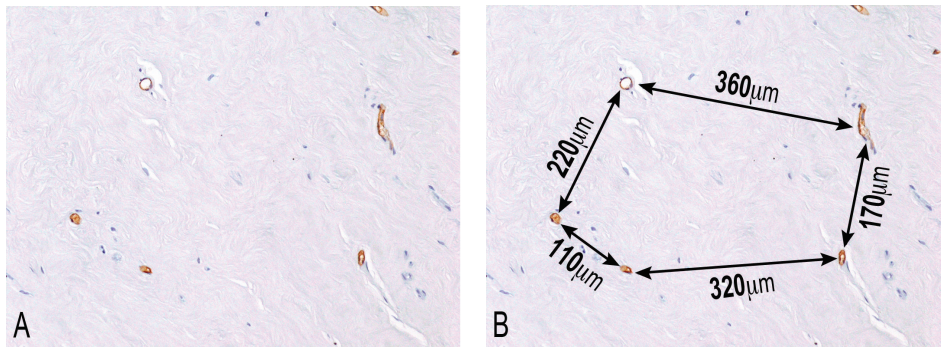
Figure 4: Fibroid phases: microvessel density. (a) In this panel, a representative photo of Factor 8 immunostaining from a fibroid in each of the 4 phases is shown. In some tumors the concentration of vessels was variable from one area to another, and within the fibroids of each phase there was a range of microvessel counts. However, the overall trend was one of decreasing vascularity with progression from phase 1 to 4. In phase 4 tumors, a prominent decrease in microvessel density was often noted within the hypocellular, hyalinized areas, as seen in the left half of the phase 4 tumor in (4). All images were taken with the 10x objective. (b) Bar graph of mean microvessel counts from each phase. Although the microvessel density varied among the individual tumors of each phase, the mean values declined from phase 1 to 4. There were 5 samples per phase group and the values represent the mean ± SEM. *Significantly different from phases 2, 3, and 4 ($P < 0.05$).

myocytes from blood vessels in phase 4 tumors was estimated to be 58.0 μm by Monte Carlo simulations, with a likely range of 50.5 to 68.4 μm. The maximum distance of myocytes from blood vessels in phase 4 tumors was estimated to be 195.6 μm, with a likely range of 144.2 to 283.4 μm.

The differences between phase 1 and phase 4 tumors were statistically significant for both average ($P < 0.001$) and maximum ($P < 0.001$) distance of myocytes from blood vessels.

4. Discussion

The results of these morphometric studies are supportive of the concepts and conclusions derived from our morphologic



(a) (b)

Figure 5: Phase 4 Fibroid. Hyalinized areas of fibroids, as in this phase 4 tumor, sometimes exhibit prominently reduced vascularity, in comparison to the adjacent areas of residual smooth muscle. Factor 8 immunostaining highlights the widely separated vessels in (a), and the distance between these vessels, measured with an ocular micrometer, is shown in (b). Note that the distances between adjacent vessels range from 110 μm to 360 μm, often exceeding the physiologic limits of cell survival, as evidenced by almost a total absence of cellularity within the intervening stroma. 10x original objective magnification.

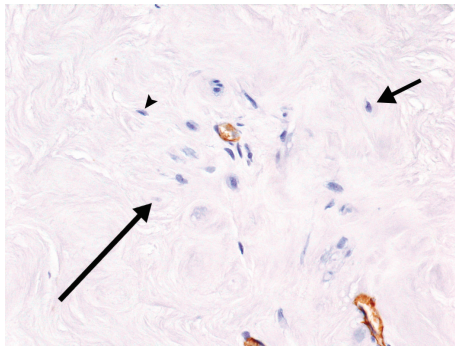


Figure 6: Phase 4 fibroid. Note that the only remaining viable cells in this field of a phase 4 fibroid are clustered around and between the two vessels. Most of these cells lie within 50–75 μm of the 2 capillaries. The cell at 2 o'clock (short arrow) is located almost 100 μm from the capillary in the center, and there are no viable cells beyond this point. The cell marked by an arrowhead is 50 μm from the central vessel, and the apparent nuclear ghost (long arrow) is 70 μm from the vessel. This suggests that in the setting of hyalinized fibroid stroma the maximum cell to vascular distance that is compatible with cell survival is in the range of 70–100 μm, and that other factors such as the local density of the stroma may also play a role. 20x, anti-Factor 8 immunostain.

studies. The demonstration of an inverse relationship between the percentage of collagenous tumor matrix and the microvessel density is central to our thesis of interstitial ischemia, which, combined with vascular ischemia, leads to myocyte atrophy and eventual tumor involution.

Our findings also show the variation in collagen content, proliferative activity, and microvessel density within each of the fibroid phases 1–4. Overlap in values is also noted between individual tumors in adjacent phases (e.g., phases 3 and 4). However, these observations of variation within phases, and overlap between phases, do not invalidate the trends that are apparent by grouping fibroids into phases based on estimation of collagen content. What the variations and overlaps do indicate is the genetic and epigenetic heterogeneity of fibroids. Thus, some tumors may continue to proliferate and grow to a large size, with relatively little collagen production. Other tumors, in contrast, may produce abundant collagen early in development, resulting in interstitial ischemia with an associated reduced rate of proliferation and subsequent involution while still small in size. The majority of tumors, however, seem to exhibit a growth pattern between these two extremes, in which a more balanced progressive growth occurs as a result of both the proliferation of transformed myocytes and the production and deposition of collagen, until the tumors ultimately involute because of the accumulation of collagen in the stroma with secondary interstitial ischemia.

Cells ordinarily lie within 20–30 μm of capillaries [6]. Proximity to vessels is essential for cell maintenance and survival since they are dependent upon diffusion of nutrients and oxygen from the blood stream. In our studies, the microvessel density of phase 1 tumors was 2.8 times the microvessel density of phase 4 tumors (92 versus 33 vessels/0.5625 mm^2 field), while the percentage of collagen in phase 4 tumors was 72.2 times that of phase 1 tumors (59.99% versus 0.83%). Based upon these empirical data, the average distance of myocytes from blood vessels in phase 1 tumors was estimated, through simulations, to be 34.9 μm, while the average distance in phase 4 tumors was estimated to be 58.0 μm. These data reinforce our morphologic observations and our hypothesis that myocytes become progressively more separated from their blood supply as the tumors progress from Phase 1 to phase 4.

In addition to the increase in diffusion distance between myocytes and blood vessels, the density of both the fibrotic interstitial tissue and the vessel walls would be expected to increase the resistance to diffusion of nutrients and oxygen. The effective diffusion of molecules to the cells is dependent upon both the microvessel permeability and the tissue diffusion coefficient [7]. Although we are unaware of studies specifically investigating diffusion in fibrotic tissue, the reduction in ground substance within dense fibrotic

tissue that is suggested histologically would probably be an additional impediment to the diffusion process.

The consequences of a reduction in the supply of nutrients and oxygen to cells are probably dependent upon both the degree of the reduction and the rapidity with which it occurs. While a sudden, marked decrease in blood flow might result in necrosis, a lesser degree of ischemia or hypoxia might induce an apoptotic reaction, and both types of cell death may be observed in fibroids. However, much of the cellular loss in fibroids appears to be related to a much slower process in which cells denied proper sustenance and oxygen because of the vascular and interstitial fibrosis undergo atrophy and injury. Ultimately, the fibrotic isolation of atrophic myocytes results in such severe deprivation of essential nutrients and oxygen that vital functions cannot be maintained and cell death occurs by inanition, or starvation, a process that we refer to as inanosis. Hypoxia is probably an important contributing factor to cell demise since it is believed that cells located more than 100 μm from the vasculature develop anoxia, that is, complete oxygen deprivation [5]. The loss of myocytes by this more gradual process of atrophy and inanosis may also contribute to the extreme collagen to muscle ratios seen in the phase 4 tumors.

Since the number of tumors examined immunohistochemically in this study was limited, additional studies are needed to confirm these results. Taken in conjunction with our morphologic studies, however, we feel that the data are supportive of the concepts of interstitial ischemia and inanosis.

We also recognize that each individual tumor can only be examined at one point in time and that certain assumptions are necessary to advance the idea of progression and ageing changes within individual fibroids because of this limitation. Thus, each tumor may pursue its own course with regard to rate of growth and the degree and rate of development of fibrosis, and cellular tumors with little fibrosis (phase-1-like) may be large, and fibrotic tumors (phase-4-like) may be small. However, the grouping of fibroids by the percentage of collagenous stroma to the percentage of residual muscle has allowed for the observation of trends that support the concepts of interstitial ischemia, atrophy, inanosis, and involution. The arbitrary classification of tumors into phases has been employed not for any implied clinical significance or utility, but rather for providing a framework for the investigation of the pathophysiologic concepts that we have proposed. Finally, it is not our intention to imply that the presence of involutional changes in fibroids causes them to disappear, or to undergo any predictable regression in size, or to pose any less of a clinical problem; in fact, the accumulation of extracellular collagenous matrix that is largely responsible for myocyte involution also appears to be a significant contributor to the increase in the size of fibroids which is a major cause of their clinical manifestations.

Disclaimer

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Conflict of Interests

All authors declare that there are no financial conflict of interests issues.

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Review Article

Uterine Fibroids: Pathogenesis and Interactions with Endometrium and Endomyometrial Junction

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Uterine leiomyomas (fibroids or myomas) are benign tumors of uterus and clinically apparent in a large part of reproductive aged women. Clinically, they present with a variety of symptoms: excessive menstrual bleeding, dysmenorrhoea and intermenstrual bleeding, chronic pelvic pain, and pressure symptoms such as a sensation of bloatedness, increased urinary frequency, and bowel disturbance. In addition, they may compromise reproductive functions, possibly contributing to subfertility, early pregnancy loss, and later pregnancy complications. Despite the prevalence of this condition, myoma research is underfunded compared to other nonmalignant diseases. To date, several pathogenetic factors such as genetics, microRNA, steroids, growth factors, cytokines, chemokines, and extracellular matrix components have been implicated in the development and growth of leiomyoma. This paper summarizes the available literature regarding the ultimate relative knowledge on pathogenesis of uterine fibroids and their interactions with endometrium and subendometrial myometrium.

1. Introduction

Leiomyomas are benign uterine tumors of unknown aetiology. These kinds of lesions seem to arise from myometrial transformation as a result of specific physiological and pathological conditions. The majority of these monoclonal estrogen-dependent uterine neoformations [1] afflict mostly women during reproductive age, and 80% of them suffer from this during their whole lifetime [2]. In the past, most women with fibroids remained undiagnosed, because they were asymptomatic. Analyses based on clinical diagnosis or diagnostic tests underestimate the true incidence; in fact, they take only into account symptomatic patients.

Cramer and Patel [3] estimated the prevalence of uterine fibroids based on clinical assessment at 33%, ultrasound scan at 50%, and histological examination of hysterectomy specimens at 77%. The reported frequency of the disease varies widely due to differences in study design. In fact, to determine the exact prevalence of fibroids, a correct clinical research should apply ultrasound scanning in a randomly sampled population [4].

Nowadays, conflicting data about the pathogenesis of leiomyomas coexist in the literature. The development of uterine myomas can be linked to predisposing risk factors, initiators and genetic mechanisms, promoters, and effectors. The aim of this work is to discuss the latest knowledge on the

pathogenesis of uterine fibroids and their interactions with the endometrium and subendometrial myometrium.

2. Pathogenesis of Uterine Leiomyoma

2.1. Risk Factors. Even if many risk factors suggested by epidemiologic studies have linked uterine leiomyomas to the effects of estrogens and progesterone levels and their metabolism, other mechanisms may be involved in fibroids pathogenesis. In fact, recently, Peddada et al. [5] have questioned the exact role of female hormones (estrogens and progesterone) in the development and growth of uterine fibroids. The authors measured the growth of fibroids in black and white women with clinically relevant fibroids using MRI technology; they demonstrated that fibroids within the same woman often have different growth rates despite having a similar hormonal milieu. In the same patients, fibroids were found to vary in size, regress, or remain stable. Each tumor appeared to have its own intrinsic growth rate, and fibroid growth appeared not to be influenced by tumor characteristics such as size and location. This study encouraged new research directions, consistent with studies showing that fibroids are monoclonal in origin with variable molecular characteristics [6–9]. Wei et al. [7] also found ethnic differences in expression of the dysregulated proteins in uterine leiomyomata. Tumor size has been related to variation in molecular markers [6–8, 10], and it has been assumed that the molecular differences reflect differences in tumor growth rates. Moreover, molecular markers also may differ between tumors from blacks and whites [7, 10]. It has been generally accepted that myomas are more prevalent in blacks than in Caucasian and Hispanic populations [11, 12]. Although the cause of the higher prevalence among black women is unclear, differences in circulating estrogen levels have been found [13]. It is still unclear [14] whether these ethnical differences are genetic or due to known variations in hormonal metabolism, diet, or environmental factors. Recently, some authors reported a statistically significant inverse correlation between serum 25-(OH) Vit D levels and fibroid prevalence in black subjects [15, 16]. Leppert et al. reported that the pathogenesis of fibroids seems to involve a positive feedback loop between extracellular matrix production and cell proliferation, and vitamin D might act to block the positive feedback [17]. It is also interesting that myomas and keloids, both more common in black women, have similar gene characteristics. Furthermore, it is well known that family history could represent a strong predisposing factor; the first-degree relatives of affected women have a 2.5 times increased risk of developing fibroids [18, 19]. However, as recently reported from Saldana et al., such bias would invalidate self-reported family history as a predictor of fibroid risk [20].

Several studies [13, 14, 18, 19] reported a rapid increase of fibroid incidence after the age of 30. This could be the result of time-related hormonal changes or an enhanced symptomatology from already existing fibroids. Furthermore, the high incidence of fibroids in the perimenopausal period could be responsible for increasing gynecologic surgery rates in women who have completed the childbearing period.

A study found that the risk of myomas increased 21% with each 10 kg increase in body weight and with increasing body mass index [21]. Shikora et al. reported similar results in women with greater than 30% body fat [22]. The adipose tissue converts adrenal and ovarian androgens into estrogens, whereas several mechanisms associated with obesity lead to decreased synthesis of sex hormone binding globulin. Consequently, the increase of biologically available estrogens could be responsible for increasing myoma prevalence and/or growth in overweight and obese women. Furthermore, Nair and Al-Hendy evaluated the association between obesity-related chronic inflammation and initiation, as well as the progression of uterine leiomyoma by using an *in vitro* model with representative cell lines of adipocytes and human uterine leiomyoma cells. They demonstrated that coculture of adipocytes and uterine leiomyoma cells results in an increased proliferation of leiomyoma cells, and they have also demonstrated that TNF-α treatment increases human uterine leiomyoma cells proliferation in a concentration-dependent manner [23].

It is not clear whether diet habits, such as consuming red meat, ham, green vegetables, or fiber, could influence the growth of myomas. It is also difficult to analyze the specific effects of physical exercise on the development of uterine myomas, as only a few observational studies have addressed this aspect so far [24, 25].

Several studies have revealed that smoking may reduce the incidence of myomas; nicotine inhibits aromatase and reduces the conversion of androgens to estrone. Smoking also exerts a powerful inducing effect on the 2-hydroxylation pathway of estradiol metabolism, which is likely to lead to decreased bioavailability at estrogen target tissues [26–28].

An early menarche, before the age of 10, has been found to be a risk factor for uterine myomas, while a menarche over the age of 16 seems to decrease the same risk [29]. Some studies stressed that a lower incidence and a reduced number of clinically apparent myomas are linked to increased parity [30–32]. This could be due to a remodeling process of the extracellular matrix (ECM) and a specific expression of receptors for peptide and steroids hormones induced by pregnancy and parturition.

Postmenopausal hormone therapy seems not to be responsible for any important stimulus to fibroid growth [33]. Likewise, conflicting data coexist about the relationship between oral contraceptives (OC) and the growth of leiomyomas. This could be related to the differing content of estrogens and the type of progesterone in each specific OC preparation [34].

Several theories about the initiators of fibroids have been proposed. Rein [35] stated that increased levels of estrogens and progesterone could result in an augmentation of mitotic rate that could be responsible for somatic mutation. Richards and Tiltman found increased concentration of receptors for estrogens (ER) in certain regions of the nonneoplastic myometrium of uterus myomatous [36]. Another interesting theory underlines that the pathogenesis might be similar to a response to injury [37]; ischemic damage could be linked to release of increased vasoconstrictive substances at the time of the menses. Smooth muscle cells of the myometrium could

react to injury with the synthesis of extracellular fibrous matrix [38]. After vascular damage, basic fibroblast growth factors are overexpressed in leiomyomas [39, 40].

2.2. Genetic Mechanism Involved in Fibroids Etiology. Historically, uterine leiomyomata have not been considered a genetic disease. However much recent clinical evidence indicates that at least some myomata have a genetic etiology. Actually, cytogenetic surveys have found that about 40% of uterine fibroids are chromosomally altered and bear cytogenetic anomalies shared by several other types of tumors. For example, studies found translocations between chromosomes 12 and 14, trisomy 12, translocations between chromosomes 6 and 10, and deletions of chromosomes 3 and 7 [41].

The HMGA2 gene was found in translocation 12:14, the most common cytogenetic abnormality, that occurs in about 20% of chromosomally abnormal lesions. This gene encodes a high mobility group DNA binding protein and embryonic proliferation modulator [42]. The HMGA2 gene is expressed in uterine leiomyoma and in other human tissues with a proliferative phenotype, such as fetal tissues, lung, and kidney, but not in the normal myometrium [43]. Markowski et al. found that the antagonism of HMGA2 *in vitro* decreased leiomyoma cell proliferation [44].

Heritable cancer syndromes can be characterized by uterine leiomyomas such as hereditary leiomyomatosis and renal cell cancer (HLRCC). This syndrome predisposes patients to benign leiomyomas of skin and uterus and early-onset renal cell carcinoma. Fumarate hydratase (FH) is the gene implied; it encodes a Kreb's cycle enzyme responsible for conversion of fumarate to malate [45]. Alport syndrome is an X-linked progressive nephropathy associated with leiomyomas due to defect in COL4A5 and COL4A6 genes [46].

Cha and colleagues [47] genotyped 1607 individuals with uterine fibroids and identified 3 susceptibility loci associated with uterine fibroids. Chromosome 10q24.33 seems to have the best association with leiomyomas; the region was mapped to the $5'$ region of the SLK gene encoding STE20-like kinase. STE20-like kinase has a role in myogenic differentiation, and, after activation by epithelial disruption, it is expressed in proliferating myoblasts. Another gene product located in the region is A-kinase anchor protein-13 (AKAP13), associated with cytoskeletal filaments. Related mutations could alter the regulation of extracellular matrix deposition and, consequently, of the fibrotic phenotype of the leiomyoma [48].

Recent studies described that 70% of fibroids contained a series of mutations in a transcriptional regulator complex subunit 12 (MED12) [49, 50]. Pérot et al. reported that MED12 is frequently mutated in typical leiomyomas (66.6%) and also that mutations are not restricted to benign tumors since highly aggressive leiomyosarcomas were also mutated. However, no mutations were detected in nonuterine leiomyosarcomas; so Pérot et al. affirmed that MED12 seems to be specific to uterine smooth muscle tumors [51]. Previously, it has been shown that MED12 is implicated in transcription activation of Wnt target genes by interacting with β-catenin [52, 53]. However a recent study combining mRNA and miRNA differential expression between fibroids and myometrium has observed a downregulation of the Wnt pathway and an upregulation of the focal adhesion pathway in leiomyomas [54]. The β-catenin immunohistochemistry data tends to indicate that the canonical Wnt pathway is not implicated in fibroids development, since β-catenin, when expressed, is located at the membrane in mutated cases; a localization which has been demonstrated to be indicative of a low transactivation activity [51, 55, 56].

The same authors [51] concluded that the Wnt/β-catenin pathway does not seem constitutively activated in MED12 mutated tumors, and they hypothesize that if MED12 mutations play a role in uterine tumor development, it is probably not through Wnt target genes activation in association with β-catenin.

2.3. Role of Mechanical Transduction and Extracellular Matrix. Research for the pathogenesis of fibroids and abnormal extracellular matrix (ECM) led to the analysis of a growth factor with profibrotic activity, transforming growth factor β (TGF-β) [17, 57]. The β3 subunit of TGF-β3 and its signal mediators are overexpressed in leiomyomas compared to normal myometrium [58]. Furthermore, the mRNA expression of multiple ECM genes in uterine leiomyomas is decreased when the TGF-β pathway is downregulated [59].

Norian et al. have examined the role of ECM, opening new directions of research. They reported that mechanical signals are transmitted from the ECM scaffold via transmembrane receptors to the internal cytoskeleton in order to maintain an isometric state. Transmembrane receptors respond to stretch, fluid shear stress, elevated hydrostatic pressure, and increased osmotic forces. In this way, myometrial cells react to, and may be protected from, external loads by the mechanical properties of the surrounding matrix through secretion of ECM. The authors [60] have demonstrated that the ECM microenvironment of leiomyoma cells is characterized by increased mechanical stress. They extended the results of their previous study [48] showing that the viscoelastic properties of the ECM contribute substantially to the increased tissue stiffness of leiomyoma. They hypothesized that since the viscoelastic properties of the ECM are complex, it is possible that the interstitial fluid may alter the repulsive forces of the glycosaminoglycans allowing them to collapse or inflate. So the authors [60] suggested that the mechanical properties of leiomyoma are a key feature of these tumors and may contribute to their growth.

2.4. MicroRNA. Epigenetic changes have also been implicated in leiomyoma formation. Studies directed at identifying epigenetic abnormalities in fibroids demonstrated abnormally hypomethylated ER-α [61]. Follow-up studies demonstrated globally abnormal genomic methylation in leiomyomas compared to myometrium [62], implicating possible epigenetic contributions to genetic susceptibility of leiomyoma development. Knowledge regarding the molecular causes of uterine leiomyomas is in its infancy. Early studies suggest common mutations that correlate with the development of leiomyomas. MicroRNAs (miRNAs) are a novel class of small nonprotein coding RNAs which regulate a high number of biological processes by targeting mRNAs for cleavage

or translational repression [63, 64]. Several miRNAs such as let7, miR-21, miR-93, miR-106b, and miR-200 are significantly dysregulated in uterine leiomyoma compared to those in normal myometrium [10, 65]. Further research will need to identify specific genes responsible for the development of leiomyomas that can be directly targeted as preventive therapy. Additional efforts need to be directed at investigating specific inhibitors of disrupted pathways involved in the leiomyoma growth in susceptible patients. The wide spectrum of clinical and genetic heterogeneity of uterine leiomyomas underscores the importance of continued investigation to determine the various molecular etiologies that result in leiomyoma development.

2.5. Estrogens. Uterine leiomyoma growth is strictly related to estrogens and their receptors. Several studies found that mRNA and protein expression levels as well as the content of ER-α and ER-β are higher in leiomyoma compared to those in normal myometrium [66, 67]. According to their hypothesis, estrogens may exert their growth-stimulatory effects on leiomyomas intermediated by cytokines, growth factors, or apoptosis factors [68]. Ishikawa et al. [69] suggested that estrogens can maintain progesterone receptor (PR) levels, and thus progesterone through its receptor may promote leiomyoma growth. Furthermore, other authors suggested that estrogens may stimulate leiomyoma growth partially by suppressing normal p53 functions [70].

Estrogens are able to regulate the expression of growth factors by activating some signaling pathways. Estrogens upregulate platelet-derived growth factor (PDGF) expression [71] in leiomyoma cells, while they downregulate activin and myostatin [72] in human myometrial explants. In addition, estrogens also downregulate epidermal growth factor (EGF) expression but upregulate the expression of EGF-R in both myometrium and leiomyoma cells [73, 74]. These estrogen actions are accomplished through the rapid activation of different kinds of kinases; some of them [75] result to be increased in both immortalized uterine smooth muscle and leiomyoma cell lines under estrogen stimulation. In addition, Park and colleagues reported that estrogens may also stimulate the proliferation of leiomyoma cells by activating ATP-sensitive potassium channels [76].

2.6. Progesterone. Progesterone interacts with its receptors PR-A and PR-B [77] playing a key role in myometrial and leiomyoma biologies [78, 79]. Several studies have stressed that PR content and mRNA levels are higher in leiomyoma than those in normal myometrium [80–84], and, in particular, Fujimoto et al. [85] described the relative overexpression of PR-B mRNA in the surface of leiomyoma.

Leiomyoma growth is influenced by progesterone interaction with some growth factors; it upregulates the EGF (mitogenic) [73] and transforming growth factor- (TGF-)β3 (bimodal action) [86] expression. On one hand, progesterone seems to downregulate IGF-I expression through PRB, while PRA appears to inhibit this function [84].

Some authors hypothesized that progesterone could stimulate leiomyoma cell growth and survival through upregulating B-cell lymphoma- (Bcl-)2 protein expression and downregulating tumour necrosis factor- (TNF-)α expression [87, 88]. Recently, Luo et al. [89] defined L-type amino acid transporter 2 (LAT2) as a novel PR target gene. Progesterone significantly induces LAT2 mRNA levels, which is blocked by cotreatment with the PR antagonist mifepristone. In the same way, Yin et al. found eighteen novel PR-binding sites, one of which is Krüppel-like transcription factor 11 (KLF11) which is minimally downregulated by progesterone [90].

2.7. Growth Factors. Several growth factors, such as vascular endothelial growth factor (VEGF), EGF, heparin binding epidermal growth factor (HB-EGF), PDGF, IGF, TGF-α, TGF-β, acidic fibroblast growth factor (aFGF), and basic fibroblast growth factor (bFGF), and their respective receptors have been demonstrated to play a role in leiomyoma growth [91, 92]. In particular, bFGF [93] and VEGF [94] have also been shown to promote angiogenesis in leiomyoma. EGF and PDGF seem to increase DNA synthesis and polyploidization in leiomyoma cells through transient activation of kinase pathways [95–97]. PDGF also modulates the rate of cell proliferation in myometrium and leiomyoma cells [98–100].

TGF-β3 induces elevated expression of ECM-related genes and decreases the expression of ECM degradation-related genes [101]. TGF-β can also activate kinase pathways (MAPK/ERK/Smad) and thereby modulate the expression of different types of genes influencing the leiomyoma growth and regression [102]. Similarly, IGF may increase cellular proliferation in uterine leiomyoma cells through activation of the MAPK pathway [103] and thus play a crucial role in leiomyoma cell growth, by upregulation of Bcl-2 protein expression in leiomyoma cells [104].

Recently, activin and myostatin have been identified in the myometrium and in leiomyoma, and Ciarmela et al. [105, 106] have hypothesized that activin-A and myostatin could regulate myometrial cell proliferation, describing higher expression levels of this molecule in leiomyoma compared to that in adjacent myometrium samples. Additionally, several less studied factors such as parathyroid hormone-related peptide [107, 108], prolactin [109, 110], endothelin-1 [111, 112], human chorionic gonadotropin [113], and pituitary tumor-transforming growth factor-1 [114] have also been implicated or hypothesized in myometrial biology.

2.8. Cytokines and Chemokines. Many cytokines, including tumor necrosis factor-α [87], erythropoietin [115], interleukin- (IL-)1 [116], and IL-6 [117], have been implicated in development of uterine leiomyoma. Even chemokines and their receptors (MIP-1α, MIP-1β, RANTES, eotaxin, eotaxin-2, IL-8, CCR1, CCR3, CCR5, CXCR1, and CXCR2 mRNA) have been shown to be mediators of the above mentioned process [118–120]. Sozen et al. [121] found that MCP-1 mRNA levels are higher in myometrium compared to leiomyoma and that estrogens and progestins decrease MCP-1 protein production, suggesting that MCP-1 may have antineoplastic activity in leiomyoma. IL-8 and IL-8 receptors type A have been identified with the elevated expression in myometrium compared to leiomyoma [122]. Hatthachote and Gillespie [116] described that this chemokine also upregulates

TGF-β1 and TGF receptor expression *in vitro* in human term myometrium.

In experimental systems, increasing carcinogen exposure tends to increase the number of tumors and their degree of malignancy. Low carcinogen exposure tends to produce benign neoplasms, whereas high exposure tends to produce both malignancies and higher numbers of tumors [3, 123, 124].

2.9. Extracellular Matrix Components. Extracellular disorganized matrix is a peculiar characteristic of fibroid growth, mainly consisting of collagen subtypes, fibronectin, and proteoglycans. Recently, a series of collagen subtypes, such as COL1A1, 4A2, 6A1, 6A2, 7A1, and 16A1, have been found expressed to a greater extent in leiomyoma cells compared to myometrial cells [125]. Leiomyomas and myometrium are characterized by variable expression of glycosaminoglycans and their protein bound forms, proteoglycans [126, 127]. Matrix metalloproteinases (MMPs) have also been implicated in leiomyoma remodeling. Bodner-Adler et al. [128] found that MMP-1 is expressed more in leiomyomas, while MMP-2 is less expressed. Alternatively, another work found MMP-1, MMP-2, MMP-3, and MMP-9 with higher activity of MMP-2 in leiomyoma compared to myometrium [126]. Recently, Bogusiewicz et al. revealed increased MMP-2 activity in leiomyomas than in surrounding myometrium [129].

3. Fibromatosis, Endometrium, and Endomyometrial Junction

The advances in pathogenetic knowledge of fibroids and the introduction of magnetic resonance imaging led to the study of endomyometrial junction [130], the interface between cyclic endometrium and the myometrium, where important vascular and physiochemical phenomena seem to take place. Tocci et al. [131] proposed that the "endometrial-subendometrial myometrium unit disruption disease" should be considered as a new entity and distinguished from adenomyosis. This condition is expressed mainly by a pathological thickening or abnormality of the subendometrial myometrium, that is, the possible site of origin of submucosal and intramural fibroids. The study also reports on the influence of abnormal thickening or disruption on human fertility and outcome of assisted reproduction techniques. The mechanism underlying zonal myometrial differentiation is not known, but growing evidence suggests that ovarian hormone action may be mediated by cytokines and uterotonins locally released by the basal endometrial layer and endometrial-myometrial T-lymphocytes. Irregular *thickening* of the junctional zone due to inordinate proliferation of the inner myometrium, junctional zone hyperplasia, is a common MR finding in women suffering from menstrual dysfunction [132].

Nowadays, it is not well established how uterine fibroids could interfere with the endometrial environment and the subendometrial myometrium and vice versa. In these patients, the interaction between the endomyometrium and fibromatosis could have a role in influencing their fertility and the risk of miscarriage. The American Society for Reproductive Medicine has reported that uterine myomas are associated with infertility in 5–10% of cases [133] and may be responsible for 2-3% of infertility cases [134]. All confounding variables may be difficult to control when searching for the impact of fibroids on infertility, and there is no definitive association between reproductive dysfunction, miscarriage and fibroids.

Some studies [135–138] indicated that the uterine tissue is susceptible to fibrinogenesis, as seen in rare occasions in response to mechanical injury in women undergoing endometrial ablation and caesarean delivery and in women affected by Asherman's syndrome. In all these cases, in fact, there is an involvement of the subendometrial myometrium, an alteration of endometrial microvascular blood flow with endometrial atrophy and abnormal activation of cytokines and chemokines, which might have an important role in the pathogenesis of uterine leiomyomas and related symptoms. Frequent mucosal injury with stromal repair reactions may release growth factors that promote the high frequency and multiplicity of uterine leiomyomas [139].

A threefold increase in percentage of nuclear area was found in the junctional zone in comparison with the outer myometrium, reflecting an increase in both size and number of nuclei. No difference in distribution of common components of the extracellular space (collagen, laminin, and fibronectin) was found between the two layers [140].

It has been reported that myomectomy can increase the pregnancy rate for patients with infertility [141]. However, the mechanisms by which this occurs are not well understood. Many hypotheses have been suggested. First, fibroids could alter uterine cavity contour, through a mechanical distortion, or they could be responsible for an abnormal uterine contractility [142, 143]. Moreover, local inflammation associated with the presence of fibroids may give rise to a hostile endometrial environment that impairs sperm transport and embryo implantation. Some authors [144, 145] reported that excessive concentrations of inflammatory cytokines could have negative effects on embryonic development and implantation. Inagaki et al. [146] demonstrated that uterine cavities containing fibroids or adenomyosis showed a state of excess inflammation, with upregulation of MMPs and inflammatory cytokines such as interleukin-1 and TNF-α. In particular, the levels of MMPs in the uterine cavity of women with leiomyoma and adenomyosis were significantly higher than those in women with a histologically normal uterus. Matsuzaki et al. [147] detected significantly lower expression levels of HOXA-10 in patients with uterine leiomyoma. HOXA-10 is one of the best-recognized sequences of signaling events in implantation [148].

A study reported that HOXA10 and HOXA11 mRNA expression were significantly decreased in uteri with submucosals myomas compared to those in controls with normal uterine cavity and to uteri with intramural myomas [149]. Although intramural myomas were not associated with a significant change in these markers of endometrial receptivity, the same authors noted a trend toward decreased endometrial HOXA10 mRNA and stromal protein expression in the intramural myoma group compared to those in the control

group. Sinclair et al. [150] evaluated the effect of leiomyoma on endometrial gene expression essential for implantation and haemostasis both *in vivo* and in primary endometrial stromal cells. They hypothesized that failure of blastocyst implantation in women with uterine leiomyomas is secondary to impaired BMP-2-mediated decidualization. BMP-2 is a growth factor that belongs to the TGF-β superfamily. It regulates cell proliferation and differentiation. Conditional ablation of BMP-2 in murine endometrium results in complete infertility because BMP-$2^{d/d}$ mice are unable to form implantation sites and demonstrate a complete lack of decidual response [151].

The authors tested the hypothesis that TGF-β3 may cause impaired decidualization in endometrial stromal cells by inducing BMP-2 resistance. They found a significant reduction in HOXA10 in leiomyoma-associated endometrial stromal treated with rhTGF-β3 supporting this hypothesis. TGF-β3 downregulated BMP receptors. The significant reduction in BMPR gene expression in response to treatment with rhTGF-β3 suggests that TGF-β3 induces BMP-2 resistance by downregulation of the BMPRs. Persistently decreased receptor expression explains the continued lack of BMP-2 response of endometrial stromal cells in culture.

Morosova et al. [152] studied common polymorphisms of MMP genes in myometrial and endometrial hyperplasia. An accelerated leiomyoma growth correlated with higher frequency of the MMP-1 2G allele. MMP-1 2G was also associated with multinodular growth, and it also tended to increase in patients with adenomyosis, suggesting that the 2G (-1607)MMP-1 genotype may be a potential risk marker of myometrial and endometrial hyperplasia.

4. Conclusions

The etiopathology of uterine fibroid remains unclear, multifactorial, and enigmatic. Classic studies showed steroid dependence of myomas for growth and development. The genetic background seems to play an important role, with cytogenetic anomalies observed in about 40% of uterine fibroids. Abnormal ECM expression, increased growth factors, cytokines and chemokines concentrations, and an extracellular disorganized matrix have been implicated in development and growth of uterine leiomyomas.

However, clinical aspects are related prevalently to the number, volume, and intrauterine localization of nodes. In particular, submucosal nodes are associated with important clinical manifestations, and it is still not well established how uterine fibroids could interfere with the endometrial environment and the subendometrial myometrium and vice versa. It has been proposed that the "endometrial-subendometrial myometrium unit disruption disease" should be considered as a new entity and distinguished from adenomyosis [131]. This condition is expressed by a pathological thickening or abnormality of the subendometrial myometrium, that is, the possible site of origin of submucosal and intramural fibroids. Recent findings suggested that myomatosis and adenomyosis share some pathogenetic features like a state of excess inflammation, increased endothelial nitric oxide synthesis with upregulation of MMP, and inflammatory cytokines such as interleukin-1 and TNF-α.

Endomyometrial junction disruption might play a crucial role on fibroid-related infertility, uterine bleeding, and growth of submucosal and intramural myomas.

Nowadays, there is a clear trend to delay the time of pregnancy, and the clinical and the social impact of uterine fibromatosis is growing and requires future studies to clarify the etiopathogenesis and elaborate new and effective therapies for this condition.

Authors' Contribution

Fernando M. Reis and Pasquapina Ciarmela contributed equally to this paper.

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Review Article

Interplay between Misplaced Müllerian-Derived Stem Cells and Peritoneal Immune Dysregulation in the Pathogenesis of Endometriosis

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In the genetic regulation of Müllerian structures development, a key role is played by Hoxa and Wnt clusters, because they lead the transcription of different genes according to the different phases of the organogenesis, addressing correctly cell-to-cell interactions, allowing, finally, the physiologic morphogenesis. Accumulating evidence is suggesting that dysregulation of Wnt and/or Hox genes may affect cell migration during organogenesis and differentiation of Müllerian structures of the female reproductive tract, with possible dislocation and dissemination of primordial endometrial stem cells in ectopic regions, which have high plasticity to differentiation. We hypothesize that during postpubertal age, under the influence of different stimuli, these misplaced and quiescent ectopic endometrial cells could acquire new phenotype, biological functions, and immunogenicity. So, these kinds of cells may differentiate, specializing in epithelium, glands, and stroma to form a functional ectopic endometrial tissue. This may provoke a breakdown in the peritoneal cavity homeostasis, with the consequent processes of immune alteration, documented by peripheral mononuclear cells recruitment and secretion of inflammatory cytokines in early phases and of angiogenic and fibrogenic cytokines in the late stages of the disease.

1. Introduction

Endometriosis is an estrogen-dependent disease [1] characterized by the ectopic presence and growth of functional endometrial tissue, glands, and stroma, outside the uterine cavity [2, 3]. It affects deeply and negatively woman's quality of life, contributing not only to suffering but also to marital and family problems, to problems related to the achievements of work tasks, and overall to disability in woman's role in modern society [4–7]. Its treatment, medical or surgical depending on each case, on the contrary, could improve and partially restore women's health-related quality of life (HRQoL), like is reported by Jia et al. [8] and Gao et al. [9]. Risk factors for this disease are nulliparity, high education level, and social class (probably because these patients undergo accurate medical controls more easily) [10], although it is widespread across countries and ethnicities, and women continue to experience diagnostic delays in primary care [6]. As is suggested by many authors [11, 12] the risk of endometriosis appears to increase for reproductive health factors that may relate to increased exposure to menstruation (i.e., shorter cycle length, longer duration of flow, or reduced parity). The risk appears to decrease for personal habits that may relate to decreased estrogen levels (i.e., smoking, exercise). Approximately 10% of women in reproductive age are estimated to be affected by this disease [13, 14] and its symptoms, which include acute or chronic pelvic pain (CPP) and abnormal bleeding [12]. Pelvic pain could be expressed as dysmenorrhea, dyspareunia, dysuria, dyschezia, and nonmenstrual chronic pelvic—abdominal muscle pain [15]. Dysmenorrhea is independent

of the macroscopic type of the lesions or their anatomical locations and may be related to recurrent cyclic microbleeding in the implants [16]. The severity of dysmenorrhea seems to be significantly correlated with the presence and extent of pelvic adhesions, whereas the severity of CPP and deep dyspareunia is correlated with deep endometriosis on the uterosacral ligaments and extent of pelvic adhesions [17]. For example, Vercellini et al. [18] analysing 1054 consecutive women with endometriosis undergoing first-line surgery found first of all a significant inverse relationship between age at surgery and moderate-to-severe dysmenorrhoea, dyspareunia, and nonmenstrual pain. Moreover, they reported a strong association between posterior cul-de-sac lesions and pain at intercourse. Similar finding was found by Arruda et al. [19] in a smaller cohort study of Brazilian women, in which endometriosis symptoms (especially CPP) were more severe in young women with delayed diagnosis. There is evidence [20] that the typical endometriosis-associated chronic pelvic pain and sensitivity to estrogen could depend, at least in part, by the growth into the ectopic endometrial tissue of a nerve supply. Affected women are at higher risk than the general female population of developing ovarian cancer, and they also may be at increased risk of breast and other cancers as well as autoimmune and atopic disorders [1]. The disease most often affects the ovaries (up to 88% of all cases), uterine ligaments, fallopian tubes, rectum, cervical-vaginal region, and urinary tract. Urinary tract involvement is rare accounting for around 1-2% of all cases [21, 22], of which 84% are found in the bladder [23]. However, endometriosis can be encountered in other abdominal organs such as the liver, pancreas, intestinal tract, spleen [24], gallbladder [25], the abdominal wall, and even the navel [26]. Endometriosis is classified depending on the number, size, and superficial and/or deep location of endometrial implants, plaques, endometriomas, and/or adhesions, as follows: stage I (minimal, 1–5 points), stage II (mild, 6–15 points), stage III (moderate, 16–40 points), and stage IV (severe, >40 points), following the revised American Society for Reproductive Medicine classification for Endometriosis (American Society for Reproductive Medicine, 1996) [27].

2. Immune Disturbance of the Peritoneal Microenvironment

Immune system seems to play a key role in the pathogenesis of endometriosis. In these patients, immune alterations occur in the PF (PF) and peripheral blood, in part comparable to those proper of autoimmune diseases. It is widely reported an increase in the number but not in the function of macrophages, abnormalities in the functions and numbers of T and B lymphocytes, a reduction in number and activity of natural killer cells, apoptosis impairment, changes of cytokines and other soluble products in the peritoneal microenvironment.

2.1. Macrophages. Macrophages are "master regulators of the innate response to injured, infected, and neoplastic tissues" [28]. The microenvironment may drive the macrophage plasticity toward a transient and reversible polarization. These polar phenotypes are not expressed together, but the activation state of tissue macrophages can change over time. Then, they may be divided into two main populations: the "classically activated" macrophages, named M1 and stimulated by IFNγ and LPS, and "alternatively activated" M2 macrophages, stimulated by IL4, IL13, IL10, and TGFβ [29]. M1- and M2-activated macrophages perform different functions by producing pro- or anti-inflammatory factors. M1 macrophages play endocytic functions via the production of cytokines such as IL1α, IL6, IL12, and TNFα and reactive oxygen (ROS) and nitric oxide (NO) species [30]. In contrast, M2 macrophages are involved in resolution of inflammation and promotion of tissue repair, by secreting anti-inflammatory and immunosuppressive cytokines, IL10 and TGFβ, proangiogenic factors, such as coagulation factor XIII and vascular endothelial growth factor (VEGF) associated with a high degree of vascularization in vivo [29]. Resident peritoneal macrophages and peripheral monocytes, recruited from the blood into the peritoneal cavity, physiologically play a pivotal role in the scavenging mechanisms [31–35]. The immune surveillance of the peritoneal microenvironment would be able to prevent ectopic endometrial cells from becoming established. Macrophages are physiologically recruited in injured tissues, where they activate the neo-angiogenic switch, sustain resistance to apoptotic stimuli, and stimulate the proliferation and invasion of precursor cells in order to prompt tissue regeneration. Macrophages recruited in the endometriotic lesions activate a similar program. Several authors indicate that infiltrating macrophages in the endometriotic lesions are activated by signals generated within the same lesions [36, 37] or possibly by the lack of hormone-dependent anti-inflammatory signals in the ectopic but none in the eutopic endometrium [38].

Once endometriosis is established, the cyclic death of endometrial cells, due to progesterone withdrawal, leads to the release of cell debris, erythrocytes, and heme-bound iron in the peritoneal cavity. Recruited macrophages perceive ongoing cell death and tissue damage; in endometriotic patients they activate a reparative/regenerative/angiogenic program that is required for lesion maintenance, growth, and spreading [28]. The persistence of cells dying as a result of progesterone withdrawal within endometriotic lesions could cyclically activate infiltrating macrophages, thus sustaining the inflammation associated to the disease. Chuang et al. [39] reported an apparent impairment in the macrophage ability to phagocytose these cells dying, probably due to the defective expression and function of the class B scavenger receptor CD36 [40]. However, it is difficult to verify whether such defect is cause or consequence of the persistent inflammation of the peritoneal cavity associated to the disease. The pathogenesis of endometriosis results therefore by combination of inappropriate or persistent polarization, leading to tissue damage (increased M1 response) and immune dysfunction (increased M2 response). This allows for persistence of ectopic endometrial tissue. The disease is associated with significant alterations in the number of tissue macrophages expressing M1 or M2 surface markers. M2 activation leads to stimulation of anti-inflammatory cytokine production and inhibition of proinflammatory cytokine expression, thus

reducing inflammation. Macrophages may act to suppress the immune response to endometriosis and provide an environment permissive to the growth and progression of endometriosis lesions. It was demonstrated that recruited macrophages largely develop an immunosuppressive phenotype M2, thereby supporting endometriotic cell survival, attachment, and invasion through matrix remodeling, angiogenesis, and lesion maintenance. The reduced phagocytic ability of peritoneal macrophages of women with endometriosis is driven by soluble factors of tissue microenvironment, that determine the macrophage phenotype and function [29]. Then, the recruitment of macrophages into the lesions represents not only an early event in the disease development but also a necessary step for the successful establishment of endometriotic lesions [28]. Macrophages from endometriotic patients and mice with implanted endometriotic lesions (but not peritoneal macrophages from human or murine controls) express typical markers of alternative activation, in particular high levels of scavenger receptors, CD206 and CD163 [41]. CD206, a PRR which belongs to the C-type lectin superfamily, contributes to remove or inactivate inflammatory signals. CD163 mediates endocytosis of haptoglobin-hemoglobin complexes, with degradation of heme-iron components that can be recycled for erythropoiesis [42]. Macrophage polarization results in differential iron management in both humans and mice, with classically activated M1 macrophages that are characterized by iron sequestration [43] and alternatively activated M2 macrophages that are able to internalize and recycle the metal [44]. It is hypothesized that the polarization of infiltrating macrophages toward M2 phenotype provokes a more effective transfer of the metal to epithelial cells, supporting the growth and the spreading of the lesions. In experimental animals, it is reported that infiltrating M2 strongly enhance the growth of endometriotic lesions, suggesting that this program is important for the natural history of the disease. In contrast, mice injected with M1 macrophages do not develop growing minute lesions [41]. Macrophage migration inhibitory factor (MIF) activates macrophages and may therefore play a role in retaining these cells into the inflammatory sites [45]. It was demonstrated [46] that an increased secretion of MIF by peritoneal macrophages of women with endometriosis and further revealed an increased expression of this factor in eutopic endometrium and initial, active, and vascularized endometriotic lesions. Moreover, either local peritoneal fluid or systemic circulating levels of MIF were found to be higher in women with endometriosis and appeared to depend on the disease's stage and major clinical symptoms (pain and infertility) [47]. Interestingly, according to Seeber et al, the association of MIF with cancer antigen (CA)-125, monocyte chemotactic protein 1 (MCP1) and leptin, can diagnose endometriosis in 48% of patients with a specificity equal to 93% [48]. MIF is now known for being a multifunctional factor with a wide spectrum of effects and cell targets. MIF plays an essential role in tumorigenesis, tissue remodeling, and angiogenesis [49]. Recent data from the literature showed an important role for MIF in cell proliferation, inhibition of apoptosis [50], stimulation of metalloproteinases, and induction of angiogenesis [51]. MIF stimulates COX2 expression in ectopic endometrial cells and elicit proangiogenic and proinflammatory phenotype of macrophages, thereby potentiating their capability to stimulate the host angiogenic response and exacerbate the immunoinflammatory reaction occurring in the implantation site [46]. In addition, it was reported that macrophage migration inhibitory factor (MIF) protein secretion and mRNA expression increase significantly in endometriotic cells in response to estradiol. In turn, MIF reciprocally stimulates aromatase protein and mRNA expression, contributing to elevation in estradiol levels. Consequently, this mechanism may establish a positive feedback loop that contributes to develop and aggravate the disease [52].

2.2. T Lymphocytes. Several studies demonstrated that defective T-lymphocyte response to autologous endometrial cells was associated with endometriosis. There is evidence that the lymphocyte proliferative response to autologous endometrial cells was decreased in women with endometriosis. The cytotoxicity of T lymphocytes against autologous endometrial cells was also reduced in women with endometriosis [72]. Another mechanism by which endometriotic cells are able to escape from immune surveillance of cytotoxic T lymphocyte is attributable to FasL expressed by endometriotic cells. FasL induces apoptosis of lymphocytes by binding to its receptor, Fas, expressed on lymphocytes. Therefore, cells that are expressing high FasL may cause apoptosis of surrounding lymphocytes and thereby escape from lymphocytes response. Like we argued in our previous work [73], PF of women with endometriosis may have a potential to induce apoptosis of cytotoxic T lymphocytes, directly or indirectly via stimulating endometriotic cells, and contribute to the survival of endometriosis. Besides cytotoxic T lymphocytes, characterized as $CD8^+$ T cells, helper T cells or, namely, $CD4^+$ T cells are further diminished in their activity in PF from patients with endometriosis, probably because PF homeostasis breakdown suppresses activation of helper T cells [72]. Probably, IL-10, one of the well-known immunosuppressive cytokines, plays a role in this mechanism: we have already evidenced high level of IL-10 mRNA expression in ovarian endometrioma samples [74, 75], and this cytokine is also associated with decreased activated $CD4^+$ T cells in endometriotic PF. Moreover IL-4 and IL-10 were shown to be upregulated in peripheral lymphocytes in women with endometriosis. Increased IL-4 expression is also seen in lymphocytes in endometriotic tissues and in PF. On the other hand, production of IFN-γ was reduced in peripheral lymphocytes in endometriosis. Likewise, production of IFN-γ in peritoneal cells and IFN-γ concentrations in PF were decreased in endometriosis [72]. The PF mononuclear cells (PFMCs), as well as endometriotic cells, secrete different patterns of cytokines [34, 35, 52, 76] which drive the differentiation programs of $CD4^+$T cells toward Th1, Th2, and Th17 and Tregs. Th1 cells are characterized by T-bet and STAT1 and 6 and the production of IL-2, IFN-γ, and TNF-α. Th2 cells are characterized by GATA-3 and STAT5 and 6 and the production of IL-4, IL-5, and IL-13. In very recent years, however, the Th1/Th2 dogma has been challenged by the introduction of two other subsets of T cells: Th17 cells and regulatory T (Treg) cells. Since the eutopic endometrium behaves like an immune regulatory tissue,

the specific activities of these immune cells are crucial. Th17 cells are characterized by RORC and produce IL-17A, IL-17F, and IL-22 [77]. Recently, Osuga et al. [72] demonstrated the presence of Th17 cells in PF of endometriosis and further that IL-17 stimulates endometriosis stromal cells proliferation, their IL-8 and cyclooxygensase-2 expression. Tregs are characterized by FOXP3 and produce IL-10 and TGF-β, suppressing activation of the immune system and thereby maintaining immune system homeostasis and tolerance to self-antigens [78–82]. A mouse model study [83] reported in late-stage of the disease a switch toward Th2 and Treg cell profiles, with an overrecruitment of $Foxp3^+$ $CD4^+$ Tregs in the draining lymph nodes. This is congruent with our previous reported results [34]. It was proposed that the preserved Treg cells seen in women with endometriosis decrease the ability of newly recruited immune cell populations to effectively recognize and target endometrial antigens during menstruation, allowing survival and implantation of shed endometrial cells [72]. As proposed by Podgaec et al. [84], the immune cells (macrophages, dendritic cells, NK, $CD4^+$, and $CD8^+$ lymphocytes) responsible for local surveillance profile could have their activity suppressed by Treg cells, a fact that would prevent the ectopic endometrial cells from being removed from the peritoneal cavity. This, together with endometrial cells decreased apoptosis rate (see after), may cause perpetuation of disease growth. Moreover, it seems that hormonal therapies for endometriosis not only affect directly the endometriotic cells but also alter the immunological environment and thus in turn contribute to the control of endometriosis. For example, GnRH analog therapy was reported to increase total T lymphocytes number in peripheral blood and T-lymphocyte activity in peripheral blood and PF.

2.3. B Lymphocytes. B lymphocytes are responsible for humoral immune response, principally producing antibodies against antigens. In the pathogenesis of endometriosis, they have been suggested to play roles by secreting autoantibodies. Focusing on the role of B lymphocytes in the pathogenesis of endometriosis, particularly autoimmune responses, could be elicited via two major types of autoantibody: antibodies that specifically respond to the endometrium and antibodies that are commonly observed in various autoimmune disorders. Autoantibodies that are frequently found in patients with various autoimmune diseases such as antinuclear antibodies, anti-DNA antibodies, and antiphospholipid antibodies have also been observed in women with endometriosis. This suggests that endometriosis is associated with abnormal polyclonal B-cell activation, a classic characteristic of autoimmune disease. The association between autoantibody and endometriosis may also explain endometriosis-related infertility, as these antibodies might bind to not only the endometrium but also embryos and sperms [72].

2.4. Natural Killer Cells. NK cells destroy target cells by releasing small cytoplasmic granules of proteins that induce apoptosis. A possible link between NK cells and endometriosis was initially arisen from a study which showed that NK cells in peripheral blood have an ability to destroy endometrial cells. The NK activity and the cytotoxicity against autologous endometrial cells were decreased in women with endometriosis and correlated with the severity of the disease. The decreased cytotoxicity to endometrial cells in women with endometriosis is mainly because of a defect in NK activity but is also partially because of a resistance of the endometrium to NK cytotoxicity [85]. Recently, Sikora et al. [86] suggested that endometriosis may be related to a defect of NK cell cytotoxicity function in the ability to eliminate endometrial cells in ectopic sites. Alternations of the innate immunity mediated by NK cells may promote impairments or disrupt functions of adaptive immunity, which can contribute to development and progression of endometriosis and infertility associated with endometriosis. The reduction of cytotoxic activity of NK cells was also observed in PF of endometriotic women. In particular, Oosterlynck et al. [85] found that PF taken from patients with endometriosis had greater suppressive effect on NK cells cytotoxicity compared to PF from healthy women, suggesting the presence of substances which suppress NK cells cytotoxic activity. Impaired NK cell cytotoxic activity may be a primary cause of development of endometriosis, by allowing endometrial cells escape from their attacks.

2.5. Apoptosis Impairment. During endometriosis a breakdown occurs in endometrial and peritoneal homeostasis caused by cytokine induced cell proliferation and dysregulation of apoptosis [34, 87–89]. Execution of the programmed cell death is a process that can be triggered by many apoptotic signals, and it occurs via two main pathways. Both pathways stimulate an intracellular cascade of events that leads to cell death. The intrinsic pathway is initiated from mitochondria, whereas the extrinsic pathway is activated by death ligands (FasL and TNFα) on the cellular surface membrane that engage their respective receptors (Fas and TNFR1/TNFR2) on the surface membrane of target cells [90].

2.5.1. Apoptosis Intrinsic Pathway. Intrinsic cell death pathway is regulated by BCL2 family proteins. This family is divided into three different subclasses based on structural and functional features. BCL2 (and its antiapoptotic orthologues) seems to inhibit apoptosis by the preservation of mitochondrial membrane integrity as its hydrophobic carboxyl-terminal domain is linked to the outer membrane. BCL2 prevents BAX/BAK oligomerization, which would otherwise lead to the release of several apoptogenic molecules from the mitochondrion. It is also known that BCL2 binds to and inactivates BAX and other proapoptotic proteins, thereby inhibiting apoptosis [91]. Numerous alterations in the apoptotic intrinsic pathway, including a significant up-regulation of the antiapoptotic molecule Bcl2 and a significant downregulation of the proapoptotic factor Bax, occur in endometriosis [46].

2.5.2. Apoptosis Extrinsic Pathway

Fas and FasL System. Fas (DR2/CD95/Apo-1) is a type I cell membrane protein (mFas) with an extracellular domain that binds FasL and a cytoplasmic domain that transduces the death signal [92, 93]. FasL (CD95L/CD178/Apo-1L) is

type II cell membrane protein (mFasL) which is inducibly expressed in lymphocytes and constitutively expressed in cells present in immune-privileged organs [94, 95]. We have previously reported [73] that Fas/FasL system is dysregulated progressively throughout the course of the disease, with the result that endometriotic cells do not undergo Fas/FasL-mediated apoptosis because they do not receive a death signal from PFMCs, thus implanting themselves and surviving outside of the uterus. Paradoxically, endometriotic cells become themselves capable of killing PFMCs, and this may allow their establishment in the peritoneum, which in turn becomes an immune privileged environment [96–99].

TNFα and TNFR1/TNFR2 System. TNFα, belonging to TNF superfamily, is synthesized as a 26 kDa transmembrane type II protein (mTNFα). 17 kDa soluble form of the cytokine (sTNFα), that retains its biological activity, rises by TACE action on mTNFα. Both forms of TNFα coexist as mono-, di-, or trimeric proteins. The last form only of both cytokines is biologically active and exerts its effects interacting with two different transmembrane receptors of TNFR superfamily, TNFR1 and TNFR2. TNFR superfamily consists of two main groups of receptors: the first group includes death receptors, characterized by the presence of the death domain (DD) in their intracellular region, whereas the second one does not have DD. TNFR1 is a 55 kDa protein which belongs to the first group of receptors and is expressed in almost all cell types. TNFR2 is a 75 kDa protein of the second group and is expressed only in certain cell types, including T cells. Whereas sTNFα binds both receptors but only activates efficiently TNFR1, mTNFα can bind and activate both TNFR1 and TNFR2 [100]. Peritoneal microenvironmental changes could depend, at least in part, also by TNFα and TNFR1/TNFR2 system. TNFα, upon binding TNFR1 or TNFR2, appears to mediate different biological activities ranging from the proliferation, differentiation, and angiogenesis to the activation of apoptosis [101]. We have showed that also this last described system is dysregulated during endometriosis and addresses immune responses according to disease's stage [101]. About this, we must consider that Zhao et al. [102] analyzed 26 single-nucleotide polymorphisms (SNPs) in the coding and the promoter region of the TNFα gene in 958 endometriosis cases and 959 controls, and they concluded that TNFα gene is not a major susceptibility gene for endometriosis. However, other data provided by another group [103] point out that the frequencies of the TNFα T/C/C haplotype allele and the TNFR2 G/G/T haplotype allele are significantly decreased in women with endometriosis compared to women without endometriosis, thus associating these haplotype alleles and polymorphisms to the disease. Moreover, an Indian population study [104] suggests an association between TNFα-C850T polymorphism and endometriosis.

2.6. Changes of Cytokines and Other Soluble Products in the Peritoneal Microenvironment. Knowledge of these factors is indispensable for the development of strategies for prevention and targeted treatment of endometriosis. Changes of the wide range of soluble products including (a) cytokines, (b) angiogenic factors, (c) adhesion molecules, (d) hormones, (e) prostaglandins (PGs), and (f) reactive oxygen species (ROS) are characteristic findings in the peritoneal microenvironment of endometriotic women [105].

2.6.1. Cytokines. The role of cytokines in the development of endometriosis is emphasized in the literature. Several researchers, in independent works, assessed the levels of cytokines involved in immune response patterns Th1 and Th2 in patients with endometriosis. Podgaec et al. [84] noted an increase in the levels of IFN-gamma and IL-10 in patients with endometriosis, evidencing the coexistence of both responses. However, it a predominance of IL-4 and IL-10 was observed, thus reflecting a polarization toward Th2 immune response [106]. In a previous study, we showed a prevalence of Th1 profile cytokines in the PF of women with endometriosis at minimal and mild stages, whereas Th2 profile cytokines prevailed in severe stages [34]. Particularly, we reported that serum and PF levels of TNF-α were very high at the early stage and decreased with the severity of the disease. TGF-β levels were high and increased with the severity of the disease, particularly in the PF. Serum and PF IL-8 as well as MCP-1 concentrations at all stages were high, yet showed an opposite behaviour in both biological fluids. In fact, IL-8 and MCP-1 serum levels were higher at early stages and decreased with the severity of the disease, whereas the PF levels increased with the worsening of the disease [34].

IL1. Data from the literature evidenced an impairment of the secretion of the IL-1 cytokine family in endometriosis. For example, a marked imbalance was reported between IL-1 and its natural inhibitor IL-1 receptor type 2 (IL1R2). This points to a deficiency in the local control of IL-1 that, in view of the cytokine's elevated levels and potent proinflammatory, angiogenic, and growth-promoting effects, may contribute to endometriosis development. sIL1R2 significantly downregulated the expression of major cell adhesion receptors (αv and β3 integrins), matrix metalloproteinases (MMP-2 and -9), and VEGF [107]. Moreover, invalid IL-1β and IL-18 maturation by interleukin-1 converting enzyme (ICE) may be an important pathogenic factor in endometriosis [108].

IL6 and IL8. Concentrations of these cytokines are elevated in the PF of endometriotic patients. It was suggested that the inflammatory process, typical of the disease, once started continues being activated constantly, perpetuating itself via high concentrations of IL-6 [109].

Significantly higher IL-6 and IL-10 levels were found in moderate-to-severe but not in minimal-to-mild endometriosis as compared to controls [110]. Moreover, serum levels of both IL-6 and IL-8 are significantly higher in patients with ovarian endometrioma, but not in the presence of deep infiltrating endometriosis [111].

IL10. Studies regarding this cytokine reported conflicting results. Some authors share the evidence that no significant differences were observed in the PF of endometriotic women in respect to controls, whereas others showed an elevation [112]. Contradiction can be explained by the velocity of

production and consumption of inflammatory products, making comparisons difficult [84]. In particular, according to these latter authors, increased IL10 production may partially contribute to the disturbed immune regulation in patients with endometriosis, because it attenuates TNF-α-induced IL6 synthesis via NF-kappaB and MAPK pathways in endometriotic cells.

IL15. The levels of this cytokine are increased in PF of women with endometriosis. However, these levels are inversely correlated with the depth of invasion and disease stage, suggesting a possible role for IL15 in the early pathogenesis of endometriosis [113].

IL17. Zhang et al. [114] related an elevation of IL17 levels in the PF of patients with minimal or mild endometriosis stages. This relation was even more positive when endometriosis at these stages was associated with infertility. Conversely, Others did not find any difference in IL17 peritoneal fluid concentration in patients with or without endometriosis.

IL18. PF levels of this cytokine are elevated in women with peritoneal, minimal-to mild-stage endometriosis [115]. Moreover, the increased concentrations of IL-18 in PF of endometriotic women do not correlate with menstrual cycle phase [109].

IL19 and IL22. Serum levels of these cytokines are decreased in women with ovarian endometrioma [116].

IL33. Peritoneal as well as serum levels of IL33 are elevated in women with endometriosis and principally in deeply infiltrating endometriosis. Elevated serum but not peritoneal IL33 levels are correlated with the intensity of preoperative painful symptoms and with the extent and severity of the deeply infiltrating endometriosis [117].

TNFα. A large body of evidence indicates that TNFα and IL1β, typical inflammatory cytokines, are involved in macrophage activation, inflammatory change, and enhanced angiogenesis to develop endometriosis [118]. A pivotal role of TNFα in endometriosis is corroborated by the finding that TNF-α-targeted suppression by specific drugs inhibits the development of endometriosis in baboons [119, 120]; TNFα has also been shown to be elevated not only in the peritoneal fluid but also in the serum of women with the disease. Indeed, there is a positive correlation between peritoneal levels of TNFα and the size and number of active lesions [121]. In addition to its proinflammatory functions, TNFα also stimulates the expression of matrix metalloproteinases in endometrial tissue [122]. Matrix metalloproteinases are known to play a role in tissue remodeling and invasion of endometriotic lesions [123]. These data suggest that TNFα may influence the establishment and progression of disease and that an antagonist of TNFα may be effective in treating patients with endometriosis.

TGFβ. There is evidence that concentrations of this cytokine are ten times higher in peritoneal fluid of patients with endometriosis compared to those without the disease. In the inflammatory process, high levels of TGFβ may occur in the regeneration process, inducing adhesion formation and the appearance of fibrotic tissue and stimulating Treg cells that are elevated to regulate the exacerbated immune response [84]. However, Meta-analyses of the available data showed that the association between TGF-β1-509C/T polymorphism and susceptibility of endometriosis was not significant [114].

2.6.2. Angiogenic Factors. The ability of ectopic endometrial cells to invade the underlying basement membrane represents a further necessary step for lesions establishment. Endometrial tissue invades even intact serosal membranes, indicating that a previously disrupted peritoneum is not a requirement. Invasion is a prerequisite for the organization of the ectopic endometrial cells in tridimensional cysts but is not sufficient: novel vessels are also necessary. Angiogenesis may play an important role in the pathogenesis of endometriosis. Endometriotic implants require neovascularization to proliferate, invade the extracellular matrix, and establish an endometriotic lesion, similar to tumour metastases. Several studies have reported, in endometriosis, an increase in levels of VEGF-A, angiogenic factor playing a major role in the progression of the disease. Thrombospondin-1 (TSP-1), an inhibitor of angiogenesis, may also be involved in endometriosis, in which vessel formation occurs. Moreover, the same authors observed an increase in VEGF-A levels and proteolytic factors, like urokinase plasminogen activator (uPA) and metalloproteinase-3 (MMP-3), in peritoneal fluid from patients with endometriosis in comparison with women without the disease. These factors may enhance the angiogenic and proteolytic capability of ectopic tissue to facilitate the implantation process [28]. MicroRNAs (miRNAs) may be the main regulators of angiogenesis. Several studies [124–129] have indicated that endometrium and PF from women with endometriosis have different expression patterns of several angiogenic and proteolytic components in comparison with endometrium and peritoneal fluid from control women, suggesting that these systems play a role in the pathogenesis of endometriosis. Braza-Boïls et al. [130] evaluated the influence of PF from women with or without endometriosis on the expression of six miRNAs that modulate angiogenesis, as well as several angiogenic and proteolytic factors, in endometriotic and endometrial cell cultures from women with and without endometriosis. All of these alterations could dysregulate miRNA expression in stromal cells of endometrial fragments migrated to peritoneum, facilitating the implantation of ectopic lesions. The study elucidates that peritoneal fluid from women with endometriosis induces the highest decrease in angiogenesis-related miRNAs and the highest increase in VEGF-A protein levels in endometrial cell cultures from patients. In conclusion, this "in vitro" study indicates that peritoneal fluid from women with endometriosis modulates the expression of miRNAs that could contribute to the angiogenic and proteolytic disequilibrium observed in this disease. Another evidence suggests that increased levels of VEGF-A may be associated with a decreased rate of pelvic adhesion formation in the course

of endometriosis [131]. Regarding to angiogenic factor polymorphisms analysis, Cosín et al. [132] suggested that the VEGF 936C/T polymorphism may be associated with the risk of endometriosis. Moreover, they showed that endometrium and PF from women with endometriosis showed an increase in VEGF levels. Other data [133] seem to suggest that an increased frequency of the +405CC polymorphism in VEGF was observed in the patients with endometriosis as compared with the controls.

2.6.3. Enzymes and Adhesion Molecules. The establishment of endometriotic lesions in the peritoneal cavity requires adhesion, migration, invasion, and proliferation of the ectopic endometrial tissue [134]. For example, the expression levels of MMP-2 and MMP-9 were higher in women with endometriosis [135]. In fact, the enzymes play important roles in the ectopic adhesion, invasion and implantation, and neovascularisation of the endometrium. MMP-2 and MMP-9, by degrading extracellular cellular matrix and promoting the release of key factors, play a critical role in the pathogenesis of endometriosis [129]. In addition, MMP-2 and MMP-9 are elevated in the urine of patients with endometriosis compared to control. An immunohistochemical study revealed that MMP-9 expression is higher in endometriosis than proliferative endometrium [136].

A substantial body of evidence suggests that a large number of mediators, including cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD106) [137] as well as proinflammatory cytokines such as TNF-α, IL1, IL6, and IL8, and chemokines such as MCP-1, play key roles in the pathogenesis of endometriosis. These factors are present in PF as well as in endometriotic implants, and TNF-α in particular is regarded as a critical regulatory molecule in eliciting inflammatory immune responses in endometriosis [138]. Interestingly, ICAM-1 is expressed not only in endometrial stromal cells in human endometrium in situ but is also markedly expressed on cultured human endometrial stromal cells [139]. Human ectopic endometrial stromal cells strongly express ICAM-1, and these cells express a higher level of ICAM-1 than eutopic endometrial stromal cells in patients with endometriosis [140]. These data suggest that there may be cross-talk between endometrial stromal cells and leukocytes in normal as well as in endometriotic endometrium and peritoneal fluid via ICAM-1 and its receptors. Furthermore, significantly increased ICAM-1 expression in ectopic endometrial stromal cells from endometriomas is enhanced by stimulation with proinflammatory cytokines such as IL-1β and IFN-γ, indicating the important role of peritoneal cytokine milieu in the regulation of ICAM-1 expression in endometriotic stromal cells and consequently in the pathogenesis of endometriosis [137]. In addition to ICAM-1, VCAM-1 is also expressed in human endometrial stromal cells. It was observed that TNFα markedly augments the expression of ICAM-1 and VCAM-1 [140]. ICAM-1 is involved in the impairment of NK cell function in endometriosis. In patients with endometriosis, elevated expression of ICAM-1 on the human endometrial stromal cells gives rise to the shedding of soluble ICAM-1 (sICAM-1) from the cell surface into the peritoneal cavity, where sICAM-1 binds to the cell membranes of NK cells and interferes with their cytotoxic function, consequently hampering NK cell-mediated removal of ectopic endometrial cells in the peritoneal cavity, and can lead to the development of endometriosis. Moreover, it was found that endometrial release of sICAM-1 is directly correlated with the number and score of endometriotic implants [141]. During menstrual phase, increased endometrial mRNA levels of αV integrin, combined αVβ3 integrins were observed in women with endometriosis. Women with endometriosis had increased peritoneal mRNA expression of VCAM-1 during menstrual compared with luteal phase [142].

2.6.4. PGs. Data from women with endometriosis and a murine model of the disease showed that expression of annexin A2 in peritoneal macrophages is inhibited by prostaglandin E2 (PGE2), and this impairs the phagocytic ability of macrophages. The level of annexin A2 mRNA in the macrophages was reduced by PGE2 via the EP2/EP4 receptor-dependent signaling pathway [143]. Endometrial PGE2 and PGF2α act as potent vasoconstrictor on the spiral arterioles. PG production, spiral arteriole vasoconstriction, and local hypoxia in turn regulate the production of chemokines, such as IL-8 (CXCL8) and CXC chemokine ligand 12 (CXCL12) stromal cell-derived factor (SDF-1) [28]. Increased PG concentrations in the PF of infertile women with endometriosis have been reported. COX-2 expression was upregulated in endometriotic tissue. Well known as a potent vasodilator, PGE2 may play a role in endometriosis-associated angiogenesis and further contribute to ectopic endometrial cell growth. Moreover, PGE2 appeared to stimulate the expression of aromatase, an essential enzyme in estrogen synthesis in ectopic endometrium, which may favor the ectopic implantation and growth of endometrial tissue. Aromatase, the rate-limiting enzyme for the synthesis of estrogen, is aberrantly expressed in endometriotic implants. Aberrant expression of COX-2 and PGE2 secretion by ectopic endometriotic implants has been reported, although the underlying mechanism is not clearly understood [49]. Epigenetic changes favoring the transcription of the aromatase gene in the endometrium allow endometrial cells to survive in ectopic locations by producing estrogens that spare them from destruction through activated macrophages. Local estrogen production hastens prostaglandin synthesis by stimulating COX-2 activity, thus creating a self-perpetuating sequence of augmented estrogen formation and enhanced inflammation. Repetitive retrograde menstruation reintroduces aromatase-positive endometrial cells endowed with the capacity to implant and invade the peritoneum [144].

2.6.5. Hormones. The biologically active estrogen estradiol (E2) is the best-defined mitogen for the growth and inflammation processes in the ectopic endometriotic tissue. Progesterone and progestins were used in therapy to limit growth and inflammation in endometriosis, but a portion of patients do not respond to treatment with progestins. Bulun et al. [145] reported that this is indicative of a resistance to progesterone

action, related to an overall reduction in the levels of progesterone receptors (PRs) and the lack of the PR isoform named progesterone receptor B (PR-B). In normal endometrium, progesterone acts on stromal cells to induce secretion of paracrine factor(s). These unknown factor(s) act on neighboring epithelial cells to induce the expression of the enzyme 17beta-hydroxysteroid dehydrogenase type 2 (17beta-HSD-2), which metabolizes the biologically active estrogen E2 to estrone (E1). In endometriotic tissue, progesterone does not induce epithelial 17beta-HSD-2 expression due to a defect in stromal cells. The inability of endometriotic stromal cells to produce progesterone-induced paracrine factors that stimulate 17beta-HSD-2 may be due to the lack of PR-B and very low levels of progesterone receptor A (PR-A) observed in vivo in endometriotic tissue. The end result is deficient metabolism of E2 in endometriosis giving rise to high local concentrations of this local mitogen [146]. Changes in estradiol homeostasis have been locally observed in endometriosis. A balance was observed between local 2-methox yestradiol production and angiogenesis, which could promote the development of endometriotic lesions [147].

A local increase in estrogens levels is characteristic of patients with ovarian endometrioma, and this condition could promote endometriotic cell proliferation [148–150]. Moreover, Pabona et al. [151] investigated the expression of Krüppel-like factor 9 (KLF9), a progesterone receptor-interacting protein, in eutopic endometrium of women with and without endometriosis. They reported that the loss of coregulation by KLF9 on WNT-signalling component expression, in human endometrial stromal cells, may account for progesterone resistance in endometriosis. According to Vinatier et al. [152], these changes may influence the development of the peritoneal surface metaplasia or of Müllerian residues.

2.6.6. ROS. 17β-estradiol (E2) is known to play important roles in the processes that control cell division, differentiation, and proliferation and is considered a major risk factor in the development and progression of endometriosis. It was reported that H_2O_2 is a signaling molecule that downregulates apoptosis in endometrial cells, supporting the fact that endometriosis, albeit a benign disease, shares some features with cancer such as decreased catalase levels [153].

3. Genetics

Many aspects of female reproductive function are strongly influenced by genetic factors, and numerous studies have attempted to identify susceptibility genes for endometriosis. Family studies of endometriosis indicate that close relatives of patients with endometriosis have an increased risk for the disease [154], suggesting that genetic components perhaps contribute to endometriosis. Recently, several lines of genetic-association studies have revealed associations between the development of endometriosis and certain genetic polymorphisms, although the genes that play a role in susceptibility to the development and progression of endometriosis are unknown [155]. It is widely accepted also that this disease has a family tendency, suggesting an important role of genetic factor in the pathogenesis: for example, Kashima et al. [156] analyzing 339 patients with endometriosis detected sisters with the same disease in 8.8% of cases. Similar finding are reported by Kennedy et al. [157]. Others [158] performed an extensive review of studies of association between genetic variation at common DNA polymorphisms and variation in disease susceptibility and reported that over 600 positive associations have been reported, including single nucleotide polymorphisms (SNPs). This, remembering also that is very important the to take into account gene-environment interactions with known epidemiologic risk factors [159–161]. Among all SNPs, Falconer et al. [162] proposed that genetic polymorphism of hormone receptors, growth factor, and human leukocyte antigen system components showed a relatively stronger correlation than the others. To better understand endometriosis-related genomic regions, Treloar et al. [53] conducted a linkage study of 1,176 families (931 from an Australian group and 245 from a UK group), each with at least two members with surgically diagnosed disease, and identified a region of significant linkage on chromosome 10q26 and another region of suggestive linkage on chromosome 20p13. Considering that endometriosis is clearly an estrogen-dependent disease, there are many reports of positive associations with numerous polymorphisms involving sex steroids production and metabolism: for example, a consistent work by Singh et al. [54] was conducted on eutopic and ectopic (ovarian) endometrium from patients with stage 3 or 4 endometriosis, comparing ectopic to eutopic endometrium; their data showed a 3–9-fold increase in intraindividual expression of CYP1A1, a 5–53-fold intra-individual increase in gamma-SYN expression, and an elevation in Estrogen Receptor β (but not α). Additionally we have to consider that CYP1A1 and gamma-SYN are dioxin-inducible genes, and the observed upregulation of them could support, at least in part, the involvement of endocrine-disrupting agents in the pathogenesis of the disease. This is compatible with the results of Wu et al. [55], suggesting that polymorphisms of dioxin receptor complex components and detoxification-related genes jointly confer susceptibility to advanced-stage endometriosis. Considering the importance of gene-environment interactions, previously underlined, the Italian group of Vichi et al. [56] suggests that glutathione transferase (GST) gene polymorphisms per se do not increase the risk of developing endometriosis, although some genetic variants could modulate in different way the effect of endocrine-disrupting polychlorinated biphenyls (PCBs) implicated in the pathogenesis of the disease. Others [57], moreover, reported a linkage peak for endometriosis in rs11592737 SNP in the cytochrome P450 subfamily C (CYP2C19). Additionally, there is no evidence that CYP17 gene and Estrogen Receptor α gene could be considered as genetic risk factors for endometriosis, at least in Chinese women [163]. Furthermore, there is evidence of a significant correlation between polymorphism of the progesterone receptor gene (PROGINS) and endometriosis [58]. Focusing on E-cadherin, Govatati et al. [59] found increased membranous form expression of this protein in endometriosis in respect to controls, and moreover that the expression seems to be genotype dependent, according to

various SNPs. Guo [60] on the contrary, reviewing 12 association studies on 5 genes (CYP17, CYP19, Androgen Receptor, Progesterone Receptor, and Estrogen Receptor) found no functional data that support a putative relationship of these genetic polymorphisms with endometriosis. Kim et al. [61] reported of a positive association between endometriosis and polymorphisms in insulin-like growth factors receptor genes: in fact, they found that women with endometriosis were observed 1.99 times more frequently to have IGF-II 820G>A SNP. A more recent study [62] of the same authors concluded that also polymorphisms in the insulin-like growth factor binding protein type 3 (IGFBP3) gene may be associated with advanced endometriosis. About this, others [63] suggest that the insulin receptor substrate (IRS)-2 G1057D polymorphism may be associated with an increased risk for endometriosis. Moreover, probably endometriosis pathogenesis may depend, at least in part, by mutation in autoimmunity genes. For example, Ammendola et al. [64] investigated on PTPN22, one of the few known shared-autoimmunity genes, and found that women carriers of the PTPN22(∗)T variant are significantly more susceptible to endometriosis than controls. Among the huge number of interleukin polymorphism, a case-control study based on Korean population [65] reported that the C627T polymorphism of the IL-2R beta gene may not be associated with the risk of endometriosis. Another study provided by Gonçalves-Filho et al. [66] Proposed that plasminogen activator inhibitor-1 4G/5G polymorphism may be associated with a risk of endometriosis-associated infertility. About the endometriosis-associated infertility André et al. [67] reported that FoxP3 polymorphisms can be associated with risk of idiopathic infertility (rs2280883 and rs2232368) and endometriosis (rs3761549), and this is congruent with other reports (see before) about the importance of $FoxP3^+$ $CD4^+$ Tregs in the pathogenesis of the disease. A more recent study [68] by the same group adds the hypothesis that FoxP3 and FCRL3 polymorphisms may have a cumulative effect in increasing the risk of developing endometriosis. Furthermore, Ruiz et al.'s results [69] demonstrated statistically significant differences in genetic variants in lysyl oxidase-like protein 4 (LOXL4) and complement component 3 (C3) in patients with endometriosis-associated infertility versus controls, and in patients with endometriosis versus controls, respectively. Medeiros et al. [70] found HMGA1 and HMGA2 gene rearrangements in the stromal component but not in the glandular component in 3 cases of polypoid endometriosis, suggesting a possible role of them in the pathogenesis of the disease. Despite the great number of work in the literature that evidenced the possible role of certain genetic variants in endometriosis susceptibility, the data are not conclusive. This consideration pushed some authors to try to resolve the dilemma, through a genome-wide association meta-analysis [71] showing three well-identified genes, which, if mutated by SNPs, seems to be associated with endometriosis: WNT4 encodes for wingless-type MMTV integration site family, member 4, and is important for the development of the female reproductive tract [164] and steroidogenesis [165]; VEZT encodes vezatin, an adherens junction transmembrane protein that is downregulated in gastric cancer [166]; GREB1 encodes growth regulation by estrogen in breast cancer 1, an early response gene in the estrogen regulation pathway involved in hormone dependent breast cancer cell growth [167]. For an accurate overview of the genetic variants reported in this chapter, refer to Table 1.

4. Organogenesis of the Müllerian Reproductive Tract

Embryogenesis phase, from 29th to 56th of development day, is defined as organogenesis, because during this period organs start to develop. The defects acquired during the organogenesis are usually more circumscribed than those of the first 28 development days (blastogenesis phase) and generally affect a single organ without compromising the survival of a developing organism. Having stated that, it seems to be essential to consider the embryological origin of various elements of genitourinary system in order to understand the pathogenesis of reproductive organs' diseases [168]. Around the 5th week of pregnancy Müllerian ducts (or paramesonephric ducts) appear as developing structures, and each part of them has a different developing pattern to form the final shape and function of the Müllerian-derived organs. The caudal extremity of the ducts is destined to merge and to constitute superior 2/3 parts of vagina and uterine cervix, the intermediate part fuses and creates uterine body, while the upper portions maintain their own independence and, opening in the coelomatic cavity (future peritoneal cavity), make fallopian tubes. In the same period, the renal system develops through the growth of urethral sketch, derived from Wolff's ducts (mesonephric ducts) within the mesenchyme of the metanephros. In similar times, the migration of the primordial germinal cells from the yolk sac leads to the formation of ovaries which arise from mesenchyme and from the epithelium of genital crest of the intermediate mesoderm, with female reproductive tract organogenetic processes different from those of mesonephros; therefore, the anomalies of Müllerian ducts are not associated, generally, with anomalies of ovary development [169].

5. Role of Hoxa Genes

It is widely accepted that during embryogenesis a key role is played by Hox (homeobox) genes: like is well reported by Zanatta et al. [170] in humans and mice, there are at least 39 Hox/HOX genes distributed in four groups lettered A, B, C, and D. These groups each comprise 9–13 genes and are distributed in the human chromosomes 7, 17, 12, and 2, respectively [171]. Two weeks after birth, a period that corresponds to the peak of the differentiation process in mice, Hoxa9, Hoxa10, Hoxa11, and Hoxa13 develop their characteristic spatial distribution throughout the Müllerian ducts: according to Taylor et al. [172] Hoxa-9 expression is limited to the fallopian tube; Hoxa-10 is expressed in the uterine epithelium, stroma, and muscle; Hoxa-11 is expressed in the cervical glands and epithelium (although it is also expressed in the uterine corpus); and Hoxa-13 is strongly expressed in the vaginal epithelium. The importance of the development and

Table 1: Genetic variants associated with endometriosis.

Author(s)	Genetic variants
Treloar et al., 2005 [53]	Significant linkage on chromosome 10q26 and chromosome 20p13
Singh et al., 2008 [54]	Upregulation of dioxin-inducible CYP1A1 and gamma-SYN and of Estrogen Receptor β
Wu et al., 2012 [55]	Polymorphisms of dioxin receptor complex components and detoxification-related genes
Vichi et al., 2012 [56]	GSTP1(Ile/Ile) and GSTM1 null genotypes, modulating the effect of PCB153, PCB180, and of total PCBs
Painter et al., 2011 [57]	rs11592737 in the cytochrome P450 subfamily C (CYP2C19)
Costa et al., 2011 [58]	PROGINS polymorphisms (A1/A1, A1/A2 and A2/A2)
Govatati et al., 2012 [59]	E-cadherin −347GA/GA and −160A/A genotypes and −347GA/−160A/+54C and −347G/−160A/+54C haplotypes
Guo, 2006 [60]	CYP17, CYP19, Androgen Receptor, Progesterone Receptor, and Estrogen Receptor genetic variants are not associated with endometriosis
Kim et al., 2011 [61]	IGF-II 820G>A polymorphism
Kim et al., 2012 [62]	AAG haplotype allele of the −672A>G, −202A>C and c.95C>G polymorphisms in the IGFBP3
Çayan et al., 2010 [63]	IRS2 G1057D polymorphism
Ammendola et al., 2008 [64]	PTPN22(∗)T variant
Lee et al., 2009 [65]	C627T polymorphism of the IL-2R beta
Gonçalves-Filho et al., 2011 [66]	PAI-1 4G/5G polymorphism
André et al., 2011 [67]	FOXP3 polymorphisms (rs3761549)
Barbosa et al., 2012 [68]	Allele FOXP3 T for genotypes FCRL3TT/FOXP3CT, FCRL3CT/FOXP3CT, FCRL3CC/FOXP3CT
Ruiz et al., 2011 [69]	Variants in LOXL4 and complement C3
Medeiros et al., 2012 [70]	HMGA rearrangements
Nyholt et al., 2012 [71]	WNT4, VEZT, GREB1 polymorphism

patterning of the uterus during embryogenesis is also supported by others [173]. Concerning these data, Hox genes are strictly involved in the differentiation of the paramesonephric duct into the mature female reproductive system, and moreover their persistent expression in the adult, as reported by Taylor et al. [172], may play a role in maintaining the developmental plasticity that is characteristic of this organ system. The late differential Hox axis formation may reflect the late differentiation of this organ system, and alterations in its expression could provoke, consequently, reproductive tract anatomic and functional anomalies. For example, it is documented in the mouse model that the losses of Hoxa-10 function provoke uterine alterations in decidualization and implantation phases, resulting in female infertility [173]; the mechanism by which Hoxa-10 altered expression causes these events is still unknown. Lim et al. [174] reported that in the absence of Hoxa-10, two prostaglandin E2 (PG)-E2 receptor subtypes, named EP3 and EP4, are inappropriately regulated by progesterone (P4). Moreover, they suggest that Hoxa-10 specifically mediates progesterone regulation of EP3 and EP4 in the uterine stroma, while epithelial cell functions seem to be not impaired. Regarding endometriosis, Painter et al. [175] conducted a rigorous genome-wide association study in 3,194 individuals with surgically confirmed endometriosis case and 7,060 controls from Australia and the UK. They found strong association signal located at 7p15.2 region, upstream of the plausible candidate genes NFE2L3 and HOXA-10. Others [176] reported that ectopic expression of Hoxa-9 in tumorigenic mouse ovarian surface epithelium cells gave rise to papillary tumors. In contrast, Hoxa-10 and Hoxa-11 induced morphogenesis of endometrioid-like and mucinous-like epithelial ovarian cancers, respectively. For this reason, it is hypothesized that inappropriate activation of a molecular program that controls patterning of the reproductive tract could address the development of different Müllerian-like features. Moreover, endometriosis progression could be caused, at least in part, by systematic repression of the genes involved in cell cycle and a specific regulation of the HOX genes [177]. Another of the most studied genes is EMX2: this is a fundamental transcription factor necessary for reproductive tract development, and its expression is strictly related to hormonal levels. About this, Daftary and Taylor [178] characterized menstrual cycle-dependent expression of EMX2 in endometrium from women with and without endometriosis, and they found that in endometriosis-free women EMX2 mRNA levels declined 50% in peri-implantation endometrium compared with levels in the proliferative phase. Moreover, they reported that sex steroids seem to regulate endometrial HOXA10 gene expression, which in turn negatively regulates EMX2. Conversely, others [179] identified

a region of significant linkage peak for endometriosis on chromosome 7. For this reason, they screened coding regions and parts of the regulatory regions of three candidate genes with a known role in endometrial development and function, INHBA, SFRP4, and HOXA10, located under or very near this linkage peak: their results, surprisingly, indicated that the coding regions of these three genes do not harbor mutations responsible for linkage to endometriosis in the study population. Given all this, we have to take into account that endometriosis could arise, at least in part, by congenital abnormalities of the Hoxa genes during the embryonic life.

6. Role of Wnt Genes

Another well-known family of genes that influence remarkably the organogenesis of the Müllerian reproductive tract is Wnt (wingless-type MMTV integration site family). In particular, Wnt4 gene produces a secreted protein that suppresses male sexual differentiation, probably repressing the biosynthesis of gonadal androgen in female subjects. Moreover, like is reported by Biason-Lauber et al. [180] Wnt4 loss-of-function mutation could result in altered development of Müllerian-derived structures, like for example, phenotype resembling the Mayer-Rokitansky-Küster-Hauser syndrome, and to androgen excess. The limiting factor of these results is that they are derived from a small cohort [181]. Basing on a mouse model, it is demonstrated that Wnt genes expression is temporal and spatial differentiated according to phases of endometrial modification that occurs in pregnancy, concurrently with another gene family of Fzd (frizzled family receptor). About this, Hayashi et al., in a well-designed study [182], reported that during peri-implantation Wnt7a, Wnt7b, and Wnt11 mRNAs were abundantly detected in the endometrial epithelia. Conversely, Wnt16 mRNA was localized to the stroma surrounding the luminal epithelium (LE) on Gestational Day 4 and remained in the stroma adjacent to the LE but not in areas undergoing the decidual reaction. Moreover, they reported that this genes regulation seems to be addressed by ovarian steroid hormones, such as progesterone stimulated Wnt11 and estrogen stimulated Wnt4 and Wnt7b mRNA production. Same opinions are shared by Sonderegger's group [183], who moreover have showed that the canonical Wnt pathway seems to be involved in nuclear recruitment of β-catenin and activation of Wnt-dependent transcription factors. These transcription factors are critically involved in development and differentiation of the diverse reproductive tissues. Furthermore, they suggest that failures in Wnt signalling are associated with infertility, endometriosis, endometrial cancer, and gestational diseases such as complete mole placentae and choriocarcinomas. Others [184] found that E-cadherin, total β-catenin, and dephosphorylated β-catenin protein expressions were significantly higher in the mid-secretory endometrium of infertile patients with endometriosis or unexplained infertility compared to both luminal and glandular epithelial endometrium of healthy fertile controls. These alterations of the physiologic expression profile of the endometrium may underlie, at least in part, the pathogenesis of the endometriosis-related infertility.

7. Hoxa and Wnt Dynamic Interplay

Like reported before, Hoxa and Wnt regulation and cell signaling pathways are utilized to pattern organs and to specify the fate of organogenesis. For this reason, it is widely accepted that the complex and accurate process of organogenesis of the Müllerian reproductive tract could be addressed, at least in part, by a strict interaction between these two gene families. This interaction is variable in the course of time according to the different phases of development, during which Hox transcription factors specify positional identity, and Wnt signaling provides spatial information and promotes asymmetric cell division [185]. Another evidence of these two gene families interplay was derived by the work of Klapholz-Brown et al. [186]. They analyzed the Wnt transcriptional effects on the stromal cells that make up the scaffold and infrastructure of epithelial tissues during key-event as development, regulation of stem cell self-renewal and differentiation, cell polarity, and morphogenesis. They confirmed the previous results and suggest that Wnt induced key transcription factors for the development, including Hox genes. Moreover, they verified that Wnt3a induced a gene, named GREMLIN2, which encodes a secreted bone morphogenetic protein (BMP) antagonist. Additionally, Wnt3a signals for the maintenance of stem cell niches, by inhibiting their differentiation and promoting their expansion in microenvironment, through the induction of high level of BMP antagonist production by nearby fibroblasts. The importance of Hox gene cluster in the correct development process is also shared by Deschamps [187], who underlined the well-conserved genetic pathways during evolution of different animal species. As suggested, Hox cluster regulation prefigured the temporal colinearity of expression of these genes in vertebrates, addressing the development of specific embryonic structures. Consequently, it is possible that any disturbance during the Müllerian reproductive tract development may lead, first of all, to altered molecular interactions, then to modified intercellular communications, and finally to readdressing of developing structures and coregulation of common downstream targets. Remembering that the form is the expression of the function, also the function of a temporally and spatially altered developing tissue will be compromised. For an accurate overview of the Hoxa and Wnt interplay mechanism reported in this chapter, refer to Figure 1.

8. The Importance of Endometrial Stem Cells

Recently, many studies in the literature support the hypothesis of the presence of adult stem cells in developed human body, within spatially selected areas named "niches" in which the surrounding microenvironment prevents the differentiation of this kind of cells. The uterus, in particular, seems to have a remarkable regenerative ability responsible for cyclical regeneration and remodelling throughout the reproductive life, responding to hormonal influence. One of the best example of this plasticity is represented by endometrial regeneration from the basal layer, which is fundamental for the replacement of the functionalis layer followed by its slough off

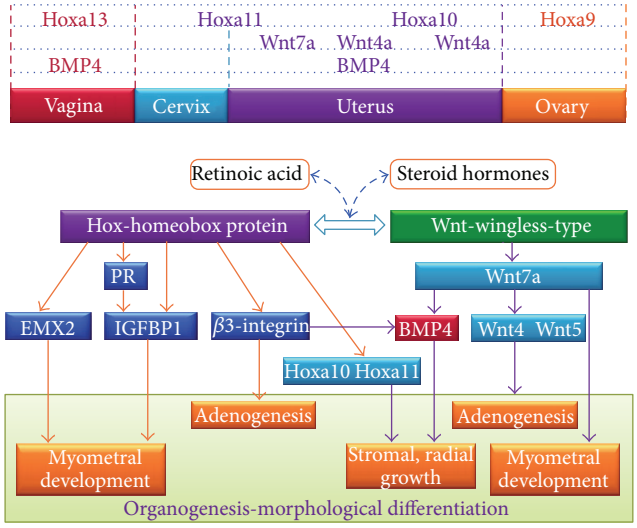

Figure 1: Hoxa and Wnt interplay mechanism. Hox—homeobox protein: this gene is part of the A cluster on chromosome 7 and encodes a DNA-binding transcription factor that may regulate gene expression, morphogenesis, and differentiation. More specifically, it may function in fertility, embryo viability, and regulation of hematopoietic lineage commitment. Wnt—wingless-type: this gene is involved in the development of the anterior-posterior axis in the female reproductive tract and also plays a critical role in uterine smooth muscle pattering and maintenance of adult uterine function. BMP—bone morphogenetic protein: the protein encoded by this gene is a member of the bone morphogenetic protein family which is part of the transforming growth factor-beta superfamily. The superfamily includes large families of growth and differentiation factors. Please refer to chapters 5 (Role of Hoxa genes), 6 (Role of Wnt genes), and 7 (Hoxa and Wnt dynamic interplay) for extensive explanation.

during menses and parturition. Moreover, Cervelló et al. [188] isolated, identified, and characterized the side population (SP) cells corresponding to the human stromal and epithelial compartments and demonstrated that they display genotypic, phenotypic, and functional features of classic endometrial somatic stem cells (SSCs) population. This hypothesis is also shared by Tsuji's group [189], who showed that BCRP1/ABCG2, known as a marker of side population cells, was strongly expressed in the vascular endothelium and the epithelium of the basal layer of the endometrium. Furthermore, others [190], basing on a mouse model, suggest the importance of CD45-positive hematopoietic progenitor cells in regenerating the uterine epithelium. Others [191], moreover, reported that endometrial SP cells exhibit preferential expression of several endothelial cell markers compared to endometrial main population (EMP) cells. Additionally, they demonstrated that a medium specific for endothelial cell culture enabled endometrial SP cells to proliferate and differentiate into various types of endometrial cells, including glandular epithelial, stromal, and endothelial cells in vitro, whereas in the same medium, EMP cells differentiated only into stromal cells. Finally, their result concludes that endometrial SP could have the possibility, more than EMP, to direct in vivo angiogenesis and endometrial cell regeneration: this is consistent with the fact that endometriotic foci show a very high potential of angiogenic factor, related and needed for the worsening of the disease. Another very important study [192] provided evidence that human embryonic stem cells (hESCs) can be differentiated into cells with a human female reproductive tract epithelial cell phenotype. These findings support the hypothesis for which stem/progenitor cells, responsible for tissue regeneration and proliferative disorders of human endometrium, may be derived from Müllerian duct. The idea of the presence of adult stem cells in endometrial tissue pushed Schwab et al. [193] to make clonal analysis of human endometrial epithelial and stromal cells: their results demonstrated that inactive endometrium contains clonogenic epithelial and stromal cells, although this clonogenicity does not vary from the proliferative to secretory stage of the menstrual cycle, or between active cycling and inactive endometrium for both epithelial and stromal cells. Another astonishing results derived from Ikoma et al.'s study [194]: they searched for the presence of donor-derived cells in endometrium from patients who received bone marrow transplantation from male donors. Surprisingly, these donor-derived cells are capable of composing endometrium in recipients, remarking plasticity of bone marrow stem cells as well as a potential origin of endometrial stem cells. Though the reader may think that these results may arise from an isolated study, others confirm these findings [195] or share the same hypothesis [196]. According to Maruyama et al. [197], we think that this endometrial plasticity could derive, at least in part, by the presence of adult stem cell niches in the basal layer of endometrium. For this reason, there is not only the possibility that stem cell activities could play a role in the physiological remodelling and regeneration of the human uterus, but, moreover, that also the pathogenesis of gynaecological diseases such as endometriosis may be linked to stem cell genetic dysregulation, altered cell-to-cell communication, and, finally, misplacement of them.

9. Theories on Aetiopathogenesis of Endometriosis

Pathogenesis of endometriosis still remains controversial: immune, hormonal, genetic, and environmental factors seem to be involved. There are several theories that have been proposed to explain the pathogenesis of endometriosis. According to the Sampson's implantation theory [198], for example, during retrograde menstruation, eutopic endometrial cells reflux throughout the tubes to the peritoneal cavity, adhere to the peritoneal wall, proliferate and form endometriotic lesions, thereby triggering and advancing the disease [2, 3, 199] by the fact that the retrograde transport of endometrial cells was actually shown, as well as by the fact that the sites of greater frequency of the disease are fallopian tubes, ovaries, and pouch of the Douglas, those most easily reached by the refluxed cells. Moreover, there is evidence that nulliparous women and women with heavy and short menses are at higher risk of developing endometriosis [200]: experimental implantation of endometrial debris in peritoneum provokes growth of endometriotic foci in the animal model and

an association between obstructed menstrual outflow and endometriosis [13]. However, this phenomenon could be observed in 90% of endometriosis-free women in reproductive age with pervious fallopian tubes and contrast with the relative low incidence of the disease [201]. Another hypothesis speculates that the endometriotic foci are derived by endometrial cells that enter in the uterine venous circulation and in this way could reach distant sites of implantation, like for example, brain [202], nasal mucosa [203], or spinal intradural [204]. This theory could explain distant sites of endometriosis, but we have to consider that the venous drainage of the uterus arrives to the lungs before to become oxygenated and pass in the arterial circulation: for this reason, according to this theory, we will have to find several cases of lung endometriosis which is not reflected in clinical facts. Another hypothesized way of dissemination of the endometrial cells is the lymphatic: there are in the literature several reports of endometriotic foci in the lymphatic node, but we think that this is not enough to support this theory. It is widely accepted also that endometrial cells could implant and proliferate in surgical scars after caesarean section or laparotomic/laparoscopic surgery [205] or in the episiotomy scars, and moreover the rupture of an endometrioma with leakage of its contents in the course of laparoscopic surgery or laparotomy could provoke dissemination in the peritoneal cavity. According to another theory endometriotic foci derive from peritoneal mesothelial cells of coelomic origin that undergo metaplasia transforming into endometrial cells. This hypothesis would explain the exceptional formation of foci in the bladder and prostate of males. Others, on the contrary, suggest that endometriosis could derive from a displacement of the primitive tissue that gives rise to endometrial cells, caused by incorrect reproductive tract organogenesis (embryonic derivation theory) [172, 206–208]. Other studies postulate that endometriotic cells may derive from "committed" stem cells that under the influence of different factors in the milieu evolve to form foci [191, 196]. Last but not least, basing on animal and experimental investigations, some authors suggest that in the pathogenesis of endometriosis play a role the exposure to environmental toxicants, such as dioxin and dioxin-like, although the mechanism(s) underlying this potential association are poorly understood [201]. Regardless of the correct etiopathogenetic theory, the implant and the proliferation of endometriotic cells seem to depend strictly on the local immune alterations present in the PF and inside of endometriotic cysts.

10. Discussion

As reported before, the elaborate process of female reproductive tract organogenesis is under the control of a wide range of genetic clusters that address spatially and temporally tissue-specific development of the pelvic structures. Among this genetic regulation, a key role is played by Hoxa and Wnt clusters, because they lead the transcription of different genes according to the different phases of the organogenesis, addressing correctly cell-to-cell interactions, allowing, finally, the physiologic morphogenesis. Hoxa and Wnt are important also later in life, because they seem to sustain endometrial plasticity according to hormonal influences and moreover preserve adult stem cells in the endometrial niches. Accumulating evidence [197] is suggesting that this particular type of stem cells is essential for the correct cyclic process of endometrial self-renewal after physiologic menses. Additionally, it was demonstrated that also other sources of stem cells, as for example, bone marrow derived pool [194], could repopulate endometrial niches under proper influences, modifying their genetic expression profile and consequently their cell phenotype and functions. Taking all this, there is the possibility that during organogenesis of Müllerian structures, Hoxa and Wnt alterations could provoke a disturbed development of female reproductive tract system, and for this reason primordial endometrial stem cells may be dislocated and disseminated in ectopic regions. These primordial endometrial stem cells, isolated or more probably organized in foci, could remain misplaced and quiescent until insults of various kinds (physical, chemical, hormonal) provoke expression of silent genes up to that moment, modifying in this way their phenotype, biological functions, and finally also immunogenicity. The discovery of endometriotic foci in fetal age [207, 208], outside the uterine cavity, supports the hypothesis that the disease originates during early organogenesis of the female reproductive tract, and develops in its clinical form in the postpubertal age as a result of hormonal influences. Moreover, Signorile et al.'s data [206] showed the ectopic presence of primitive endometrium, expressing both CA125 and oestrogen receptor, in 11% of female foetuses (4/36). Focusing on this evidence, we have to consider also that endometriosis affects approximately 10% of women in reproductive age [13, 14], and moreover that retrograde menstruation could be observed in 90% of endometriosis-free women in reproductive age with pervious fallopian tubes. Taking all together, all these pieces of evidence allow us to argue that the ectopic presence of primitive endometrium in female foetuses and the presence of endometriotic implants in women of reproductive ages are quite similar. Additionally, the locations of the ectopic primitive endometrium resembled the common locations for endometriosis in women [1, 3]. After certain stimuli, endometriotic cells could proliferate and form the classic ectopic foci and endometrioma, preferentially (but not only) in the peritoneal cavity. It is widely accepted that in the development of endometriosis, a key role is played by alteration in immune peritoneal microenvironment that may provoke failure of the peritoneal cavity scavenging mechanism in removing immunogenic endometriotic cells by macrophages. Like is reported before, modifications in the peritoneal microenvironment attract peripheral mononuclear cells, recruited from the blood into the peritoneal cavity, which secrete different pattern of cytokines, driving the following event of the disease. Once the endometriotic foci are established, in fact, the strict interaction between endometriotic and immune cells addresses toward a prevalence of Th1 profile cytokines in the PF at minimal and mild stages, whereas Th2 profile cytokines prevailed in severe stages [31]. Moreover, in the progression of the disease a key role could be played by impaired ratio of Th17 and Tregs populations [78–82].

11. Conclusion

Dysregulation of Wnt and/or Hox genes may affect cell migration during organogenesis and differentiation of Müllerian structures of the female reproductive tract, with possible dislocation and dissemination of primordial endometrial stem cells in ectopic regions, which have high plasticity to differentiation. We hypothesize that during postpubertal age, under the influence of different stimuli, these misplaced and quiescent endometriotic cells could acquire new phenotype, biological functions, and immunogenicity. So, these kinds of cells may differentiate, specializing in epithelium, glands, and stroma to form a functional ectopic endometrial tissue. This may provoke a breakdown in the peritoneal cavity homeostasis, with the consequent processes of immune alteration documented by peripheral mononuclear cells recruitment and secretion of inflammatory cytokines in early phases and of angiogenic and fibrogenic cytokines in the late stages of the disease.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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Review Article

Angiogenesis and Endometriosis

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A comprehensive review was performed to survey the role of angiogenesis in the pathogenesis of endometriosis. This is a multifactorial disease in which the development and maintenance of endometriotic implants depend on their invasive capacity and angiogenic potential. The peritoneal fluid of patients with endometriosis is a complex suspension carrying inflammatory cytokines, growth factors, steroid hormones, proangiogenic factors, macrophages, and endometrial and red blood cells. These cells and their signaling products concur to promote the spreading of new blood vessels at the endometriotic lesions and surroundings, which contributes to the endometriotic implant survival. Experimental studies of several antiangiogenic agents demonstrated the regression of endometriotic lesions by reducing their blood supply. Further studies are necessary before these novel agents can be introduced into clinical practice, in particular the establishment of the safety of anti-angiogenic medications in women who are seeking to become pregnant.

1. Introduction

Endometriosis is a benign sex hormone-dependent gynecological disease, characterized by the presence and growth of endometrial tissue outside the uterus; it affects 10% of women of reproductive age and is associated with infertility and pain [1, 2]. The symptoms can impact on general physical, mental, and social well-being [3]. Despite many investigations about endometriosis, the pathogenesis of the disease remains unclear [3]. The disease derives from retrograde menstruation of endometrial cells which implant on peritoneal surfaces and induce an inflammatory response. The success of the ectopic implants depends on other pathological processes such as neoangiogenesis, fibrosis, adhesion formation, avoidance of apoptosis, immune dysfunction, and neuronal infiltration [1, 2, 4–7].

During normal reproduction, cyclic angiogenesis is orchestrated by the endocrine system, providing physiological signals for follicular maturation, corpus luteum function, endometrial growth, and remodeling [8]. Endometriosis is a multifactorial disease in which angiogenesis also plays an important role [9–13]. The angiogenic potential of both the endometrium and the peritoneal environment influences lesion establishment [9–12]. Indeed, endometriotic lesions require an adequate blood supply to survive in their ectopic sites.

The goals of endometriosis treatment alternate between alleviation of pelvic pain and successful achievement of pregnancy in infertile patients. Antiangiogenic drugs hold a promise for both indications and present a distinct perspective in endometriosis treatment.

The aim of this paper is to review the literature evidence of the important role of angiogenesis in the pathogenesis of endometriosis and to establish the rationale for anti-angiogenic agents as a new therapeutic option in the treatment of endometriosis patients.

2. Methods

2.1. Search Strategy. A literature search was performed to survey the role of angiogenesis in the pathogenesis of endometriosis. Articles were identified through the following electronic databases: MEDLINE (until January 2013) and

the Cochrane Central Register of Controlled Trials (The Cochrane Library until January 2013). A combination of Medical Subject Headings (MeSH) and text words was used to generate the list of citations: (endometriosis OR "endometriotic lesions") AND (angiogenesis OR "angiogenic factors" OR vasculogenesis OR "antiangiogenic drugs"). All pertinent articles were examined and their reference lists were reviewed in order to identify other studies for potential inclusion in this review. No institutional review board approval was required because only published data were analyzed.

2.2. Selection Criteria. Randomized controlled trials (RCTs), patient preference trials, observational studies, case reports, and proceedings of scientific meetings were included in this review, whereas abstracts were excluded. Only publications in English were considered in our selection. The abstracts of studies identified in the search were reviewed to exclude irrelevant or repeat citations. The reviewers were not blinded to the names of investigators or sources of publication.

3. Results

3.1. Angiogenesis in Endometrium and in Endometriotic Implants. Endometriotic lesions are typically characterized by a dense vascularization that occurs through angiogenesis process [1, 9, 14]. In normal eutopic (intrauterine) endometrium, it has been suggested that vessel elongation, rather than branch point sprouting, is the primary mechanism for rapid vessel growth during the proliferative phase [15], but the precise mechanism in endometriosis lesions has not been evaluated to date. Recruitment of new capillaries from existing, adjacent peritoneal microvessels was postulated [10]; however, the derivation of new blood vessels from circulating endothelial progenitor cells (EPCs), the so-called "vasculogenesis," also appears to be important in the pathogenesis of endometriosis [14]. The endometrium is a dynamic tissue exhibiting populations of clonogenic epithelial and stromal stem cells [16–18] that require active cyclic angiogenesis. Bone-marrow-derived EPCs can be detected in developing endometriotic lesions [19] and those lesions show increased expression of factors and chemokines that participate in EPC recruitment, such as hypoxia-inducible-factor- (HIF-) 1α and stromal-cell-derived-factor- (SDF-) 1 [14, 20]. Moreover, the presence of hypoxia, endothelial injury, and inflammation and the expression of ER-α contribute to the mobilization and recruitment of EPCs from the bone marrow into endometriotic lesions [14, 21–27].

Endometriotic lesions can produce cytokines and growth factors that regulate their proliferation and vascularization. Interleukin- (IL-) 1β, the dominant IL-1 secreted by activated peritoneal macrophages, plays an important role in the neovascularization of endometriotic lesions [28, 29]. Cultured human endometrial stromal cells (HESC) from women with endometriosis secrete IL-6 and IL-8 robustly [30]. IL-6 is a potent multifunctional protein, which promotes endometrial cell proliferation [31] and angiogenesis [32]; its secretion is elevated in ectopic endometrial tissue and its concentrations are high in peritoneal fluid of patients with endometriosis [33]. IL-8 is a proinflammatory cytokine that induces chemotaxis of neutrophils and has a potent stimulatory effect on angiogenesis [34, 35].

Activin A is a growth factor member of the transforming growth factor β superfamily with effects on inflammation and angiogenesis [36–38]. The human endometrium is both a source and a target of activin A, which is able to modulate the expression and secretion of IL-8 and vascular endothelial growth factor (VEGF), from human endometrial stromal cells [39]. VEGF is among the most potent and specific angiogenic factors. Its effects include endothelial cell proliferation, migration, organization into tubules, and enhanced permeability, all of which participate in the angiogenic cascade [40]. Endometrial VEGF expression is enhanced by estradiol and its concentrations are correlated with neovascularization and increased vascular permeability during late proliferative phase [41]. Cyclic changes in VEGF expression throughout menstrual cycle are observed with maximal expression during the secretory phase and menstruation [9, 41, 42]. VEGF was observed in the epithelium and in stromal cells of endometriotic implants, being more expressed in the epithelium [18, 42]. Moreover, endometriotic cells can synthesize and secrete VEGF [42].

Activated peritoneal macrophages and neutrophils also have the capacity to produce and secrete VEGF [18, 43, 44]. Some studies demonstrated that the expression and concentration of VEGF are increased in tissue from endometriotic patients [45–49]. Endometriomas and red implants show the highest concentrations of VEGF [45, 46]. The expression and secretion of VEGF from human endometrial stromal cells are modulated by activin A [30].

3.2. Peritoneal Fluid from Patients with Endometriosis. The peritoneal fluid of patients with endometriosis is a complex suspension carrying inflammatory cytokines, growth factors, steroid hormones, proangiogenic factors, macrophages, and endometrial and red blood cells [42, 43, 50–52]. Leukocytes circulating in the peritoneal fluid of patients can produce and release high amounts of VEGF [18, 43, 44]. Moreover, the peritoneal fluid concentrations of VEGF in patients with endometriosis correlate with the stage of the disease [42]. Other proangiogenic factors, namely, IL-8 [30, 53–56], hepatocyte growth factor (HGF) [57, 58], erythropoietin [59], angiogenin [60], macrophage migration inhibitory factor [61], neutrophil-activating factor [62], and TNF-α [63, 64], are all found at increased concentrations in the peritoneal fluid of patients with endometriosis. This proangiogenic milieu is reinforced by reduced concentrations of antiangiogenic factors, such as adiponectin [65] and interferon-gamma-induced protein 10 (IP-10) [66, 67], although levels of the endogenous VEGF antagonist soluble Flt-1 were reported to be increased in the pelvic fluid of endometriosis cases [68].

3.3. Agents with Antiangiogenic Properties. As one of the most potent angiogenic factors, VEGF is postulated to be involved in the progress of the ectopic lesions in endometriosis [22, 67]. Vascularization and VEGF and its

Table 1: Antiangiogenic agents.

	Antiangiogenic agents	Functional activity (*in vivo* and *in vitro* studies)
VEGF blockers and inhibitors	Soluble truncated VEGF receptors (Flt-1)	Inhibited the growth of human endometrium in mice
	Anti-human VEGF antibody	Inhibited the growth of human endometrium and decreased the number of endometriotic lesions
	TNP-470 (lodamin)	Inhibited the number of endometriosis lesions, suppressed the mobilization of circulating endothelial cells and endothelial progenitor cells
	Endostatin and anginex	Inhibited the number of endometriosis lesions
	Bevacizumab (recombinant humanized monoclonal antibody that inhibits VEGF)	Inhibited the development and cell proliferation in endometriotic lesions, reduced vascular density, increased apoptosis, and reduced VEGF levels
	Sorafenib (an orally active multikinase inhibitor)	Interfered with the activity of the VEGF receptor reducing the microvessel density and lesion volume of endometrial implants
	Romidepsin (a histone deacetylase inhibitor)	Inhibited VEGF gene transcription, protein expression and secretion of VEGF
	Lipoxin A4 (LXA4, an endogenous eicosanoid)	Reduced the endometriosis lesion size and downregulated inflammation-associated proteins, including IL-6 VEGF and matrix metalloproteinase 9
	4-Hydroxybenzyl alcohol (HBA, a naturally occurring phenolic compound)	Inhibited the initiation of the angiogenic process by downregulating VEGF and matrix-metalloproteinase-(MMP-) 9 expression and by affecting endothelial cell migration
	Parecoxib (selective COX-2 inhibitor)	Reduced lesion size, microvessel density, the number of macrophages, and the expression of VEGF
	Epigallocatechin gallate (major constituent of green tea)	Decreased endometriotic lesion size, microvessel diameter and density, and VEGF mRNA expression
	SU6668	Suppressed angiogenesis and vessel maturation in endometriotic lesions.
	Macrophage migration inhibitory factor (MIF) antagonist	Reduced the expression of VEGF, cell adhesion receptors, MMP-2, MMP-9, IL-8, cyclooxygenase (COX)2
Other anti-angiogenic agents	Xanthohumol (a prenylated flavonoid)	Inhibited the formation of new blood vessels
	Rapamycin (an immunosuppressant drug)	Inhibited neovascularization and cell proliferation
	Retinoic acid	Decreased the volume of endometriotic implants
	Progestogens (progesterone, dydrogesterone, or its metabolite dihydrodydrogesterone)	Reduced proliferation of endometrial stromal cells and suppressed the transcription of VEGF-A and the microvessel density
	Statins (atorvastatin, lovastatin)	Inhibited the inflammatory and angiogenic genes COX-2 and VEGF in endometriotic stromal cells
	Dopamine agonists	Reduced microvessel density and angiogenic gene expression

receptor expression are particularly high in deeply infiltrating endometriosis, supporting the hypothesis that antiangiogenic therapy (Table 1) could represent a new and promising modality of treatment of this symptomatic disease manifestation [13]. Classic treatments of endometriosis rely on the use of hormonal drugs with undesirable menopausal side effects or surgery, with its risks of complications, frequent recurrence, and common need for adjuvant medical therapy. New agents, like antiangiogenic factors, offer a different perspective in endometriosis therapy, but their development will necessitate the monitoring of potential side effects.

3.4. VEGF Blockers and Inhibitors. Soluble truncated VEGF receptors (Flt-1) and affinity-purified goat antibodies to human VEGF-A inhibited the growth of human endometrium fragments implanted into nude mice [69]. In similar studies, treatment with anti-human VEGF antibody resulted in a significant decrease in the number of lesions of endometriosis in the nude mouse model [70]. The angiogenesis inhibitors TNP-470, endostatin, and anginex inhibited the number of endometriosis lesions present in a mice model [70]. Lodamin, an oral nontoxic formulation of TNP-470, suppressed the mobilization of circulating endothelial cells and endothelial progenitor cells and inhibited the growth of endometriotic lesion in a mouse model of endometriosis, demonstrating a potential clinical use of antiangiogenic therapy for endometriosis [19].

Bevacizumab, a full-length recombinant humanized monoclonal antibody that inhibits VEGF, inhibited the

development and cell proliferation in endometriotic lesions, reduced vascular density, increased apoptosis, and reduced VEGF levels in peritoneal fluid in a murine model of endometriosis [71]. Bevacizumab reduced the volume of endometriotic implants but did not show any detrimental effect on ovarian reserve in a rat model of induced endometriosis [72].

Sorafenib, another anti-angiogenic agent, is an orally active multikinase inhibitor that interferes with the activity of the VEGF receptor, along with other tyrosine kinase receptors. This drug reduced the microvessel density and lesion volume of endometrial implants in a rat model of induced endometriosis [72].

Hypoacetylation of histone H4 is associated with downregulation of the p53 and von Hippel-Lindau proteins and the upregulation of HIF-1α. All three effects promote VEGF gene expression [73]. Romidepsin, a histone deacetylase inhibitor, may be a potential therapeutic candidate against angiogenesis in endometriosis. This agent inhibited VEGF gene transcription, protein expression, and secretion of VEGF in an *in vitro* study with human immortalized epithelial endometriotic cells [74].

Lipoxin A4 (LXA4) is an endogenous eicosanoid which participates in the regulation of inflammation. This lipid can block migration of endothelial cells and VEGF-stimulated angiogenesis [75]. In endometriosis induced in BALB/c mice, LXA4 reduced the endometriosis lesion size and downregulated inflammation-associated proteins, including IL-6 VEGF and matrix metalloproteinase 9 [76]. 4-Hydroxybenzyl alcohol (HBA) is a naturally occurring phenolic compound, found in many plants, including carrots [77]. HBA exhibits an anti-inflammatory activity and the development of new blood vessels [78]. HBA inhibited the initiation of the angiogenic process by downregulating VEGF and matrix-metalloproteinase-(MMP-) 9 expression and by affecting endothelial cell migration *in vitro* and *in vivo* [78, 79].

Parecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, reduced lesion size, microvessel density, the number of macrophages, and the expression of VEGF and led to atrophy and regression of endometrial implants in a rat model of peritoneal endometriosis [80].

The major constituent of green tea, *Epigallocatechin gallate*, also appears to have antiangiogenic properties since its use decreased endometriotic lesion size, microvessel diameter and density, and VEGF mRNA expression in an experimental SCID mouse model of endometriosis [81]. Moreover, this extract from green tea increased apoptosis in the endometriotic lesions [81]. Another study confirmed that *Epigallocatechin gallate* blocked VEGF expression of hamster endometrial cells *in vitro* and inhibited angiogenesis and blood perfusion of endometriotic lesions *in vivo*, inducing regression of the endometriotic lesions [82]. These antiangiogenic and proapoptotic proprieties of green tea suggest that it might be used as a complementary treatment in endometriosis, but its potential benefit remains to be evaluated in clinical trials. Combined inhibition of VEGF, fibroblast growth factor, and platelet-derived growth factor by inhibitor SU6668 suppresses angiogenesis and vessel maturation in endometriotic lesions in an animal model [22].

Macrophage migration inhibitory factor (MIF), which is markedly upregulated in active endometriosis lesions [83], also contributes to angiogenesis. An MIF antagonist suppressed the development of endometriotic lesions *in vivo* reducing the expression of VEGF, cell adhesion receptors, MMP-2, MMP-9, IL-8, and cyclooxygenase- (COX-) 2. Moreover, MIF antagonist demonstrated a proapoptotic action in the nude mouse model [84].

3.5. Other Antiangiogenic Agents. Retinoic acid, known to have anti-angiogenic proprieties, decreased the volume of endometriotic implants in mouse [85] and rat [72] models of induced endometriosis. Xanthohumol, a prenylated flavonoid isolated from hops, demonstrated the capacity to inhibit the formation of new blood vessels in developing peritoneal and mesenteric endometriotic lesions which were surgically induced in BALB/c mice, without affecting the histomorphology of the uterus or ovary [86]. Rapamycin, an immunosuppressant drug with antiangiogenic effects, induced regression of endometriotic lesions by inhibiting neovascularization and cell proliferation in an *in vitro* model [87].

Progestogens (progesterone, dydrogesterone, or its metabolite dihydrodydrogesterone) reduced proliferation of endometrial stromal cells and suppressed the transcription of VEGF-A and the microvessel density in human ectopic endometrial lesions in a mouse model, regulating important-factors for the establishment of ectopic lesions [88]. Dienogest reduced IL-1β production from peritoneal macrophages and implant volume in a rat model of endometriosis [89].

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase with intrinsic antioxidant, anti-inflammatory, and anti-angiogenic properties [90]. Atorvastatin inhibited the inflammatory and angiogenic genes COX-2 and VEGF in endometriotic stromal cells [91]. Cell proliferation and angiogenesis were inhibited by lovastatin in a dose-dependent manner in a three-dimensional *in vitro* model of endometrium [92].

The dopamine agonist cabergoline exerts antiangiogenic effects through VEGFR-2 inactivation inhibiting the growth of established endometriosis lesions [93]. Moreover, cabergoline treatment results in a significantly lower expression of VEGF and VEGFR-2 in endometriotic lesions [94]. Quinagolide, binding to dopamine D2 receptor, downregulated VEGF/VEGFR2, inhibited neoangiogenesis, and reduced the size of active endometriotic lesions [95].

4. Conclusion

A comprehensive synthesis of the complex pathogenesis of endometriosis remains elusive, but we know that this is a multifactorial disease in which the development and maintenance of endometriotic implants depend on their invasive capacity and angiogenic potential (Figure 1).

As angiogenesis represents a critical step in the establishment and pathogenesis of endometriosis, this process has been viewed as a potential new target for therapeutic intervention. In this review, experimental studies of

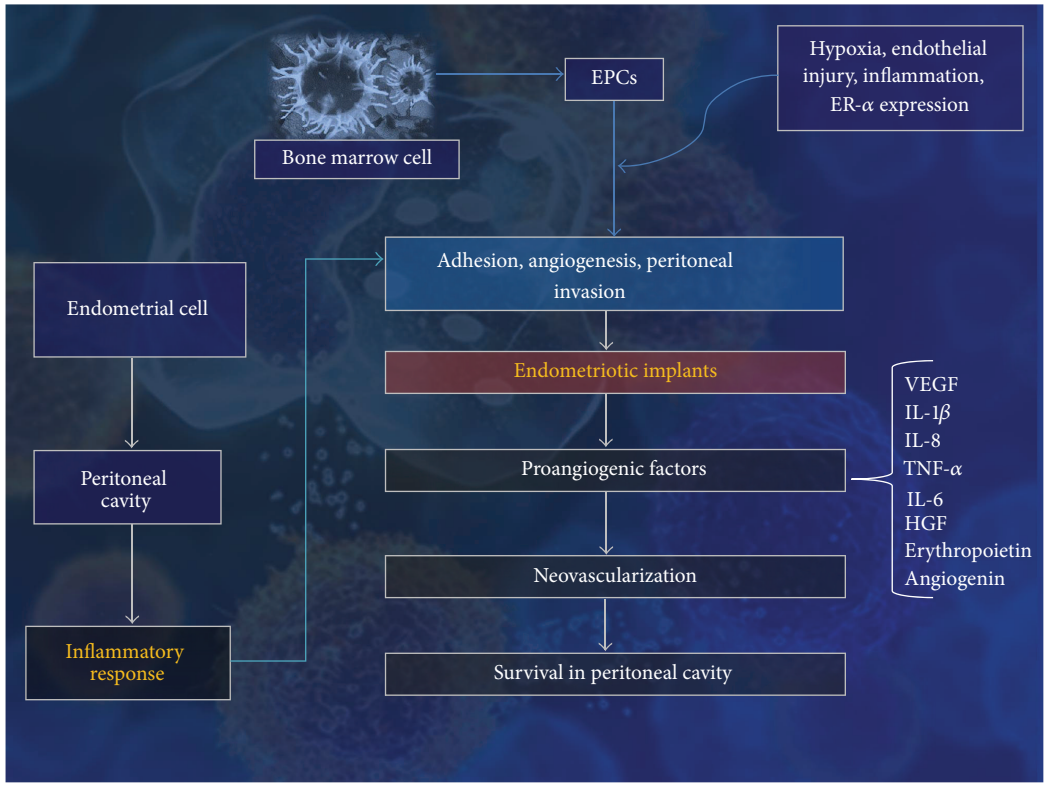

Figure 1: Angiogenesis in the pathogenesis of endometriosis. EPCs, endothelial progenitor cells; VEGF, vascular endothelial growth factor; IL, interleukin; TNF, tumor necrosis factor; HGF, hepatocyte growth factor.

several anti-angiogenic agents demonstrated the regression of endometriotic lesions by reducing their blood supply (Table 1). Further studies are necessary before these novel agents can be introduced into clinical practice, in particular the establishment of the safety of anti-angiogenic medications in women who are seeking to become pregnant. Precautions such as those instituted for the prescription of retinoic acid should be considered to avoid the possible consequences of impaired blood vessel formation to the developing embryo and placenta. With this provision, anti-angiogenic treatments offer novel perspectives and mechanisms and promise more effective adjuvant therapies for patients with endometriosis.

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Clinical Study

Gene Expression of Leptin and Long Leptin Receptor Isoform in Endometriosis: A Case-Control Study

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In this study, leptin/BMI ratio in serum and peritoneal fluid and gene expression of leptin and long form leptin receptor (OB-R_L) were assessed in eutopic and ectopic endometria of women with endometriosis and controls. Increased serum leptin/BMI ratio was found in endometriosis patients. Leptin and OB-R_L gene expression was significantly higher in ectopic versus eutopic endometrium of patients and controls. A positive, significant correlation was observed between leptin and OB-R_L transcripts in ectopic endometria and also in eutopic endometria in endometriosis and control groups. A negative and significant correlation was found between OB-R_L mRNA expression and peritoneal fluid leptin/BMI ratio only in endometriosis. These data suggest that, through a modulatory interaction with its active receptor, leptin might play a role in the development of endometrial implants.

1. Introduction

Endometriosis is defined as the presence of endometrial glands and/or stroma outside the uterus. In women with endometriosis, the eutopic endometrium has specific characteristics that favor tissue survival, adhesion, and growth outside the uterine cavity. Several studies have demonstrated that endometriosis is associated with abnormal peritoneal and endometrial production of proinflammatory cytokines and growth and angiogenic factors [1, 2].

Leptin, a hormone produced mainly by adipocytes, is expressed in endometrium [3] and has been implicated in the regulation of sex hormone production, ovulation, endometrial cell physiology, and early embryo development and implantation [4]. It may also play a role in endometriosis through its inflammatory and angiogenic properties. Nevertheless, studies evaluating serum and peritoneal fluid (PF) levels of leptin in patients with endometriosis report conflicting results: some describe increased levels [2, 5–10], while others report no differences between patients with endometriosis and controls [7, 11–14]. Moreover, the possibility of an association between PF leptin levels and severity of endometriosis is also controversial, with some studies suggesting a negative correlation [2, 6, 8] and others showing a positive correlation with more severe forms of peritoneal endometriosis [5, 7, 13, 15].

Interestingly, only a few studies so far have evaluated leptin receptor gene and/or protein expression in endometrial tissue of women with endometriosis [16–18]. Lima-Couy et al. [16] evaluated the three isoforms of leptin receptor—total (OB-R_T), long (OB-R_L), and short (HuB219.3)—in the eutopic endometrium of patients with moderate and severe

endometriosis. Those authors observed increased receptor expression in the period corresponding to embryo implantation, with no difference between patients and controls. Some authors [17, 19] have reported expression of leptin receptor in both eutopic and ectopic endometria.

Therefore, the aims of the present study were (a) to assess leptin and OB-R_L gene expression in ectopic and eutopic endometria of women with endometriosis and in eutopic endometrium of non-endometriosis controls, (b) to determine the leptin/BMI ratio in serum and PF in both groups, (c) to assess the immunoreactive presence of OB-R_L in endometrium and endometriotic implants, and (d) to investigate the relationship among these variables.

2. Materials and Methods

2.1. Subjects. The sample was selected among patients undergoing gynecological laparoscopy for infertility, pelvic pain, ovarian pathology, or tubal ligation (TL), between September 2007 and March 2009. Twenty-eight women with pelvic endometriosis and 17 women without laparoscopically proven endometriosis or other pelvic pathology were consecutively selected from this group. Infertility was defined as inability to achieve pregnancy after one year of unprotected sexual intercourse. Chronic pelvic pain was defined as noncyclical pelvic pain of sufficient severity to cause functional disability or lead to medical care, lasting six months or longer (American College of Obstetricians and Gynecologists). Endometriosis was confirmed by histology in all patients with suspected lesions at laparoscopy. Endometriosis was classified according to the revised classification of the American Society of Reproductive Medicine [20]. Peritoneal endometriotic lesions were observed in all patients, and superficial ovarian endometrioma was also found in two of them.

Inclusion criteria were (i) premenopausal status, (ii) need for laparoscopy, and (iii) no use of hormonal medication in the previous three months. The sole exclusion criterion was body mass index (BMI) above 35. Participants presented neither metabolic comorbidities, such as diabetes, dyslipidemia, abnormal renal, or hepatic function, nor clinical evidence of systemic diseases or pelvic inflammatory disease. The study protocol was approved by the Research Ethics Committee at Hospital de Clinicas de Porto Alegre (IRB-equivalent), and written informed consent was obtained from all subjects.

2.2. Study Protocol. All participants underwent physical examination, including measurement of height and weight and estimation of BMI. Laparoscopy with biopsy of endometriotic implants and a concomitant biopsy of the eutopic endometrium were performed preferentially in the second half of the menstrual cycle. However, in about 20% of participants, laparoscopy and biopsy were performed in the proliferative phase. A single sample of superficial peritoneal endometriotic tissue was obtained from the largest lesion. Subcutaneous adipose tissue samples (approximately 1 cc) were also collected from the periumbilical region before the end of the procedure. Eutopic endometrial samples were collected using curettage. The same surgeon performed all laparoscopic evaluations (AN).

Table 1: Characteristics of women with endometriosis and normal pelvis controls.

	Endometriosis	Controls
N	28	17
Age (years)[a]	32 ± 7	33 ± 5
BMI (kg/m^2)[a]	25.6 ± 4.5	25.2 ± 3.5
Estradiol (pg/mL)		
Proliferative	122 (37–160) (n = 5)	64 (18–144) (n = 4)
Secretory	104 (56–226) (n = 23)	91 (55–132) (n = 13)
Progesterone (ng/mL)		
Proliferative	0.2 (0.18–0.79) (n = 5)	0.42 (0.21–0.86) (n = 4)
Secretory	3.6 (0.56–11.25) (n = 23)	4.15 (0.59–9.1) (n = 13)

[a] Age and BMI are expressed as mean ± SD.
No significant difference in age, BMI, estradiol, and progesterone between endometriosis and control groups (Student's t-test).
BMI: body mass index.

Peripheral venous blood samples were collected immediately before anesthetic induction for laparoscopy. PF samples were collected from the Douglas pouch immediately after the start of the procedure. All samples were kept on ice for transport to the laboratory and stored in aliquots at −80°C until assayed. Endometriotic and endometrial samples were fractioned: one portion was immediately frozen in liquid nitrogen and stored at −80°C until mRNA extraction, while the other was fixed in 10% buffered formalin and embedded in paraffin for subsequent histological diagnosis and immunohistochemistry, as previously described [21].

Based on histological and laparoscopic findings, three types of tissue were studied: (1) eutopic endometrium from nonendometriosis controls, (2) eutopic endometrium from patients with endometriosis, and (3) ectopic endometrium from patients with endometriosis.

2.3. Serum and Peritoneal Fluid Measurements. Serum estradiol and progesterone concentrations were assayed by electrochemiluminescence (Roche Diagnostic, Mannheim, Germany). Serum and peritoneal leptin levels were determined using a Human Leptin ELISA kit (LINCO Research, St. Charles, MO, USA).

2.4. RNA Isolation. Endometrial and adipose tissue total RNA extraction was carried out in phenol/guanidine isothiocyanate (Trizol, Invitrogen Life Technologies, Foster City, CA, USA) as previously described [21, 22]. Concentration and quality of total RNA were assessed using a GeneQuant spectrophotometer (Pharmacia Biotech, Cambridge, England).

2.5. Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Protocol. Reverse transcription of 1 μg of

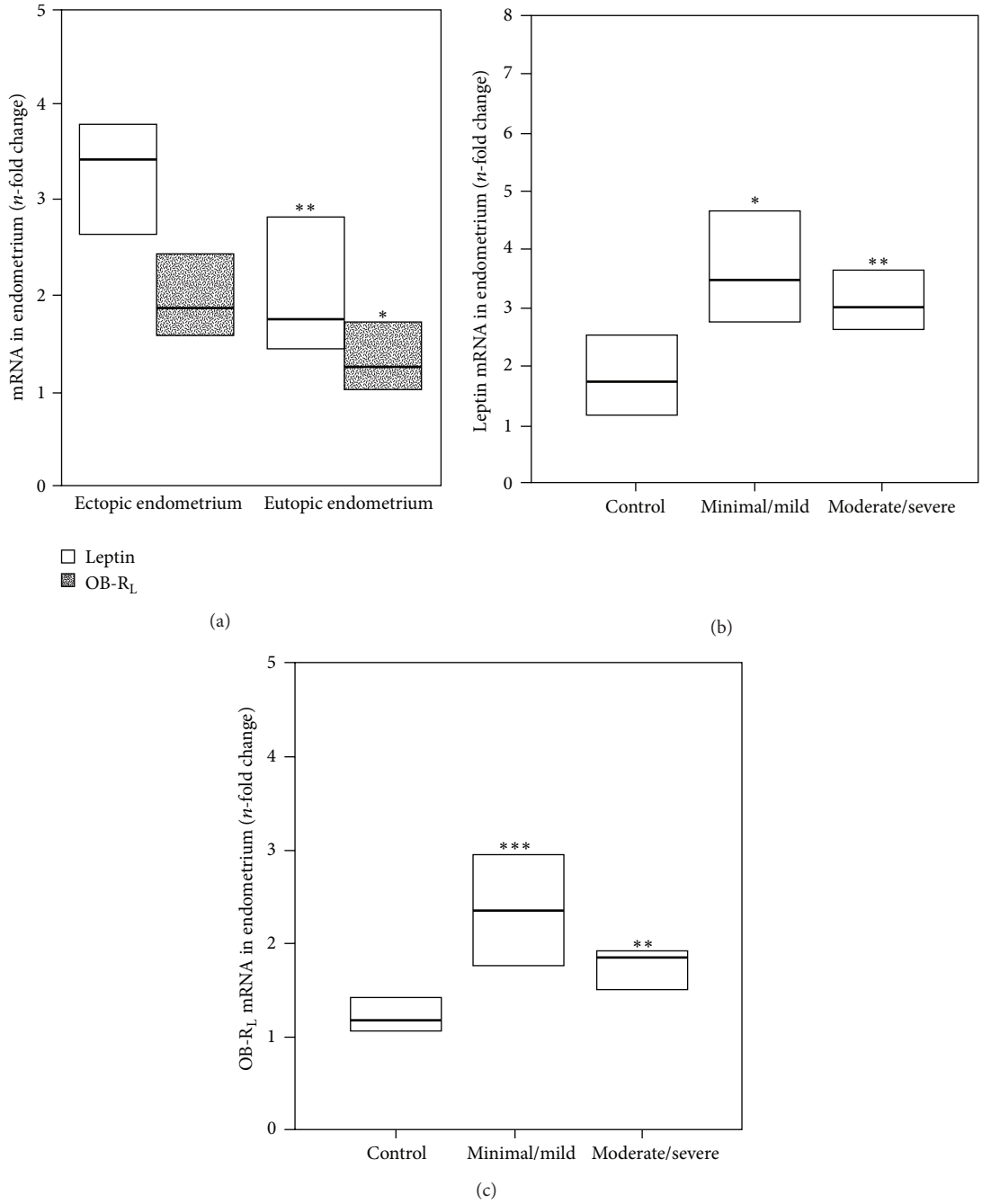
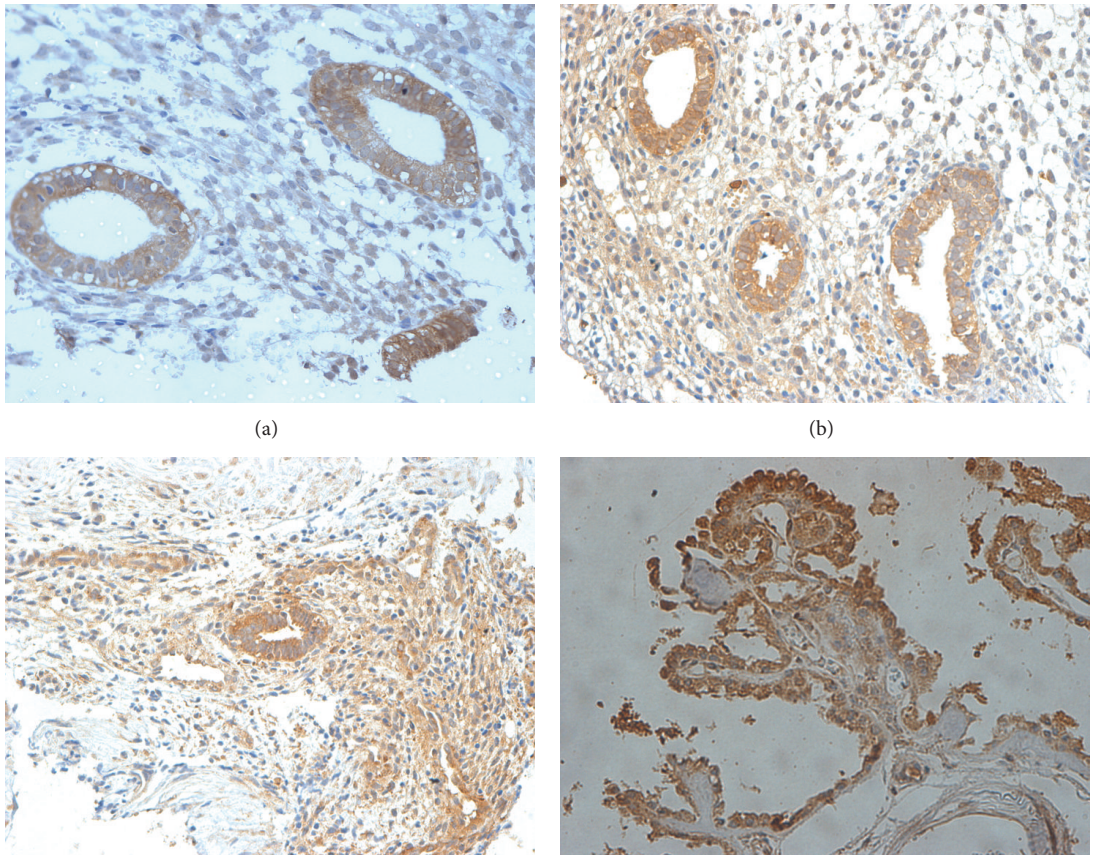


Figure 1: Leptin and long leptin receptor isoform (OB-R_L) gene expression in endometriosis. (a) Ectopic endometrium and eutopic endometrium of patients with endometriosis. Values are expressed as *n*-fold difference in relation to the calibrator sample (ΔΔCt method). $^{**}P < 0.001$ (leptin mRNA ectopic versus eutopic endometrium); $^{*}P < 0.05$ (OB-R_L mRNA ectopic versus eutopic endometrium) (Wilcoxon signed-rank test). (b) Leptin gene expression and (c) long leptin receptor isoform (OB-R_L) in eutopic endometrium of non-endometriosis controls and ectopic endometrium of minimal/mild and moderate/severe endometriosis. Values are expressed as n-fold difference in relation to the calibrator sample (ΔΔCt method). Asterisks indicate significant difference in comparison to controls. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ (Mann-Whitney *U*).

total RNA into cDNA was carried out using the Superscript II First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Foster City, CA, USA), according to the manufacturer's instructions, in a PCT-100 Programmable Thermal Controller (MJ Research Inc., Watham, MA, USA).

Real-time PCR was performed in triplicate in a 7500 Fast real-time PCR System thermal cycler with 7500 Fast System Sequence Detection 1.4 Software (Applied Biosystems, Foster City, CA, USA). Experiments were performed by monitoring in real time the increase in fluorescence of the SYBR Green dye as previously described [23–25]. Primers



(a) (b) (c) (d)

Figure 2: Immunostaining of long leptin receptor isoform (OB-R_L) in eutopic endometrium. (a) Non-endometriosis controls, (b) eutopic and (c) ectopic endometria of patients with endometriosis, and (d) positive control (choroid plexus). Original magnification: 400x.

were designed by Primer Express 3.0 Software for real-time PCR (Applied Biosystems, Foster City, CA, USA) and acquired from Invitrogen (Life Technologies, Foster City, CA, USA). Primer sequences were designed to target two exons of an mRNA sequence with respect to known splice variants and single-nucleotide polymorphism positions. The forward and reverse primer sequences designed for leptin (NM_000230.2) were (5′ to 3′) TCCCCTCTTGACCCATCTC and GGGAACCTTGTTCTGGTCAT, respectively. These primers anneal between residues 858 to 876 (forward) and 967 to 948 (reverse), producing a PCR product of 110 bp. The forward and reverse primer sequences for leptin receptor (NM_001003679.2) were (5′ to 3′) AGGAAGCCCGAAGTTGTGTT and TCTGGTCCCGTCAATCTGA, respectively. These primers anneal between residues 3,617 to 3,636 (forward) and 3,716 to 3,698 (reverse), resulting in an amplicon of 100 bp. Beta-2 microglobulin (NM_004048.2) was used to normalize mRNA quantitation. CTATCCAGCGTACTCCAAAG and ACAAGTCTGAATGCTCCACT (5′ to 3′) forward and reverse B2M primer sequences anneal between residues 119 to 138 (forward) and 283 to 264 (reverse), resulting in an amplicon of 165 bp. cDNA samples (1.0 ng/μL) were mixed with a predetermined forward and reverse primer volume (0.9 and 0.7 μL for leptin, 0.9 and 0.3 μL for leptin receptor, and 0.7 and 0.9 μL for B2M) and 12.5 μL of 2X Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a total of 25 μL. Protocol conditions consisted of denaturation at 94°C for two minutes followed by 50 cycles (30 s at 94°C and 30 s at 60°C). Primers generated amplicons that produced a single sharp peak during melting curve analysis. Data were analyzed by relative quantitation using the comparative C_T method [26]. Validation assays for endometrium and adipose tissue were performed by amplification of the target and reference genes, separately, using serial dilutions of an mRNA sample. Both target and reference mRNAs exhibited equal amplification efficiency. The $\Delta\Delta C_T$ method calculates changes in gene expression as relative fold difference between an experimental and calibrator sample, correcting nonideal amplification efficiencies [27].

2.6. Immunohistochemistry. Formalin-fixed, paraffin-embedded endometrial samples were cut into 5 μm slices, which were stained by immunohistochemistry using the avidin-biotin-peroxidase method [28], as previously described [29, 30]. Following deparaffinization and rehydration with a

Table 2: Serum and peritoneal fluid leptin/BMI ratio according to stage of endometriosis.

	Controls	Endometriosis	P	rASRM		P^a
				Stage I/II	Stage III/IV	
Serum leptin/BMI	0.41 (0.22–0.71) $n = 17$	0.61 (0.41–0.95) $n = 28$	0.04	0.56 (0.28–0.99) $n = 13$	0.78 (0.43–0.96) $n = 15$	0.08
Peritoneal fluid leptin/BMI	0.44 (0.28–0.73) $n = 17$	0.7 (0.45–1.18) $n = 23$	0.07	0.54 (0.28–1.36) $n = 12$	0.71 (0.59–1.15) $n = 11$	0.12

Data are presented as median and interquartile range (Mann-Whitney).
[a]Controls versus rASRM stage I/II versus rASRM stage III/IV (Kruskal-Wallis).
rASRM stages: revised American Society for Reproductive Medicine classification (15).

graded series of ethanol, immunohistochemistry was performed using peroxidase reaction. Sections were incubated with 3% H_2O_2 at room temperature for five minutes in order to suppress endogenous peroxidase activity. The samples were then incubated in a humidity chamber at room temperature for 30 minutes with a 0.5 μg/mL goat antihuman leptin receptor antibody C20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (w/v) replaced the primary antibody in negative controls. Samples of the choroid plexus were used as positive controls. After 20-minute incubation with the linker, streptavidin-peroxidase was used for five minutes to stain the slices. Subsequent to each incubation step, the tissues were washed three times with PBS 50 mM Tris–HCl buffer. Slices were counterstained with Mayer's hematoxylin and mounted. A positive reaction was characterized by the presence of granular brown staining in the cytoplasm. The intensity of immunostaining in epithelium and stroma was evaluated by two independent observers and classified as negative, weak, moderate, or intense and converted into arbitrary units on a semiquantitative scale of 0 to 3 [31].

2.7. Statistical Analysis. The sample size for detecting significant differences in serum leptin levels was estimated as 12 women with endometriosis and 12 controls, based on the study by Matarese et al. [2] and considering a power of 80% and alpha of 5%. Data are presented as mean ± SD or median and interquartile range. Comparisons between group means were analyzed by Student's t-test. Median values were compared using the Mann-Whitney U test. Comparisons of median values involving three groups were analyzed using the Kruskal-Wallis test. The chi-square test was used to compare qualitative variables. Pearson's rank or Spearman's correlation coefficients were calculated using a two-tailed significance test for variables with a Gaussian or non-Gaussian distribution, respectively. The nonparametric Wilcoxon signed-rank test was used if required based on the number of subjects and the nonhomogeneous features of each group. The gamma test was used for qualitative comparison of more than two groups.

All analyses were performed using the Statistical Package for the Social Sciences 16 (SPSS, Chicago, IL, USA). Data were considered significant at $P < 0.05$.

3. Results

Participants age ranged from 21 to 50 years. In one nonendometriosis control, endometrial biopsy was of insufficient quality for interpretation. Therefore, the results of gene expression and immunohistochemistry refer to data from 28 patients with endometriosis and 16 controls.

Endometriosis was classified as stage I (minimal) in 13 patients, stage III (moderate) in six, and stage IV (severe) in nine. Laparoscopy was performed for infertility investigation in 15 patients (33.3%), tubal ligation in 13 (28%), chronic pelvic pain in nine (20%), adnexal pathology in five (11%), association of infertility and chronic pelvic pain in two (4.5%), and association of tubal ligation and chronic pelvic pain in one (2.2%) patient. Table 1 presents the clinical profile of the endometriosis and control groups. Circulating levels of estradiol and progesterone in the proliferative and secretory phases of the menstrual cycle in each group also appear in Table 1. While progesterone levels were higher in the secretory than in the proliferative phase, no differences were observed in estradiol and progesterone levels between the endometriosis and control groups. Serum leptin/BMI ratio was similar in the proliferative and secretory phases of the cycle in the endometriosis (0.56 (0.27–1.24) versus 0.61 (0.41–0.91) resp., $P = 0.97$) and control (0.33 (0.21–0.53) versus 0.37 (0.18–0.68) resp. $P = 0.77$) groups. PF leptin/BMI ratio was also found to be similar in the proliferative and secretory phases in the endometriosis (1.15 (0.35–1.97) versus 0.62 (0.39–1.04) resp. $P = 0.64$) and control (0.46 (0.12–0.72) versus 0.39 (0.26–0.74) resp., $P = 0.73$) groups. Therefore, posterior analyses were performed including all patients, without considering the cycle phase.

As shown in Table 2, the serum leptin/BMI ratio was significantly higher in the endometriosis group than in controls. A trend toward significantly higher PF leptin/BMI ratio was also observed in the endometriosis group ($P = 0.07$). There were no significant differences in serum and PF leptin/BMI ratio when controls were compared to patients with minimal/mild or moderate/severe endometriosis.

Leptin mRNA and OB-R_L were detectable in all samples of ectopic endometrium. In the eutopic endometria of patients and controls, leptin mRNA and OB-R_L were

detectable in 25 out of 28 and 16 out of 17 tested samples (89% and 94%, resp.). Figure 1(a) shows that leptin mRNA expression was significantly higher in ectopic lesions than in the eutopic endometrium of patients with endometriosis ($P < 0.001$). OB-R_L mRNA expression was also significantly higher in ectopic lesions as compared to the eutopic endometrium of the endometriosis group ($P < 0.05$).

Leptin (Figure 1(b)) and OB-R_L mRNA (Figure 1(c)) expressions were also significantly higher in ectopic endometrium when endometriosis patients were stratified into minimal/mild and moderate/severe endometriosis groups as compared to the eutopic endometrium of non-endometriosis controls. Conversely, no differences were found between endometriosis stages. Leptin and OB-R_L transcripts were also similar in the eutopic endometria of patients with endometriosis and controls (data not shown). In addition, no differences were found in leptin and OB-R_L gene expression in subcutaneous fat samples of nonendometriosis controls (leptin mRNA 1.95 (1.45–2.34) and OB-R_L mRNA 2.35 (2.02–2.57)) and samples of patients with endometriosis (2.21 (1.58–2.84) and 2.13 (1.98–2.67) resp.).

A positive and significant correlation was observed between leptin and OB-R_L transcripts in the ectopic endometrium of patients with endometriosis ($R = 0.57$, $P < 0.01$) and in the eutopic endometrium of both endometriosis and control participants (endometriosis: $R = 0.52$, $P < 0.01$; nonendometriosis controls: $R = 0.57$, $P < 0.02$).

Figure 2 shows immunostaining of OB-R_L in representative biopsies of eutopic endometrium from the control and endometriosis groups in representative biopsies of ectopic endometrium and in positive control samples of the choroid plexus. Cytoplasmic staining was observed in both the stromal and epithelial compartments of women with different stages of endometriosis and controls. Intensity of OB-R_L immunostaining in moderate/severe endometriosis samples was similar to the OB-R_L immunostaining of minimal/mild endometriosis samples, for both epithelial ($P = 0.153$, gamma test) and stromal cells ($P = 0.767$, gamma test).

A negative and significant correlation was observed between OB-R_L transcripts and PF leptin/BMI ratio in ectopic endometrium ($R = -0.49$, $P = 0.019$, Spearman's correlation). This was not observed in the eutopic endometrium of controls ($R = 0.06$, $P = 0.8$).

4. Discussion

In the present study, we found a significantly higher serum leptin/BMI ratio in the endometriosis group, as well as a significantly higher expression of leptin and OB-R_L transcripts in the ectopic endometrium compared to the eutopic endometrium of patients with endometriosis and normal pelvis controls. These results suggest a putative role of leptin in the development of endometrial implants.

Despite the strong relationship between leptin and BMI, only a few studies have analyzed the leptin/BMI ratio instead of leptin concentrations in women with endometriosis [13, 32]. Using the leptin/BMI ratio allowed us to control the influence of individual body weight on leptin secretion, thus increasing the accuracy of results.

In the present study, we found a trend toward higher levels PF leptin/BMI ratio in the presence of endometriosis. Some investigators have also reported higher PF leptin in women with endometriosis as compared to controls [2, 6, 9, 10, 12]. Concerning the severity of endometriosis, whereas this aspect was not correlated with serum and PF leptin/BMI ratio in the present study, it was inversely correlated with PF leptin levels in the study by Mahutte et al. [8]. Wertel et al. [13], Bedaiwy et al. [5], and Gungor et al. [7] found a positive correlation between endometriosis severity and leptin levels, while Barcz et al. [11] did not observe any correlation. Such discrepancies are not surprising, given the fact that these studies differ widely with regard to patient characteristics (including age, BMI, and endometriosis severity), endpoints, and stratification (or not) of the primary endometriotic lesion by anatomical location. Nevertheless, despite the specificities of each study, all seem to indicate (at least with the current commercially available kits) that PF leptin alone is not a good marker to screen for the presence, location, or severity of endometriosis. In fact, recent studies have shown that combining the serum concentration of various proteins that are differentially expressed in women with and without endometriosis, including leptin, would greatly increase diagnostic accuracy as compared to assaying each protein alone [33, 34].

Concerning the associations between leptin levels and gene expression and the menstrual cycle phases, our results are consistent with previous findings that showed no significant differences in leptin levels between follicular and luteal phases [5, 6]. Previous studies have shown higher leptin expression during the implantation period [16] and total and long-form leptin receptor gene expression throughout the menstrual cycle, with increased expression in the early luteal phase [3]. In the present study, laparoscopies were scheduled preferentially in the secretory phase in order to evaluate the association between leptin and endometrial differentiation, rather than proliferation. However, because some participants had their samples collected in the proliferative phase, we observed that serum and PF leptin/BMI ratios were comparable in the two phases in both endometriosis and control participants. In addition, the estradiol and progesterone levels recorded in each cycle phase (proliferative or secretory) were similar in endometriosis patients and controls.

It should be noted that our control group did not include strictly normal women, but rather patients without pelvic disease at laparoscopic inspection. Some previous studies have investigated the association between leptin, infertility, and chronic pelvic pain. Wertel et al. [13] have shown that serum and peritoneal fluid concentrations of leptin were similar in fertile and infertile patients with endometriosis, as well as in patients with unexplained infertility and tubal ligation. Tao et al. [14] also found no difference in peritoneal fluid leptin levels of patients with endometriosis and infertility compared to a group with fallopian-associated infertility and controls with myoma. According to Barcz et al. [11], infertile patients had higher leptin levels than patients with chronic pelvic pain, regardless of the presence of endometriosis. However, fertility

was not tested in all patients with chronic pelvic pain in that study. Bedaiwy et al. [5] observed higher peritoneal fluid leptin levels in patients with endometriosis versus patients with unexplained infertility or those undergoing laparoscopy for tubal ligation or reversal of tubal ligation. There was no difference in leptin levels between the unexplained infertility and tubal ligation/reversal of tubal ligation groups. In a subgroup of endometriosis patients presenting pelvic pain, a positive correlation was found between peritoneal fluid leptin concentration and severity of symptoms, except when infertility was the main presenting symptom. Thus, the presence of infertility associated with endometriosis does not seem to influence leptin concentrations, but leptin might play a role in thepathophysiology of pain associated with endometriosis. It is important to note that, in our study, 68% of patients underwent laparoscopy for tubal ligation. These patients had a laparoscopically normal pelvis and were symptomfree. Five patients complained of infertility, and only two reported pelvic pain. All suspicious lesions were evaluated by histopathology. Thus, our control group may be regarded as adequate for the purpose of this study.

We observed that expression of leptin and OB-R_L transcripts was significantly higher in ectopic versus eutopic endometrium of patients with endometriosis and normal pelvis controls. In addition, we detected leptin mRNA in almost all eutopic and ectopic endometrium samples, a finding that might be attributable, at least in part, to the method used in our study, as real-time RT-PCR is more sensitive to smaller amounts of mRNA.

Our choice to analyze only the long form of the leptin receptor was based on the evidence that this isoform has the highest transcriptional activity [35]. Lima-Couy et al. [16] assessed the total, long, and short isoforms of leptin receptor in eutopic endometrium of patients with moderate and severe endometriosis and observed an increase in receptor expression during the period of embryo implantation, with no differences between eutopic endometrium from patients with endometriosis and controls.

Other authors have identified leptin and OB-R transcripts in eutopic endometrium. Kitawaki et al. [3] identified the long form of the receptor in 84% of endometrial samples versus 85% in our study, including eutopic endometria of patients and controls. In an in vitro study, González et al. [36] demonstrated that the presence of leptin and leptin receptor mRNA in endometrial epithelial cells and embryos could be related to the embryo implantation process. Kitawaki et al. [3] also showed fluctuations in the expression of OB-R in endometrium, with a peak in the early secretory phase.

OB-R_L mRNA expression was negatively correlated with PF leptin/BMI ratio in ectopic endometrium. This observation is in agreement with a previous study, which found that treatment of stromal cell cultures with leptin reduces OB-R_L [17], suggesting modulation between leptin and its receptor in endometriosis. Later, the same authors demonstrated that, under hypoxic conditions, leptin gene expression was increased in both eutopic and ectopic endometrial stromal cells and that this process is likely to be mediated directly by hypoxia-inducible factor-1α (HIF-1α). Considering that leptin may promote cell proliferation, a low-oxygen environment in endometriotic implants could lead to stimulation of leptin gene expression, increasing the proliferation of endometrial stromal cells, with subsequent implantation of these cells in the peritoneum [18]. Our finding of a significant negative correlation between OB-R_L mRNA expression and peritoneal fluid leptin/BMI ratio in endometriosis also suggests that peritoneal fluid leptin/BMI ratio might have greater influence on the molecular regulation of OB-R_L receptor than the circulating leptin/BMI ratio. However, the experimental design of the present study does not allow us to confirm this possibility. Further *in vitro* studies are needed in order to determine the effect of different leptin concentrations on OB-R_L gene expression in isolated endometriotic cells.

Leptin is thought to play a role in endometriosis through its inflammatory and angiogenic properties. Using a rat model of endometriosis, Styer et al. [37] demonstrated that disruption of leptin signaling by administration of the pegylated leptin peptide receptor antagonist (LPrA) or nonfunctional leptin receptor ($Lepr^{dB}$) inhibits the establishment and development of endometriosis-like lesions that resemble peritoneal endometriotic foci. The administration of recombinant VEFG to these animals led to an increase in the formation of endometrial glands, however, at a lower density in relation to controls. Therefore, leptin signaling seems to be a necessary component of lesion proliferation, initial vascular recruitment, and maintenance of neoangiogenesis in a murine model of endometriosis. Recently, Oh et al. [19] showed in an *in vitro* model of cell culture from endometrioma and endometrial tissues of women without endometriosis that the expression of leptin receptor was significantly higher in endometriotic epithelial cells than in epithelial and stromal cells of the normal endometrium or in endometriotic stromal cells. In addition, leptin treatment stimulated the proliferation of only endometriotic epithelial cells. In contrast, inhibition of JAK2/STAT3 and ERK signaling pathways of the leptin receptor induced a blockage of growth in these endometriotic epithelial cells through leptin stimulation. The increase in leptin and leptin receptor expression in the ectopic endometrium of women with endometriosis may lead to increased leptin signaling in implants, resulting in proliferation, neoangiogenesis, and maintenance of ectopic endometrial tissue [37].

The higher serum leptin/BMI ratio observed in the endometriosis group as compared to the control group may be due to endometriotic implants rather than adipose tissue, since leptin and OB-R_L transcripts were similar in the fat tissue of endometriosis and control participants.

We were unable to confirm previous findings reporting more elevated PF leptin in milder disease [2, 6, 8]. However, the biological activity of the disease might be related to type rather than extent of lesion. In this sense, Gazvani et al. [38] showed that red lesions are more biologically active than white or black lesions. Further studies are needed to specifically study the relationships between leptin and its receptor transcripts according to the type of lesions found at laparoscopy.

One limitation of our study concerns the purity of cell populations in the ectopic endometrium samples. Tissues obtained from lesions contain a mixture of cell types, including leukocytes and peritoneal fibroblasts, in addition to ectopic endometrium [39]. Thus, there is a small risk that peritoneal cells may account for some of the results observed.

5. Conclusions

The present data suggest that serum leptin/BMI ratio is associated with the presence of endometriosis. Nevertheless, the clinical applicability of the leptin/BMI ratio for prediction of endometriosis still requires confirmation. Moreover, the increased expression of leptin and OB-R_L in ectopic endometrium suggests a modulatory interaction between leptin and its active receptor and a role of leptin, an inflammatory and angiogenic cytokine, in the initiation or development of endometrial implants.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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