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THE ROLE OF INTERSTITIAL CAJAL-LIKE CELLS (TELOCYTES) IN THE PATHOGENESIS OF UTERINE FIBROIDS.

BADANIA NAD ROLĄ KOMÓREK ŚRÓDMIĄŻSZOWYCH TYPU CAJALA (TELOCYTÓW) W ETIOPATOGENEZIE MIĘŚNIAKOWATOŚCI MACICY.

PhD thesis

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I am profoundly grateful to my tutor, Prof. Krzysztof Gil,
for involving in scientific life and learning by own example…
I am endlessly grateful to my parents
for who I am thanks to Them…

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1. INTRODUCTION

Telocytes (current synonyms Interstitial Cajal-like cells (ICLC), fibroblast-like cells and PDGFRα^{+} (plated-derived growth factor receptor alpha positive) cells), a new type of cells of mesenchymal origin, were described by Popescu over two decades ago. Despite plenty of publications worldwide, the unique nature of these cells still deserves attention from the scientific community. The number of studies and publications devoted to a new class of cells is invariably growing with each year. In addition, three hundred thirty four articles are published in PubMed (Date: 09.06.2019), focused on telocytes (TCs).

In 2005 L.M. Popescu's group from Bucharest, Romania, focused on interstitial (stromal) cells in the connective tissue of many organs of humans and laboratory mammals, which named interstitial Cajal-like cells. This group named these cells as ICLC because of their apparent similarity with the canonical gastrointestinal cells of Cajal (ICC), the gut pacemaker cells, which were first discovered as "interstitial neurons" by Santiago Ramon y Cajal in 1889 [1].

In 2008, M.S. Faussone-Pellegrini and her team from Florence, Italy, described ICLC in the muscle coat of the human gut, considerably emphasized on a difference ICLC and gastrointestinal cells of Cajal by ultrastructure and immunopositivity. In 2010 the acronym ICLC was replaced with a more appropriate name one and introduced to scientific world for the first time in the paper "TELOCYTES − a case of serendipity: the winding way from Interstitial Cells of Cajal (ICC), via Interstitial Cajal-Like Cells (ICLC) to TELOCYTES" in the Journal of Cellular and Molecular Medicine. From that time, this novel cell type became known as telocytes (using the Greek affix "Telos") [2-4]. TCs have been found in more than 50 different places inside the human body and are distributed in vertebrates (fish, reptiles, birds, mammals, including human) [5-7].

The telocyte (TC) has a small, oval-shaped cellular body, containing a nucleus, surrounded by a small amount of cytoplasm. The cellular body average dimensions are, as measured on TEM (transmission electron microscopy) images, 9.39μm ± 3.26 μm (min = 6.31 μm; max = 16.42 μm). The nucleus occupies about 25% of the cell volume and contains clusters of heterochromatin attached to the nuclear envelope. The perinuclear cytoplasm is rich in mitochondria, occupying about 5% of the cell body [2-5].

Telocytes have a variable number of telopodes (Tps) (very long cellular extensions), which are probably the longest cellular prolongations in the human body. Tps are made by an alternation of dilated portions, named podoms (250–300 nm), containing mitochondria and endoplasmic reticulum and podomers (~80 nm) with thin segments [6; 8]. TCs are clearly different from neurons, dendritic cells or fibroblasts and myofibroblasts [2; 31]. The shape of the TCs depends on the number of their telopodes: piriform for one prolongation, spindle for two Tps, triangular for three, stellate, etc. Their spatial appearance is that of a polyhedron with a different number of vertices, depending on their Tps number [81].

Based on transmission electron microscopy (TEM) analysis, Huizinga *et al.* proposed eight basic ultrastructural criteria for TC identification in 1997 ("**gold standard**"). Later Popescu and his group added two more criteria and formed "**platinum standard**" of diagnosis for TCs [7; 30; 82].

A considerable amount of telocytes' behavior is explained by their contacts with various surrounding cells: specific direct (homocellular and heterocellular junctions) and/or indirect (chemical, paracrine/juxtacrine signaling, microvesicles and exosomes, sex hormone and microRNAs) [3; 4; 31]. Homocellular junctions allow them to keep an architecture of tissue, generating 3D (three dimensional) networks. Moreover, they contain elements of the cytoskeleton such as microfilaments, microtubules and vimentin [7].

Connections between TCs-exosomes-intercellular junctions cytoskeleton form the equivalent of a primitive nervous system [56; 83-85]. Heterocellular contacts TCs make with a variety of cells: smooth muscle cells, nerves, immunocytes (macrophages, mast cells and lymphocytes), stem cells, melanocytes in the eye [27], erythrocytes in the spleen [71] and with Schwann cells in the heart [15]. Gherhiceanu *et al*. reported that TCs make contact with virtually all types of cells in the human heart. His team suggested that heterocellular contacts occur by means of minute junctions (point contacts, nanocontacts and planar contacts) and the mean intermembrane distance is within the macromolecular interaction range (10–30 nm) [15]. Of note, TCs establish close contacts, stromal synapses (connective connections), with tracheal mast cells and in the trigeminal ganglion [76; 77].

Telocytes also surrounded stem cell niches with Tps and heterocellular contacts. In addition, they establish physical contacts with nerve endings, blood vessels and different types of progenitor cells. Accumulating studies have shown that TCs play an indisputable role in neo-angiogenesis. As they have cytoskeleton elements (myosin-14, periplakin), this predicts that they could be responsible for detecting smooth muscle cell stretch during the enlargement of the uterus in pregnancy [7; 14; 85].

Telocytes release at least three types of extracellular vesicles (exosomes, ectosomes and multivesicular cargos) from their Tps and, occasionally, from the cell body [86] and secrete interleukins (IL-2, IL-6, IL-10 and IL-13), growth factors (vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF)), nitric oxide, macrophage inflammatory protein 1α and 2 (MIP-1α and MIP-2), Monocyte Chemoattractant Protein 1 (MCP-1), Growth-Related Oncogene/Keratinocyte-derived Chemokine (GRO-KC). Three major classes of elements in telocyte secretomes include growth factors, chemoattractants, and cytokines/chemokines, indicating that TCs may regulate stem cell growth and differentiation, microenvironmental formations [87-90].

Immunohistochemistry combined with TEM is the most applicable method to identify TCs [6; 7]. Despite the fact that has not yet been found a specific marker for TCs, usually for primary identification scientists use CD34 [91]. Important to note that CD117/c-kit, that firstly used for the primary identification of TCs, has been excluded for some organs or its parts [91] and differs between TCs populations (possible site dependent) [4; 92]. Nowadays, the best available choice is a combination of four immunohistochemical markers: CD34, c-kit, vimentin and PDGFRα [3; 85; 93]. TCs might act as 'hormonal sensors', and their function may be in part under hormonal control. These receptors are specific for TCs localization. For example, TCs of gallbladder are negative for both types of receptors and gallbladder muscular contractions are not regulated by sex steroids [89]. In addition, as CD34+ cells may lose CD34 expression and acquire other marker expressions "in vivo" and "in vitro" [94, 95].

TCs are also involved in the electrical modulation of excitable tissue, such as the smooth muscle of the gut and uterus. They can spontaneously initiate electrical activity and modulate of glandular and immune activity [96]. Ion channels, such as T-type calcium and small-conductance calcium-activated potassium channels, are present in TCs [4].

In the female reproductive system, TCs were described in the myometrium, endometrium, Fallopian tubes and placenta [6; 56; 89; 97]. In addition, these cells were revealed in the human and animal uterus in pregnant and non-pregnant conditions [18; 30; 57; 58; 98]. TCs are located in the neuromuscular spindles and participate in the control of muscle tone and motor activity. They form homo- and heterocellular contacts with adjacent cells and might even be able to control and regulate their activity, participating in tissue remodelling/renewal [6]. They produce electric slow waves that trigger and coordinate smooth muscle contractions in the uterus. The decreasing in TCs caused dysregulation of

oviduct motility, suggesting that tubal TCs impairment leads to the infertility of tubal origin and even tubal ectopic pregnancy [99; 100].

In human myometrium, patch-clamp recordings of TCs revealed a calcium-dependent hyperpolarization-activated chloride inward current, but absence of L-type calcium channels, which was postulated to modulate myometrial smooth muscle contractions [7; 101]. T-type calcium channels are present in TCs from human myometrium, which in pregnancy and labour participate in the generation of endogenous bioelectric signals responsible for the regulation of the surrounding cell behavior. It might be the missing link for describing the molecular mechanisms by which TCs are involved in mechanical stretching during uterine enlargement in pregnancy. The expression of α-subunit of T-type calcium channels in TCs is less intense in the case of nonpregnant myometrium [6; 102]. Steroid hormones and oxytocin might mediate the higher expression of T-type calcium channels in TCs derived from pregnant myometrium. As TCs have steroid hormone receptors, this might lead to frequent and sustained contractions that are able to trigger birth [6]. In fetal cardiac myocytes, T-type Ca^{2+} channels were suggested to play role in the regulation of cardiomyocyte size [102].

Myometrial TCs have large input resistance, ranging between 1.2 and 12 GΩ. They failed to produce the regular slow waves of depolarization described in classical ICCs, although some irregular excursions of membrane potential ranging from 10 to 35 mV. TCs did not generate action potentials in response to depolarizing current. Only passive electric potentials were recorded, when current pulses were applied [94].

The four different studies were conducted on gene expression profile of TCs in the last several years. Researchers focused on TCs-specific or TCs-dominated gene profiles in chromosome 1, 2, 3, 17 and 18 using global comparison between TCs and other cell types found in the mouse lung tissue [103]. TCs had a strong number of up- and down-regulated

genes in all patterns. Important to note that number of down-regulated genes was 2–3 times higher than up-regulated in all observed chromosomes. It had been mostly suggested that these cells are involved in cellular signaling, cell expansion and movement (migration, adhesion, migration and division), embryogenesis, morphogenesis and tissue homoeostasis (including immune homeostasis), tissue remodeling and repair, maintenance of oxidative microenvironment preventing tumorigenesis and anti-inflammatory responses [103–109].

TCs express significant amount of pro-angiogenic microRNAs (miR126, miR130a, let-7-family, miR-10, miR-155, miR-503, miR-126, miR-27b, miR-503, and miR-100), also miR-21, miR-22, miR-29 and miR-199a, both stromal specific and vascular smooth muscle specific (miR-143/145). These cells do not express miR-193 and have lack of expression of cardyomyocyte-specific miRs (miR-1 and miR-133a or miR-208) [4; 55; 110; 111].

Up to now, the presence with possible integration into physiological and pathological processes of TCs is still hotly observed and described by numerous scientists. Moreover, its dynamics were observed in such diseases as systematic sclerosis, Crohn's disease, myocardial infraction, gallstone disease, psoriasis, acute salpingitis, liver fibrosis and primary Sjögren's syndrome [33; 49; 55; 93; 95; 100]. One common tendency was observed in all mentioned diseases: reduction in number of TCs in pathological focuses [95]. The presence of TCs in uterine fibroids and its role have not been clear verified yet.

Uterine fibroid (UF) is still the most widespread gynecological disease, affected up to 80% of all women in their reproductive age [112], characterized by exceeded production of extracellular matrix (ECM). The prevalence of clinically significant fibroids peaks in the perimenopausal years and declines after menopause [113]. The incidence of uterine leiomyoma (UL) in Poland is not precisely defined in fact, epidemiological studies indicate a large variation of the results (from 20% to 40% of women) [114].

Uterine fibroids contain their own specific vasculature [115-117]. Large fibroids have a “vascular capsule”, while smaller fibroids are usually avascular [118; 119]. The vascular network separates the lesion from the surrounding myometrium. Walocha *et al*. distinguished two types of vascularizations of intramural UF. The first vascularization is characterized by the formation of a dense capsule of peripheral vessels, while the central part of the fibroid is only scantly vascularized. In the second vascularization, foci of an intensive regression of the tumor are separated from the surrounding tissue by strong vascular septa [118]. The vascular density of UF is a constant mark that does not change in response to hormonal therapy [120; 121], while the microvessel density is decreased in UF compared to that in the unaffected myometrium [120; 122-124], leading to the development of interstitial ischemia. Holdsworth-Carson *et al.* showed that the vascular density does not change in seeding fibroids, whereas in large UF, it does change. They suggested that local ischemia is not common for all types of UF [125].

A neurogenic component is invaluable for uterine homeostasis, playing a major role in the pathophysiological mechanisms of chronic pelvic pain and co-occurring in diseases such as endometriosis, adenomyosis, inflammatory pelvic disease or leiomyomata [126; 127]. It is perhaps no secret that abundant nitric oxide-synthesizing nerves play a role in inflammatory reactions and oxidative stress. As a result, these nerves are undoubtedly involved in the pathophysiology of UF [128]. Moreover, fibroids usually have a highly vascularized pseudocapsule characterized by the presence of nerve fibers [129; 130].

2. AIM OF STUDY

The aim of study was to determine the location of TCs in uterine fibroids and to clarify their possible role in the architecture of leiomyomata. In addition, to examine the autonomic nervous system and identify telocytes in normal myometrium and uterine leiomyomas in order to reveal diversity in a gross structural organization between normal and affected myometrial tissue. We intended to determine the link between myometrial TCs and vascularization in a human leiomyoma, and to clarify their possible role in the angiogenesis. We compared the collagen deposits in unaffected and unaffected by fibroid tissue, stressed an involvement of uterine telocytes in fibrosis development.

Results of the current research are presented in further publications:

1. **Aleksandrovych V**, Pasternak A, Basta P, Sajewicz M, Walocha JA, Gil K. Telocytes: facts, speculations and myths (Review article). Folia Med Cracov. 2017;57(1):5-22. (review, Points of MNiSW: 10.000)
2. **Aleksandrovych V**, Białas M, Pasternak A, Bereza T, Sajewicz M, Walocha J, Gil K. Identification of uterine telocytes and their architecture in leiomyoma. Folia Med Cracov. 2018;58(3):89-102 (original article, Points of MNiSW: 10.000)
3. **Aleksandrovych V**, Kurnik-Łucka M, Bereza T, Białas M, Pasternak A, Cretoiu D, Walocha J, Gil K. The autonomic innervation and uterine telocyte interplay in leiomyoma formation. Cell Transplantation, (March 2019). doi:10.1177/0963689719833303. (original article, IF=2.885; Points of MNiSW: 25.000)
4. **Aleksandrovych V,** Bereza T, Białas M, Pasternak A, Walocha J, Pityński K, Gil K. Identification of PDGFRα+ cells in the uterine fibroid – link between angiogenesis and uterine telocytes. Archives of Medical Science. 2019: accepted (original article, IF=2.344; Points of MNiSW: 30.000)

3. ABSTRACT OF PHD THESIS

The first mention about uterine fibroids dates back to the time of Hippocrates. However, there are still wide gaps in the understanding of their pathogenesis. No single theory explains the background of the oldest uterine pathology, which affects more than 50% of women worldwide. By contrast, a newly depicted cell type called telocyte was only recently identified in the past twenty years. The unique features of telocytes coupled with experimental evidence from numerous studies lead us to create new hypotheses of the uterine fibroid pathogenesis. The results of current research will be discussed in our thesis. We explored the important telocytes interactions in the context of the uterine fibroid architecture. Telocytes not only interact with a variety of histological structures but have been the first line cells respond to hypoxia, thereby attracting our attention.

The aim of the study was to identify uterine telocytes in the foci of fibroids and elucidate their role in fibrosis development, local angiogenesis, and autonomic nerves interplay. Firstly, we performed the primary identification of telocytes using immunolabelling with further markers: c-kit, CD34, PDGFRα, tryptase. Immunofluorescent labeling was also used for all samples: the corpus and uterine cervix (exo- and endocervix) as well as leiomyoma foci. The use of mast cell tryptase staining enabled c-kit-positive mast cells to be distinguished from c-kit-positive TCs. TCs were considered cells that were c-kit positive and tryptase negative concurrently with the characteristic morphology in tissue samples. Additionally, cells double positive for CD34 and PDGFRα with characteristic morphology and localization were also recognized as TCs. We also intended to grossly evaluate the structure of the fibroid, comparing collagen and muscle components in the foci of fibroids as well as in unchanged myometrium using hematoxylin and eosin and Masson's trichrome stainings. The performed study revealed the existence of uterine telocytes in all observed uterine tissues (fibroids, unchanged

myometrium from the corpus of the uterus and uterine cervix) mostly located in close vicinity of blood vessels, between muscle fibers.

Our next step was to focus attention on the interaction between uterine telocytes and autonomic innervation. Neuronal structures were identified by immunolabeling for neuronal markers: protein gene product PGP 9.5, inducible nitric oxide synthase (iNOS), choline acetyltransferase (ChAT), and tyrosine hydroxylase (TH). For the primary identification of telocytes we performed the same immunostaining as mentioned above. The gross organization of myometrial tissue has been analyzed by routine histology as well. The autonomic innervation in the foci of fibroids was mainly characterized by an increase in the number of iNOS and ChAT-immunopositive neurons. Uterine telocytes are also located close to nerve fibers, emphasized their putative interplay with neuronal elements. The close vicinity of TCs with nerve endings confirmed the unique involvement of these cells in neuronal regulation in the uterus; however, the role of the cell-cell interaction with nerve fibers needs further explanation.

The next issue we explored in the context of the pathogenesis of uterine fibroid was a linkage between telocytes and angiogenesis processes. Based on literature data telocytes are characterized by high sensitivity to hypoxia (more likely than fibroblasts). We picked up two additional specific markers for vessels formation (CD31 and sFlt-1 (anti-angiogenic factor)), and combine with immunostaining specific for TCs used before in order to reveal the correlation of telocytes with the expression of angiogenic markers. The declined number of CD34+/PDGFRα+-cells in leiomyoma was accompanied by a prevalence of collagen deposits, poor vascularization, a high immunopositivity of the myometrium to sFlt-1 (anti-angiogenic factor). We concluded that uterine telocytes, sensitive for angiogenic factors (PDGF and VEGF) and ischemia, have been declined and even disappeared during fibrosis and are mainly located in close vicinity of blood vessels. They

might play a role in the angiogenic response to hypoxia, universal for the whole human body.

The variety of heterocellular contacts between telocytes and surrounding cells/anatomical structures in uterine fibroids allows us to focus our attention on its importance. Each leiomyoma is accompanied by hypoxia process, exceeded production of extracellular matrix and formation of “vascular capsule”. Concluding, the local declining of telocytes in fibroids also reflects its sensitivity to hypoxia and indirect involvement in basic processes leading to fibrosis. In addition, the reduced density of uterine telocytes correlates with an elevated number of NOS-positive neurons.

Uterine telocytes are present in all parts of the human uterus, close to blood vessels, nerves, between smooth muscle cells and also in the capsule of the fibroid. Connection points in the pathophysiology of uterine fibroid and properties of telocytes allow us to underline the importance of telocytes in the pathogenesis of uterine fibroids.

Key words: uterine fibroids; extracellular matrix; telocytes; CD34; angiogenesis; nerve fibers.

4. STRESZCZENIE PRACY DOKTORSKIEJ

Pierwsze wzmianki o mięśniakach macicy sięgają czasów Hipokratesa. Pomimo to do tej pory nie udało się wyjaśnić wyczerpująco patogenezy tej najstarszej patologii macicy, która dotyka ponad 50% kobiet na całym świecie.

W ostatnich latach opisano nowy typ komórek śródmiąższowych nazwanych telocytami. Unikalne cechy telocytów w połączeniu z doniesieniami na temat ich lokalizacji i roli w patologii różnych narządów pozwoliły nam na postawienie hipotezy, że komórki te mogą być elementem złożonej patogenezy mięśniaków macicy. Ponieważ, wg ostatnich doniesień, telocyty rozmieszczone są w bezpośrednim sąsiedztwie różnych struktur histologicznych, z którymi tworzą liczne połączenia, a ponadto są komórkami tzw. pierwszej linii, które bardzo wcześnie reagują na niedotlenienie, postanowiliśmy zbadać ich rozmieszczenie, interakcje międzykomórkowe i możliwą rolę w mięśniakowatości macicy.

Celem badania było zidentyfikowanie telocytów macicy w ogniskach mięśniaków i wyjaśnienie ich roli w rozwoju procesów włóknienia, miejscowej angiogenezy oraz wzajemnych oddziaływań z nerwami autonomicznymi macicy.

W pierwszym etapie przeprowadziliśmy identyfikację telocytów za pomocą znakowania immunofluorescencyjnego ze specyficznymi dla nich markerami: c-kit, CD34, PDGFRα oraz tryptazy mastocytarnej w wycinkach z szyjki i trzonu macicy oraz tkanki zmienionej mięśniakowato. Zastosowanie barwienia przeciwko tryptazie mastocytarnej umożliwiło odróżnienie komórek tucznych od dodatnich również względem c-kit telocytów - za telocyty uznano komórki, które były c-kit pozytywne i tryptazo-ujemne oraz równocześnie miały charakterystyczne dla telocytów cechy morfologiczne. Równolegle, przeprowadziliśmy identyfikację telocytów w oparciu o inny zestaw markerów - komórki podwójnie dodatnie dla CD34 i PDGFRα o charakterystycznej morfologii i lokalizacji

również zostały uznane za telocyty. Ponadto oceniono strukturę tkanki zmienionej mięśniakowato - oprócz rutynowej analizy histologicznej porównano zawartość kolagenu i składników mięśniowych w ogniskach mięśniaków, jak również w niezmienionej mięśniówce macicy, stosując barwienie hematoksyliną i eozyną oraz metodą Trichrome wg Massona. Przeprowadzone badania wykazały obecność telocytów we wszystkich obserwowanych tkankach macicy (mięśniaków, niezmienionego mięśniówki macicy z trzonu macicy i szyjki macicy) zlokalizowanych głównie w pobliżu naczyń krwionośnych oraz także pomiędzy włóknami mięśniowymi. Jednocześnie zaobserwowaliśmy spadek gęstości telocytów w tkankach zmienionych mięśniakowato w stosunku do tkanek macicy niezmienionych patologicznie.

Nasze następne badania skupiły się na interakcji pomiędzy telocytami macicy a strukturami unerwienia autonomicznego macicy. Struktury neuronalne identyfikowano przez znakowanie immunologiczne markerów neuronowych dla: produkt genu białkowego PGP 9,5, indukowalnej syntazy tlenku azotu (iNOS), acetylotransferazy cholinowej (ChAT) i hydroksylazy tyrozynowej (TH). W celu identyfikacji telocytów przeprowadziliśmy barwienie immunologiczne opracowane we wcześniejszym etapie badań. Autonomiczne unerwienie w ogniskach mięśniaków charakteryzowało się głównie wzrostem liczby neuronów immunopozytywnych dla iNOS i ChAT. Zaobserwowaliśmy, że telocyty w macicy są również zlokalizowane w pobliżu włókien nerwowych, co podkreśla ich sugerowane wzajemne oddziaływanie z elementami neuronalnymi. Chociaż zaobserwowane przez nas bliskie sąsiedztwo telocytów z zakończeniami nerwowymi wskazuje na potencjalne zaangażowanie tych komórek w zjawiska regulacji czynności neuronów autonomicznych lub chociażby przekazywanie sygnałów w macicy, to rola tej interakcji z włóknami nerwowymi wymaga jednak dalszych badań.

Kolejnym zagadnieniem, które zbadaliśmy w kontekście patogenezy mięśniaków macicy, było powiązanie między telocytami a procesami angiogenezy. Na podstawie danych literaturowych telocyty charakteryzują się wysoką wrażliwością na niedotlenienie (bardziej wrażliwe niż fibroblasty). Dlatego wybraliśmy dwa dodatkowe markery, specyficzne dla tworzenia nowych naczyń (CD31 i sFlt-1 (czynnik antyangiogenny)) i połączyliśmy je barwieniem immunologicznym specyficznym dla telocytów, w celu ujawnienia korelacji telocytów z ekspresją markerów angiogennych. Zmniejszonej liczbie komórek CD34+/PDGFRα+ (telocytów) w mięśniakach towarzyszyło występowanie większej ilości złogów kolagenu, słabe unaczynienie oraz wysoka immunopozytywność mięśniówki macicy dla sFlt-1 (czynnik antyangiogenny). Stwierdziliśmy, że gęstość telocytów w macicy, ponieważ są to komórki wrażliwe na czynniki angiogenne (PDGF i VEGF) i niedokrwienie, obniża się w mięśniakowatości, a nawet częściowo komórki te zanikają podczas tworzącego się włóknienia i lokalizują się wtedy głównie w pobliżu naczyń krwionośnych. Mogą one prawdopodobnie odgrywać jakąś rolę w angiogennej odpowiedzi na miejscową hipoksję, co jednak wymaga dalszych badań.

Telocyty macicy są obecne we wszystkich częściach ludzkiej macicy, w pobliżu naczyń krwionośnych, nerwów, między komórkami mięśni gładkich, a także w torebce mięśniaka. Różnorodność heterokomórkowych kontaktów między telocytami i otaczającymi komórkami/strukturami anatomicznymi mięśniaków macicy sugeruje ich ważną rolę w rozwoju tego procesu patologicznego. Każdemu mięśniakowi towarzyszy proces niedotlenienia, nasilona produkcja macierzy zewnątrzkomórkowej i tworzenie „torebki naczyniowej". Miejscowe zmniejszenie liczby telocytów w mięśniakach odzwierciedla również jego wrażliwość na niedotlenienie i pośredni udział w podstawowych procesach prowadzących do zwłóknienia. Ponadto zmniejszona gęstość telocytów macicy koreluje ze zwiększoną liczbą neuronów iNOS dodatnich. Uzyskane

wyniki badań wskazują na znaczącą rolę telocytów w procesach patogenetycznych prowadzących do rozwoju mięśniaków macicy.

Słowa kluczowe: mięśniakowość macicy; macierz pozakomórkowa; telocyty; CD34; angiogeneza; unerwienie autonomiczne.

5. SUMMARY OF ARTICLES

5.1. SUMMARY OF ARTICLE 1 "Telocytes: facts, speculations and myths (Review article)."

For the purpose of this review, all available articles describing morphological, immunological, electrophysiological and functional features of uterine telocytes, were analyzed. The first section focused on ultrastructural standards of TCs identification, paying attention to the distribution in different organs of the human body with appropriate references. The telocytes have been observed in more than fifty different anatomical units of our body.

In addition to emphasized unicity of these cells, has been detailed explained with which cells they interact by forming heterocellular contacts: smooth muscle cells, nerves, immunocytes (macrophages, mast cells, and lymphocytes), stem cells, melanocytes in the eye, erythrocytes in the spleen, Schwann cells in the heart. Gherhiceanu et al. reported that TCs make contact with virtually all types of cells in the human heart. His team suggested that heterocellular contacts occur by means of minute junctions (point contacts, nanocontacts, and planar contacts) and the mean intermembrane distance is within the macromolecular interaction range (10–30 nm). Moreover, TCs establish close contacts, stromal synapses (connective connections), with tracheal mast cells and in the trigeminal ganglion.

The own genetic and microRNA profiles of TCs was additionally accompanied by the newest data from its immunopositivity and electrophysiological characteristics. The last section covered the role of TCs in the pathophysiological mechanisms, mentioned current observed models and diseases. The possible existence TCs in stem-cell niches, as well as its interaction with vascularity and nerve component, have been connected with describing of "mesenchymal cell niche" and "blood-myocardium barrier". Conductor

function in fibrotic mechanisms and contractility of smooth muscles was presented in intestinal, uterine and myocardial contexts.

This review illustrates the multifunctional nature of telocytes, focusing attention on its role in angiogenesis and fibrosis, regenerative medicine and myometrial contractions, the pathogenesis of diseases and pregnancy and allows to update the knowledge on this topic. It also summarizes particular features of TCs in different organs and systems, emphasizing their involvement in physiological and pathophysiological processes.

5.2. SUMMARY OF ARTICLE 2 "Identification of uterine telocytes and their architecture in leiomyoma"

Introduction. The uterus is often affected by leiomyoma, especially in reproductive age, not rare leading to infertility. Despite modern methods of examination and therapy, the pathogenesis of the myoma still has gaps. It is known that percentage of uterine TCs has been changed during pregnancy. Also, its morphology has changed too. TCs have not been observed in uterine fibroids, while its role in the fibrosis development and local homeostasis was previously hotly discussed. The aim of the study was to determine the location of TCs in different parts of the human uterus (exo- and endocervix, corpus and focus of fibroid) and to clarify their possible role in the architecture of leiomyomata.

Material and methods. The samples were taken from 19 surgically treated patients with uterine fibroids. Control group consisted of 15 patients suffered from other disease of pathology. Routine histology was performed and followed by Masson Trichrome staining for collagen deposits assessment. Tissue specimens were immunolabelled, with telocytes markers: c-kit, CD34 and PDGFRα. Slides were examined using an MN800FL epifluorescence microscope (OptaTech, Warszawa, Poland) equipped with a Jenoptik Progress C15Plus color camera. Digital images were collected at either 200× or 400× magnification. Qualitative analysis of cells was provided in 10 consecutive high-power fields of vision (400×) using the computer-based image analysis system Multiscan 18.03 software (CSS, Warszawa, Poland). The semiquantitative analysis of telocytes density was performed, using a simple scale with one, two or three pluses.

Results. Masson's trichrome staining revealed the prevalence of collagen deposits in UL compared with all other observed samples. C-kit/tryptase double immunolabelling was performed for distinguishing mast cells from uterine teloctyes. Double positive cells for c-

kit and tryptase were assumed as mast cells, while c-kit-positive/tryptase-negative as telocytes. CD34-positive and PDGFRα-positive cells were detected as uterine TCs.

TCs were detected in the cervix, corpus of the uterus and leiomyoma. The endocervix contains more c-kit-positive, CD34-positive and PDGFRα-immunopositive cells compared with the exocervix. These cells formed bundles mainly located longitudinally (parallel to the cervical canal). No differences in TC density were noted in all parts of the uterine cervix between myomatous and healthy uterus. The density of telocytes in fibroid foci was reduced compared with normal myometrium (Table 1). In the corpus of the uterus, TCs were located in close vicinity to blood vessels and inside muscle bundles. Subjective qualitative analyses exhibited a reduction in TC density in fibroids compare with both types of unaffected myometrium (adjacent and from healthy uterus).

Table 1. Relative frequency of c-kit-positive/tryptase-negative, CD34-positive and PRGFRα-positive cells in different parts of human uterus that was not affected or affected by leiomyoma. 0 = absence of telocytes, (+) = very few, + = few, + + = moderate density, + + + = multiply density.

	c-kit+/trypatse-	CD34+/ PDGFRα+
Normal Uterus		
Exocervix	(+)	(+)
Endocervix	+	+
Corpus	+++	+++
Myomatous Uterus		
Exocervix	(+)	(+)
Endocervix	+	+
Corpus	++	++
Fibroid	+	+

Conclusions: The current study proved the existence of telocytes in different parts of the human uterus (cervix, corpus, focuses of fibroids) — both affected and not affected by leiomyoma. Qualitative analysis revealed the reduction of TCs in fibroid foci, whereas the prevalence of collagen deposits was detected using routine histology. We attempted to explain the place of TCs in myoma architecture and focused on basic connecting points (production of extracellular matrix and dimensional organization of myometrium. The reduced density of telocytes is important for the pathomechanisms of myometrial growth, demonstrating its value as a main component of the uterine architecture.

5.3.SUMMARY OF ARTICLE 3 "The autonomic innervation and uterine telocyte interplay in leiomyoma formation"

Introduction. The autonomic innervation of the uterus is involved in multiple pathophysiological processes in both humans and animals. The neurogenic component is invaluably important for uterine contractility and blood flow regulation. Previous studies of the innervation of the mammalian female reproductive system have revealed the great importance of the autonomic nervous system, especially adrenergic fibers. The uterus contains abundant nitric oxide-synthesizing nerves that could be either autonomic and/or sensory, that involved in the pathophysiology of uterine fibroids, due to their participation in inflammatory reactions and oxidative stress. There are no previous descriptions of the interplay between TCs and autonomic innervation in leiomyomata. The aim of the study was to examine the autonomic nervous system and identify TCs in normal myometrium and uterine leiomyomas in order to reveal diversity in a gross structural organization between normal and affected myometrial tissue.

Material and methods. The samples were taken from 15 surgically treated patients with uterine fibroids. Control group consisted of 15 patients underwent elective surgery for other reasons and had no pre- or intraoperative signs of uterine fibroids. Uterine telocytes were identified by immunopositivity for c-kit, CD34 and PDGFRα. Nerves were revealed by immunolabelling for neuronal markers: protein gene product PGP 9.5, inducible nitric oxide synthase (iNOS), choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH). The gross organization of myometrial tissue have been analyzed by routine histology. Slides were examined using an MN800FL epifluorescence microscope (OptaTech, Warszawa, Poland) equipped with Olympus DP74 digital CCD camera.

Results. In the foci of fibroids, nerve fibers immunoreactive for PGP 9.5 were particularly parallel to each other and formed bundles around myometrial nodules. Most of them were

placed in the fibroid pseudocapsule. Normal myometrium had individual nerve fibers positive for PGP 9.5. The results demonstrated that the density of iNOS and ChAT-immunopositive neurons in the uterine fibroids was higher than that in the control samples. Nerves with immunopositivity for TH were detected in the foci of leiomyoma, adjacent myometrium and normal myometrium. In unaffected myometrium single, thin fibers, without strict orientation in space manifested themselves as TH-positive neurons. In contrast, in fibroids, spindle-shaped nerve fibers were densely located parallel to the muscular bundles. In samples of adjacent myometrial tissue, its orientation had less strict character - individual fibers repeated the eccentric lines framing the muscle fibers. The density of telocytes in the fibrosis foci was lower than that in the normal myometrium. Double immunostaining for such neuronal markers as iNOS and PGP 9.5 combined with a telocyte marker CD34 revealed double-immunolabeled cells (Fig. 1).

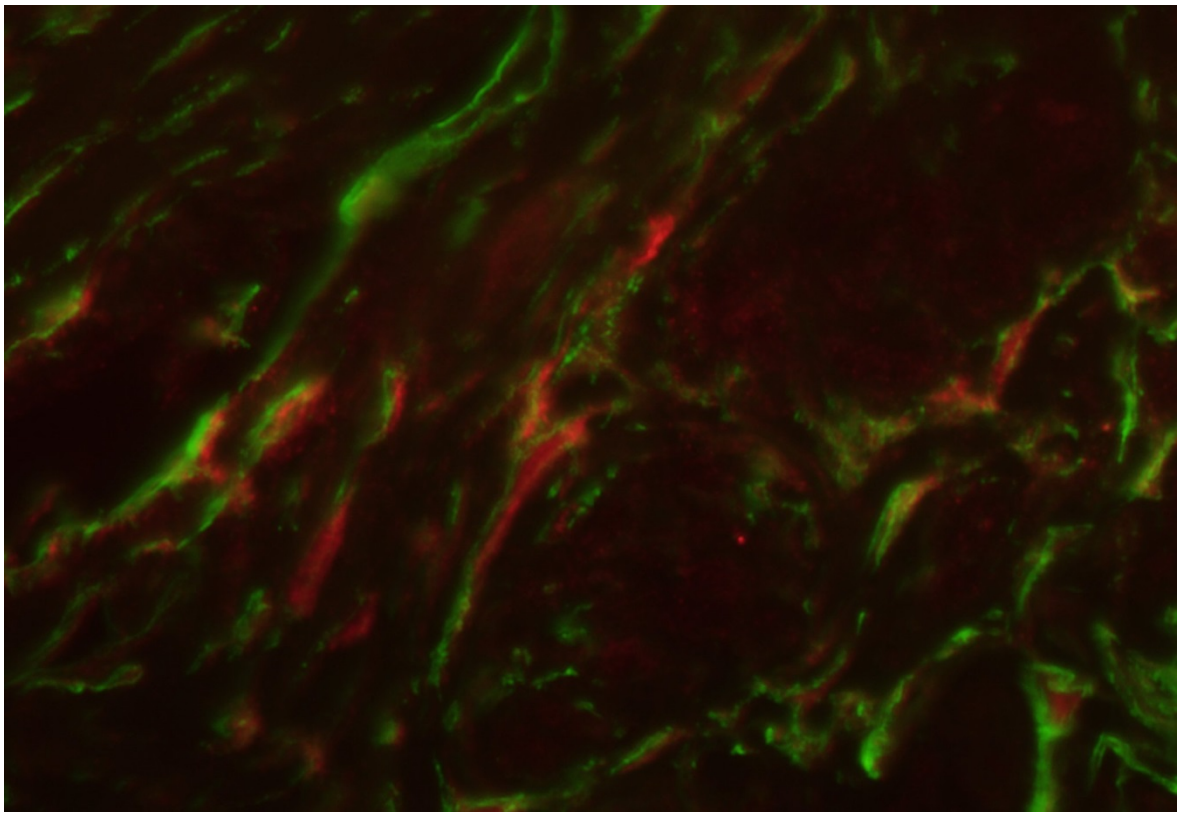

Fig. 1 Myometrial tissue sample stained for CD34 (green, Alexa Fluor 488) and PGP 9.5 (red, Alexa Fluor 594). Nerve fibers (red network) are crossed by telocytes (marked by green) throughout or/and located in their vicinity. Total magnification: × 400.

Conclusions. Our results suggest that autonomic innervation and telocytes are involved in the microenvironment imbalance characteristic of uterine leiomyoma. Since NOS-positive nerves play an important role in oxidative stress modulation, they might lead to a decrease in the number of telocytes, which are crucial components in the pathogenesis of leiomyoma formation.

5.4.SUMMARY OF ARTICLE 4 "Identification of PDGFRα+ cells in the uterine fibroid – link between angiogenesis and uterine telocytes"

Introduction. Uterine leiomyoma is strongly associated with local hypoxia, stimulated in turn production of extracellular matrix as well as angiogenic response in the myometrial tissue. Hypoxia stimulates production of soluble fms-like tyrosine kinase 1 (sFlt-1) or VEGFR-1 (VEGF receptor-1), anti-angiogenic-related factor. it declines an apoptosis in myometrial cells with no further effect on leiomyoma cells. Among cells, having receptors to PDGF and VEGF and characterized by high sensitivity ho hypoxia, we can mark such cells as telocytes. The aim of the current study was to determine the link between myometrial TCs and vascularization in a human leiomyoma, and to clarify their possible role in the angiogenesis

Material and methods. Tissue samples from 20 patients with leiomyoma and 15 without were observed using routine histology, histochemistry and immunofluorescence (CD117, CD31, CD34, PDGFRα, tryptase, sFlt-1). Slides were examined using an MN800FL epifluorescence microscope (OptaTech, Warszawa, Poland) equipped with Olympus DP74 digital CCD camera. TCs were evaluated with respect to the relative frequency (arbitrarily graded as very few = (+), few = +, moderate density = + +, multiple density = + + +). The percentage of collagen deposits and muscle tissue have been analyzed in specimens, stained with Masson trichrome. The vascular density was evaluated by the analysis of CD31 and sFlt-1 (VEGFR-1) immunopositivity in all myometrial samples.

Results. The study group was characterized by the prevalence of collagen deposits in comparison with the control group (Table 2). The amount of muscle fibers was lower in compare with the adjacent myometrium. Conversely, the percentage of muscle fibers was higher in the normal myometrium, while collagen was scanty presented in this type of tissue. In addition, the more significant difference in contents of both components was

revealed in the myometrium, surrounded myometrial fibroids. The prevalence of muscle fibers correlates with poor organization of collagen deposits, that was approximately in 2.5 times lower in compare with muscles (Table 2).

Table 2. The percentage of the collagen and muscle fibers in different types of myometrium.

	Affected uterus		**Unaffected uterus**
	Fibroid	**Adjacent myometrium**	**Normal myomaterium**
Collagen (%)	44 ± 15	18 ± 6	24 ± 10
Muscle fibers (%)	33 ± 13	45 ± 13	43 ± 10

The declining of the telocytes density in the foci of fibroids correlated with a poor vascularization inside leiomyoma. The expression of sFlt-1significantly raised inside a fibroid. In leiomyoma the decrease of telocytes and blood microvessels density were accompanied by prevalence of collagen deposits, unlike the unaffected myometrium (Table 3).

Table 3. Relative frequency of c-kit-positive/tryptase-negative, CD34–positive and PRGFRα-positive, CD31-positive and sFlt-1-positive cells in different parts of human unaffected and affected by leiomyoma uterus. 0 = absence, (+)=very few, + =few, + + =moderate density, + + + =multiple density.

	c-kit+/ trypatse-	CD34+/PDGFRα+	CD31	VEGFR-1 (sFlt-1)
Normal Uterus				
Unaffected myometrium	+++	+++	+++	+
Myomatous Uterus				
Adjacent myometrium	++	++	++ +	+
Focus of fibroid	+	+	+	++

Uterine telocytes had elongated oval-shaped cellular bodies and were distributed among the intertwined myometrial fibers and in close vicinity to blood vessels (Fig. 2). The general pattern of their localization resembled parallel eccentric lines. However, in some parts, they reflected directional smooth muscle bundles and also were scantily presented in the foci of leiomyoma.

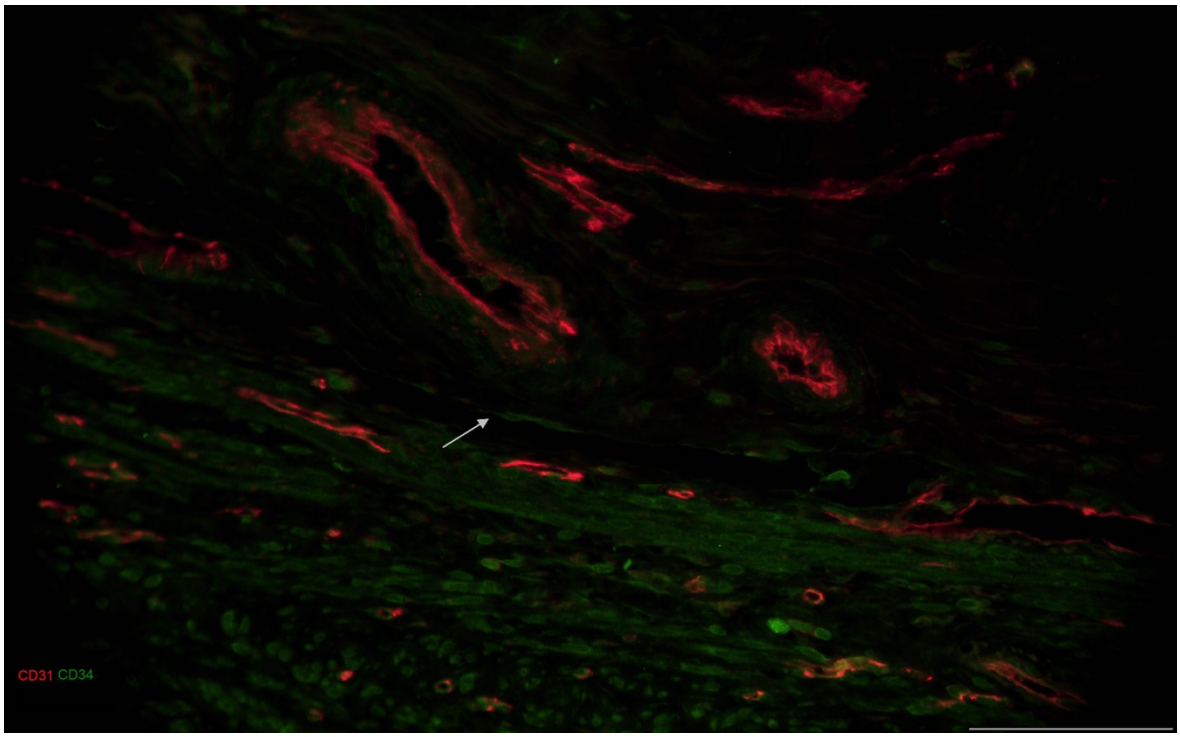

Fig. 2 Sample of leiomyoma stained for CD31 (red, Alexa Fluor 594) and CD 34 (green, Alexa Fluor 488). Double immunopositive structures were identified as vessels of different caliber, while cells with elongated bodies located between muscle fibers and close to blood vessels are identified as telocytes (one of them is marked by arrow on the image). Scale magnification bar: 50μm.

Conclusions. The decreasing of vascularization and TCs density in the foci of leiomyoma, accompanied by rising of the expression of VEGF receptor-1 (sFlt-1) amount. Close vicinity of TCs to blood vessels and its immunosensitive for growth factors' receptors reflect a putative role in the local angiogenesis.

6. ARTICLES

Article 1 (pages 31-48): Aleksandrovych V, Pasternak A, Basta P, Sajewicz M, Walocha JA, Gil K. Telocytes: facts, speculations and myths (Review article). Folia Med Cracov. 2017;57(1):5-22.

Article 2 (pages 49-62): Aleksandrovych V, Białas M, Pasternak A, Bereza T, Sajewicz M, Walocha J, Gil K. Identification of uterine telocytes and their architecture in leiomyoma. Folia Med Cracov. 2018;58(3):89-102.

Article 3 (pages 63-73): Aleksandrovych V, Kurnik-Łucka M, Bereza T, Białas M, Pasternak A, Cretoiu D, Walocha J, Gil K. The autonomic innervation and uterine telocyte interplay in leiomyoma formation. Cell Transplantation, (March 2019). doi:10.1177/0963689719833303.

Article 4 (pages 74-104): Aleksandrovych V, Bereza T, Białas M, Pasternak A, Walocha J, Pityński K, Gil K. Identification of PDGFRα+ cells in the uterine fibroid – link between angiogenesis and uterine telocytes. Archives of Medical Science. 2019: accepted

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Identification of uterine telocytes and their architecture in leiomyoma

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Abstract: Introduction: Uterine leiomyoma is the most widespread benign tumor affecting women of childbearing age. There are still gaps in the understanding of its pathogenesiss. Telocytes are unique cells described in greater than 50 different locations inside the human body. The functional relationship of cells could clarify the pathogenesis of leiomyomata. In the current study, we focused on the identification of telocytes in all regions of the human uterus to explain their involvement in leiomyoma development.
Materials and Methods: Tissue samples from a healthy and myomatous uterus were stained for c-kit, tryptase, CD34 and PDGFRα to identify telocytes. Routine histology was performed to analyze tissue morphology and collagen deposits.
Results: Telocytes were detected in the cervix, corpus of the uterus and leiomyoma. The density of telocytes in fibroid foci was reduced compared with normal myometrium.
Conclusions: Our results demonstrated the existence of telocytes in all parts of the human body affected and unaffected by leiomyoma of the uterus. In addition, telocytes were also present in leiomyoma foci. Our results suggest that the reduced density of telocytes is important for the pathomechanisms of myometrial growth, demonstrating its value as a main component of the myomatous architecture.

Key words: telocytes, uterus, extracellular matrix (ECM), stem cells, collagen, CD34, PDGFRα.

Introduction

The uterus is a unique myometrial organ that undergoes structural and functional remodeling. Successful implantation plays a crucial role in female reproductive processes. Rich vascularization, a network of autonomic innervation, sensitivity to hormonal regulation, and fluctuation in growth factors and cytokines, forms a physiological background of myometrial tissue contractility and growth. Unfortunately, the human uterus is commonly affected by leiomyomas and adenomyosis in childbearing age that lead to severe medical and social problems, such as infertility [1].

The most widespread benign monoclonal tumor of the female reproductive system is uterine leiomyoma (UL) originating from the Müllerian duct [2–4]. Despite a long history of discovery even in the time of Hypocrates and numerous explanations for thousands of years, the nature of fibroids is still not well understood [3]. Uterine fibroids exhibit heterogeneous cellular phenotypes but are consistently characterized by excessive production of extracellular matrix (ECM) that abnormally forms in fibroid foci [5–8]. The ECM consists of fibroblasts that are often termed myofibroblasts [9]. These cells produce collagen and other components of the matrix, but their inappropriate function causes fibrosis [10]. Most collagen deposits include type I [5] and type III collagen [11]. A similar trend is noted for the expression of its messenger ribonucleic acid (mRNA) [12]. Clear detailed observations of cell-cell interactions inside fibroids and adjacent myometrium reveal the pathomechanisms of ECM composition.

For the past twenty years, the scientific community has been intently discussing a new type of cells — interstitial Cajal-like cells (ICLC), which are also referred to as fibroblast-like cells or PDGFRα-positive cells. Since 2010, these cells have been identified as telocytes (TCs) in nomenclature with unique features of identification [13–15]. TCs exhibit an oval-shaped body with several long extensions, called telopodes, which represent a cell form and exhibit variability in homo and heterocellular contacts [16, 17]. Their thick and thin parts (podoms and podomers, respectively) are variable in size in pregnant and nonpregnant myometrium (Table 1) [13, 15, 18–21].

TCs are completely different from fibroblasts and mesenchymal stem cells in a variety of features, including phenotype. Their gene profile contains thousands of up- and downregulated genes compared with other cells. Some of these genes are involved in tissue remodeling. Collagen type IV is upregulated in cultured TCs [22]. These cells were described in several diseases [23–25]. Systematic sclerosis (SSc) was accompanied by ultrastructural alterations (swollen mitochondria, cytoplasmic vacuolization and presence of lipofuscinic bodies) of TCs that are reduced in the skin, correlating with disease subsets and stages. Moreover, the same changes were

described in the gastric wall (submucosa and muscle layers), the myocardium and the lung [26]. Manetti *et al.* observed limited and diffuse cutaneous SSc in early and advanced stages and concluded that the damage and loss of TCs might be caused by an ischemic injury as TCs appear to be more sensitive to ischemia compared with other stromal cell types, such as fibroblasts, myofibroblasts and mast cells [27]. A reduction in TCs in organs affected by SSc might be a cause of uncontrolled fibroblast/myofibroblast activity [26, 27].

Table 1. Differences in TC morphology in pregnant and nonpregnant myometrium.

	Pregnant myometrium	Nonpregnant myometrium
Length of telopodes (Tps)	Normal	Longer
Podomers of telocytes	Thinner (75.53 ± 1.81 nm)	Thicker (81.94 ± 1.77 nm)
Podoms of telocytes	Thicker (316.38 ± 17.56 nm)	Thinner (268.60 ± 8.27 nm)
Evidence of exosomes/shedding microvesicles (SMVs)	Normal	Lower
Diameter of extracellular vesicles measured in the myometrial interstitium	58–405 nm	65–362 nm
*Median value	151 nm	170 nm
Exosomes: SMVs	20 vs. 168	26 vs. 89
Mean diameter of exosomes/SMVs	No difference	No difference

The aim of our study was to determine the location of TCs in different parts of the human uterus (exo- and endocervix, corpus and focus of fibroid) and to clarify their possible role in the architecture of leiomyomata.

Material and Methods

Subjects

Nineteen patients with symptomatic UL were scheduled for elective surgery (laparoscopic hysterectomy) and selected for the study group (19 women, mean age 59.5 ± 14.6 years). Patients with UL exhibited detectable tumors in the uterus during gynecological examination before the operation. They presented with mild, recurrent episodes of vaginal bleeding and pain. The control group consisted of 15 patients (15 women, mean age 57.6 ± 12.8 years) who underwent elective surgery for other

reasons and had no pre- or intraoperative signs of uterine fibroids. Hysterectomy was performed according to the standard procedure. Postsurgery histological examination did not reveal any signs of UL. Tissue samples from the foci of fibrosis and adjacent myometrium were obtained from the study group for further observation. Samples of unaffected myometrium were also prepared from the control group. All patients were surgically treated at the Institute of Gynecology and Obstetrics at the Jagiellonian University Medical College in 2018.

Ethical approval

The study was conducted in accordance with the moral, ethical, regulatory and scientific principles governing clinical research. All surgical samples were retrieved with the approval of the Jagiellonian University Bioethical Committee using procedures that conformed to the Declaration of Helsinki guidelines (protocol number 122.6120.40.2016).

Tissue processing

Fresh hysterectomy specimens were collected and rinsed thoroughly with PBS (phosphate-buffered saline, 0.01 M, pH = 7.4), fixed in 4% phosphate-buffered paraformaldehyde, and routinely processed and embedded in paraffin. Serial sections were cut and mounted on poly-L-lysine-coated glass slides.

Routine histology

The sections were deparaffinized, rehydrated and stained with either hematoxylin–eosin (H&E) to evaluate the gross tissue organization or Masson trichrome staining to detect collagen deposits.

Immunofluorescence

Indirect double immunofluorescence after heat-induced epitope retrieval was used for simultaneous visualization of two antigens. After deparaffinization and rehydration, the slides were incubated for 30 min in PBS with appropriate normal serum at room temperature followed by overnight incubation at 4°C in a solution of PBS with appropriate normal serum containing primary antibody (or mixture of primary antibodies) and 0.3% Triton X-100 (Sigma, USA). After 5 washes (10 min each) in PBS, the specimens were then incubated for 1 h at room temperature with secondary antibody (or mixture of secondary antibodies) diluted in PBS. Finally, the slides were washed twice in PBS (10 min each) and cover-slipped

with Fluorescence Mounting Medium (Dako, Denmark). Labeled specimens were analyzed immediately. The primary antisera and secondary antibodies used are listed in Table 2.

Table 2. Type, sources and dilution of antibodies.

Antibody	Catalog number and company	Dilution
Primary antibodies		
Polyclonal rabbit anti-c-kit	A4502, Dako	1 : 100
Monoclonal mouse anti-CD34	M7165, Dako	1 : 100
Polyclonal goat anti-PDGFR alpha	AF-307-NA, R&D Systems	1 : 100
Monoclonal Mouse anti-tryptase	M7052, Dako	1 : 100
Secondary antibodies		
Alexa Fluor 488 Goat Anti-Mouse	115-545-146, Jackson ImmunoResearch	1 : 400
Alexa Fluor 594 Goat Anti-Rabbit	111-585-144, Jackson ImmunoResearch	1 : 400
Alexa Fluor 594 Donkey Anti-Goat	705-585-003, Jackson ImmunoResearch	1 : 400
Alexa Fluor 488 Rabbit Anti-Mouse	315-545-045, Jackson ImmunoResearch	1 : 400

Microscopic examination

Slides were examined using an MN800FL epifluorescence microscope (OptaTech, Warszawa, Poland) equipped with a Jenoptik Progress C15Plus color camera. Digital images were collected at either 200× or 400× magnification. Qualitative analysis of cells was provided in 10 consecutive high-power fields of vision (400×) using the computer-based image analysis system Multiscan 18.03 software (CSS, Warszawa, Poland). All samples were assessed by two independent specialists (each blinded to the other) without any knowledge of the clinical parameters or other prognostic factors to avoid bias. The use of mast cell tryptase staining enabled c-kit-positive mast cells to be distinguished from c-kit-positive TCs. TCs were considered cells that were c-kit positive and tryptase negative concurrently with the characteristic morphology in tissue samples. Additionally, cells double positive for CD34 and PDGFRα with characteristic morphology and localization were also recognized as TCs. In all sections, the immunoreactive cells identified were evaluated with respect to the relative frequency (arbitrarily graded as very few = (+), few = +, moderate density = + +, multiply density = + + +). The percentage of collagen deposits and muscle tissue were analyzed in specimens stained with Masson trichrome. The collagen and muscle fiber volume ratio was assessed in ten different fields of each sample.

Results

The histopathological observation of the human myomatous and unaffected uterus using hematoxylin and eosin and Masson's trichrome staining was performed (Fig. 1, 2). Corpus of myomatous uterus presented as foci of UL and adjacent myometrium. Immunofluorescent labeling was also used for all samples: the corpus and uterine cervix (exo- and endocervix) as well as leiomyoma foci. We assessed

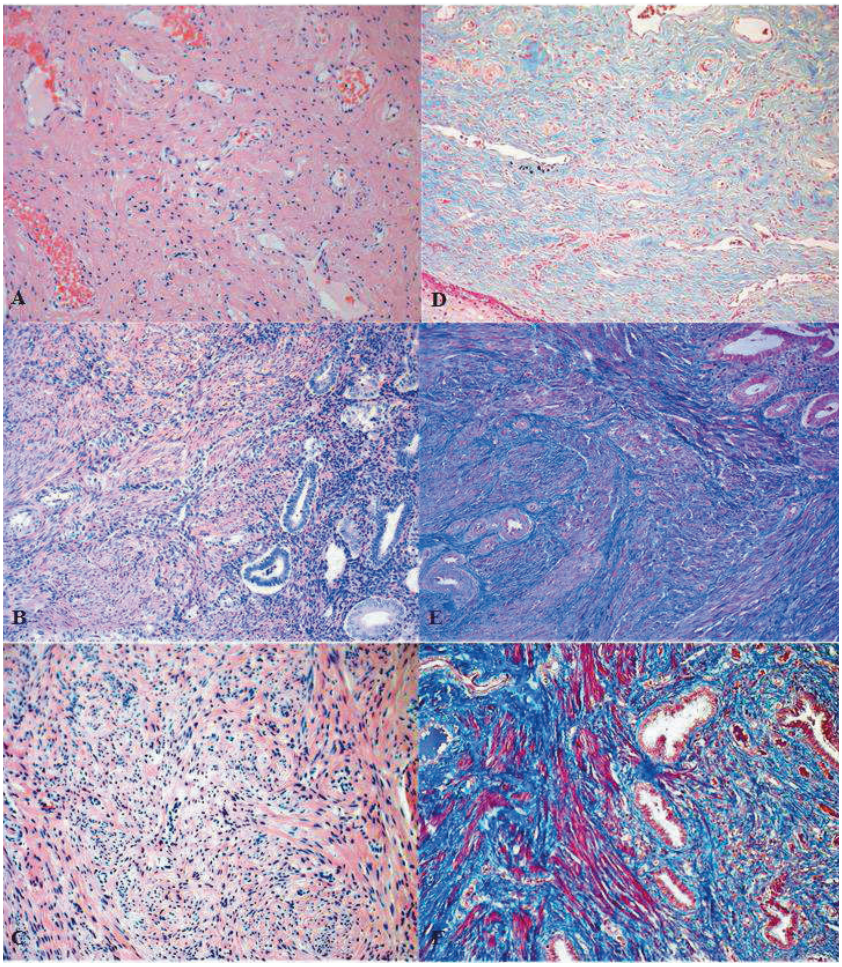

Fig. 1. Hematoxylin-eosin and Masson's trichrome stained sections of human uterus unaffected by leiomyoma. The sections from exocervix (A, D) endocervix (B, E) and myometrium from the uterus body (C, F). On Masson's trichrome staining, collagen deposits appear as blue, and muscle fibers appear as red. Total magnification: 200×.

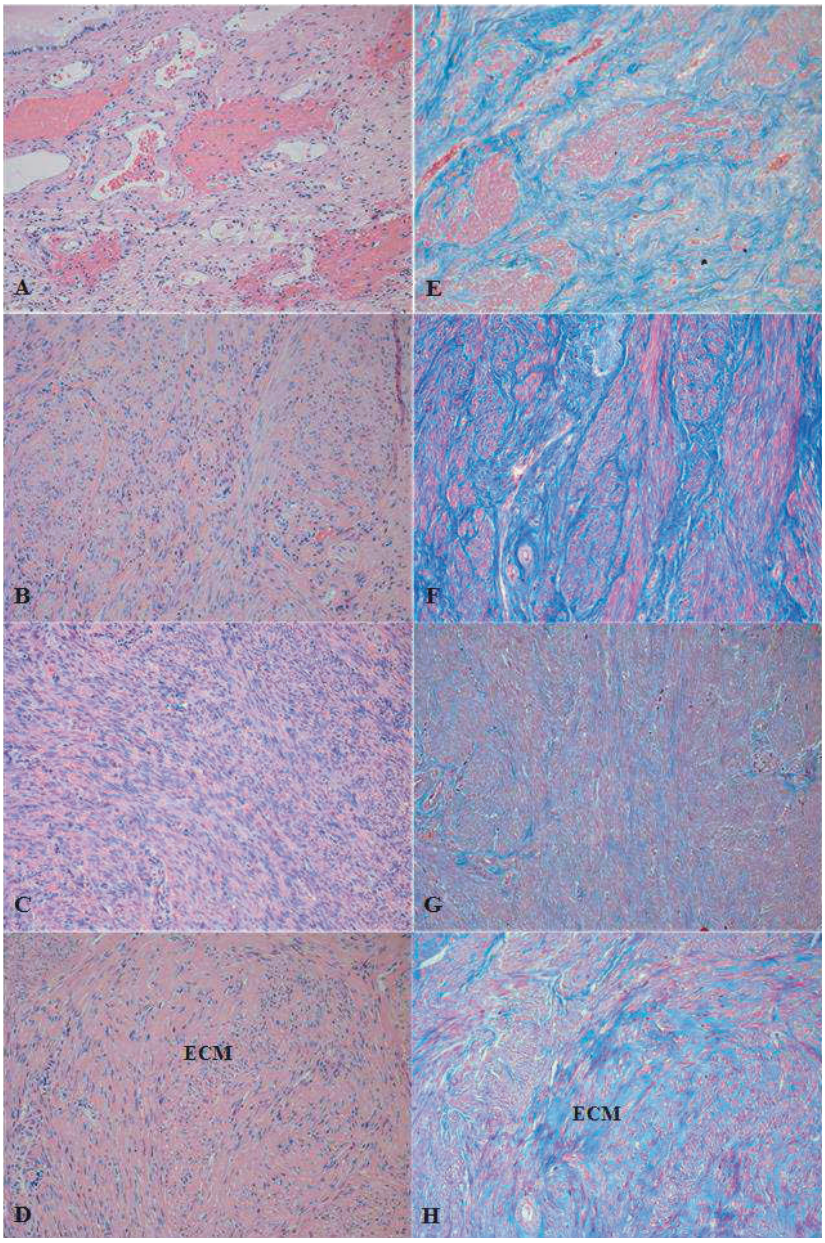

Fig. 2. Hematoxylin-eosin and Masson's trichrome stained sections of human myomatous uterus. Sections from the exocervix (A, E) endocervix (B, F), leiomyoma foci (D, H) and adjacent myometrium from the same uterus (C, G). On Masson's trichrome staining, collagen deposits appear as blue, and muscle fibers appear as red. Fragments of disordered smooth-muscle cells separated by abundant extracellular matrix (ECM). Total magnification: 200×.

mostly currently proven markers: CD34, PDGFRα and canonic c-kit (Fig. 3, 4). Double immunolabeling for c-kit and tryptase was used for the identification of mast cells and subsequent signs of inflammation. The c-kit-positive/mast cell tryptase-negative cells were considered TCs. CD34-positive and PDGFRα-positive cells were detected as uterine TCs.

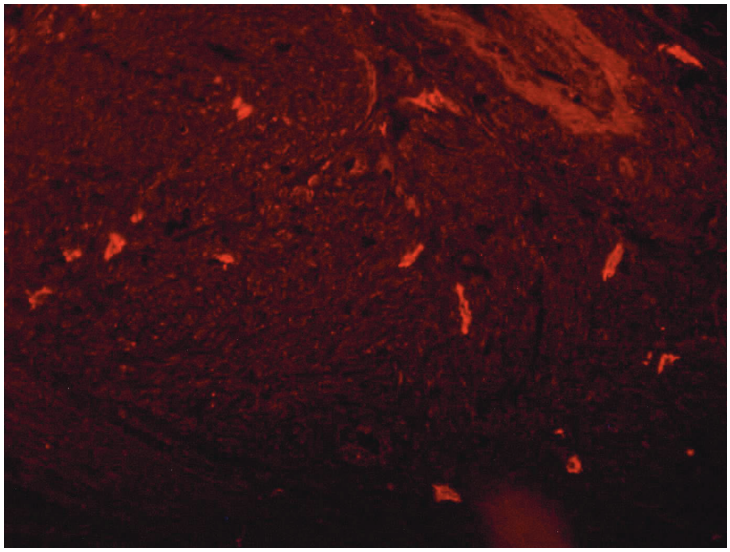

Fig. 3. Sample from the a leiomyoma focus stained for c-kit (red, Alexa Fluor 594). Total magnification: 400×.

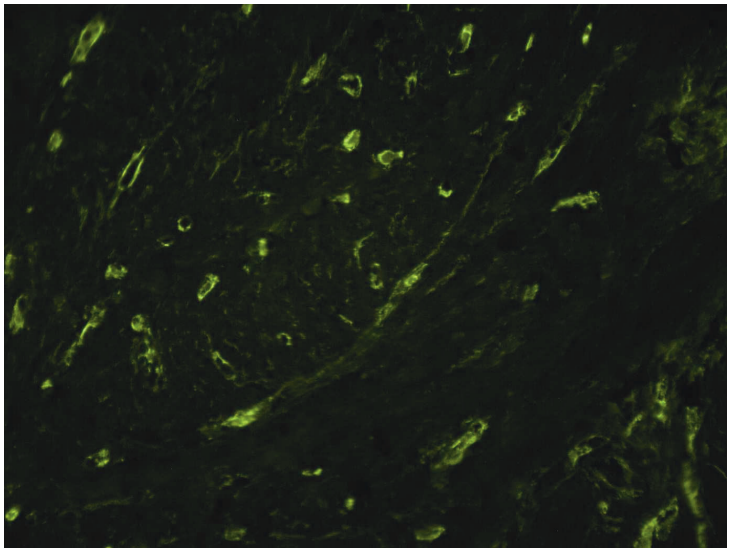

Fig. 4. Sample from a leiomyoma focus stained for CD34 (green, Alexa Fluor 488). Total magnification: 400×.

Hematoxylin and eosin staining demonstrated than UL were mainly composed of smooth muscle cells and fibrous connective tissue. Smooth muscle cells exhibited a uniform spindle size and shape with rhabditiform nuclei. The adjacent myometrium and fibroid foci were cytologically identical, but the latter exhibited circumscription, nodularity and denser cellularity. Masson's trichrome staining revealed the prevalence of collagen deposits in UL compared with all other observed samples.

We found that cells with the characteristic morphology and immunopositivity were located in all parts of the human uterus. These cells exhibit a triangular or spindle body with long, slender, moniliform cytoplasmic extensions. The endocervix contains more c-kit-positive, CD34-positive and PDGFRα-immunopositive cells compared with the exocervix. These cells formed bundles mainly located longitudinally (parallel to the cervical canal). No differences in TC density were noted in all parts of the uterine cervix between myomatous and healthy uteruse.

In the corpus of the uterus, TCs were located in close vicinity to blood vessels and inside muscle bundles. The general pattern of their localization resembled parallel eccentric lines in UL. We stressed that CD34-immunopositive and PDGFRα-immunopositive cells were observed in leiomyoma foci as well as in adjacent and control myometrium (Fig. 5). In all sections, immunoreactive cells were evaluated with respect to the relative frequency (arbitrarily graded as very few = (+), few = +, moderate density = + +, multiply density = + + +) (Table 3). Subjective qualitative analyses exhibited a reduction in TC density in fibroids compare with both types of unaffected myometrium (adjacent and from healthy uterus).

Table 3. Relative frequency of c-kit-positive/tryptase-negative, CD34-positive and PRGFRα-positive cells in different parts of human uterus that was not affected or affected by leiomyoma. 0 = absence of telocytes, (+) = very few, + = few, + + = moderate density, + + + = multiply density.

	c-kit+/trypatse–	CD34+/ PDGFRα+
Normal Uterus		
Exocervix	(+)	(+)
Endocervix	+	+
Corpus	+++	+++
Myomatous Uterus		
Exocervix	(+)	(+)
Endocervix	+	+
Corpus	++	++
Fibroid	+	+

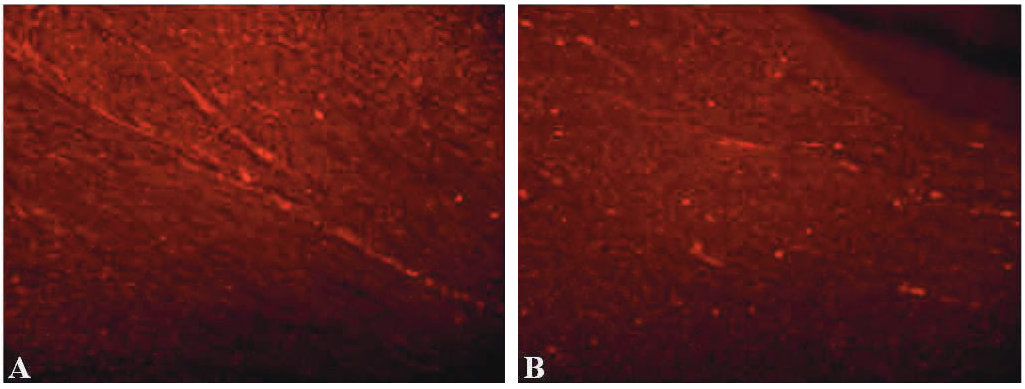

Fig. 5. Uterine samples stained for PDGFRα (red, Alexa Fluor 594) from uterus unaffected by leiomyoma myometrium (A) and fibroid focus (B). Telocytes with their longitudinal extensions are mostly located among the intertwined myometrial fibers and in close vicinity to blood vessels. Total magnification: 400×.

Discussion

This study presents evidence for the presence of TCs in different parts of the uterus, including exocervix, endocervix, and corpus in human healthy uterus, as well as fibroid foci in myomatous uterus. Identification was based on morphological and immunocytochemical criteria in fluorescence microscopy. We assumed that CD34- and PDGFRα-positive cells are TCs. In addition, c-kit-positive cells and trypatase-negative cells were also recognized as TCs.

Homo and heterocellular contacts between TCs and smooth muscle cells, nerves, immunocytes (macrophages, mast cells and lymphocytes), stem cells, melanocytes, erythrocytes and Schwann cells highlight their involvement in creating of 3D structure of tissue and facilitating muscle contractions and immune responses. The crucial role of these cells in the physiology and pathophysiology of organs has been described and hypothetically focused on their involvement in pathomechanisms of various diseases. Uterine TCs could represent a key cell type in the uterine leiomyoma that exhibits its own architecture despite its monoclonal origin [28–30].

The main feature of each uterine fibroid is excessive production of ECM that could play a role in the storage of cytokines, chemokines, growth factors, angiogenic and inflammatory response mediators that subsequently stimulate cell growth and differentiation [9]. Uterine smooth muscle cells and fibroblasts produce several growth factors that are present in different amounts in fibroids and adjacent myometrium. The ECM of leiomyomas demonstrates the focal localization of basic fibroblast growth factor (FGF)-2 and insulin-like growth factor (IGF)-I. The amount of epidermal growth factor (EGF) is significantly reduced in UL compared

with normal myometrium. Dixon *et al.* illustrated a hormonal regulation of growth factor production based on the suggestion that EGF secretion could be regulated by progesterone (and not estrogen) [31].

Richter *et al.* assessed the correlation between collagen type I deposits and TC distribution in heart muscle [32]. Fibroblasts produced collagen type I upon stimulation by growth factors. In the normal human heart, TCs were identified in close vicinity to thin collagen fibrils. In contrast, in heart failure, some parts of myocardium have been replaced by focuses of fibrosis that are grossly characterized by excessive amounts of type I collagen. In these areas of the heart, no TCs were identified. Zhao *et al*. stressed that TCs in the myocardium are important for maintenance of the physiological integrity of heart muscle [33]. Of note, a direct correlation was observed between collagen type I deposits and the presence of TCs. Moreover, the number of TCs and Tps was positively correlated with degraded collagen type I [32]. Tps were characterized by shrinkage and shortening in areas of abundant ECM. We observed the same results in the myometrium by comparing the density of uterus TCs in fibroid foci characterized by excessive amounts of ECM and normal myometrium. In UL, an increased ECM density correlates with rare cell observations that represent typical morphological and immunohistochemical features of TCs.

Another important feature of leiomyoma is the origination of the cell population. The myometrium itself has a regenerative capacity. Ono *et al*. observed two groups of leiomyoma-derived cell populations: side and main populations. The first population was undifferentiated and rarely expressed steroid hormone receptors and smooth muscle cell markers. After some time, these cells naturally express all receptors and become similar to the main population, which is common for fibroids. Ono *et al.* stressed the importance of paracrine factor-mediated signals from steroid receptor-positive cells adjacent to leiomyoma-derived side population cells [34]. We suggest that TCs dominate among adjacent cells. TCs exhibit place-dependent specificity for estrogen and progesterone receptors. TCs in the gallbladder are negative for both types of receptors [35] but positive in myometrium, Fallopian tubes or human urinary bladder [36–38]. TCs could play the role of effector cells in paracrine cooperation between steroid hormones and side population cells, as reported by the Ono scientific group [34]. We also want to emphasize that undifferentiated cells in UL are an important component of fibroid cell architecture and appear to be somatic stem cells.

Telocytes were detected in the stem cell niche of different organs, such as the heart, lung, skeletal muscle, and skin [39–43]. Heterocellular contacts between these two types of cells explain the possible involvement of TCs in tissue regeneration and repair. The first explanation of microenvironment-controlled stem cell activity, which is referred to as the niche, was provided 30 years ago [44]. Since that time,

numerous studies observed interplay between stem cell behavior and the surrounding tissue. Stem cells not only respond to multiple stimuli but also have an impact on the organism; it is therefore important to consider each stem cell interaction in both directions [45]. Gherghiceanu *et al.* focused on TC involvement in cardiac stem cell homeostasis [43]. Popescu *et al.* observed TCs-stem cells complexes in subepithelial niches of the bronchiolar tree [42]. Perlea *et al.* discussed possible detection of TCs in dental pulp stem niches [40], whereas Ye *et al.* assessed the genetic profile of murine lung TCs and their functional role in the stem cell niche [41]. We hypothesized that the bilateral interaction between uterine TCs and stem cells could represent a step in the pathogenesis of leiomyoma.

Our study proved the existence of telocytes in different parts of the human uterus (cervix, corpus, focuses of fibroids) — both affected and not affected by leiomyoma. Qualitative analysis revealed the reduction of TCs in fibroid foci, whereas the prevalence of collagen deposits was detected using routine histology. We attempted to explain the place of TCs in myoma architecture and focused on basic connecting points. We intend to continue our research to clarify the versatility of myometrial TCs and their fascinating properties.

Conflict of interest

None declared.

Author contribution

Veronika Aleksandrovych, Krzysztof Gil: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, study supervision, and final approval of the manuscript. Magdalena Białas: histology, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, and final approval of the manuscript. Artur Pasternak: searching bibliographic databases, editing and revising of the manuscript, analysis and interpretation of data, and final acceptance of the manuscript. Jerzy Walocha, Tomasz Bereza and Marek Sajewicz: analysis and interpretation of data and final acceptance of the manuscript.

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Original Article

The Autonomic Innervation and Uterine Telocyte Interplay in Leiomyoma Formation

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Abstract

The autonomic innervation of the uterus is involved in multiple pathophysiological processes in both humans and animals. Pathological conditions such as adenomyosis or inflammatory pelvic disease are usually accompanied by significant alterations in uterine innervation. In the current study, we focused on autonomic innervation of uterine fibroids, the identification of recently described interstitial cells, telocytes, and the possible interplay between these structures. In this work, uterine telocytes were identified by immunopositivity for c-kit, CD34, and PDGFRα. Nerves were revealed by immunolabeling for neuronal markers: protein gene product PGP 9.5, inducible nitric oxide synthase (iNOS), choline acetyltransferase (ChAT), and tyrosine hydroxylase (TH). The gross organization of myometrial tissue has been analyzed by routine histology. The results demonstrated that the density of iNOS and ChAT-immunopositive neurons in the uterine fibroids was higher than that in the control samples. The density of telocytes in the fibrosis foci was lower than that in the normal myometrium. Our results suggest that autonomic innervation and telocytes are involved in the microenvironment imbalance characteristic of uterine leiomyoma. Since NOS-positive nerves play an important role in oxidative stress modulation, they might lead to a decrease in the number of telocytes, which are crucial components in the pathogenesis of leiomyoma formation.

Keywords

leiomyoma, telocytes, protein gene product 9.5 (PGP 9.5), nitric oxide synthase (NOS), PDGFRα

Introduction

Uterine innervation is derived from two components: afferent (interoceptive type) and efferent (autonomic type)[1]. The uterine autonomic nerve fibers release noradrenaline (from sympathetic endings) and acetylcholine (from parasympathetic fibers). The characteristic local feature is that axonal endings are not in close contact with myocytes, but neurotransmitters are secreted in the perifascicular space[2]. The neurogenic component is invaluably important for uterine contractility and blood flow regulation. It plays an important role in the pathophysiological mechanisms of chronic pelvic pain and co-occurs in such diseases as endometriosis, adenomyosis, inflammatory pelvic disease and leiomyomata. Foci of adenomyosis are characterized by a decline up to a disappearance of nerve fibers around lesions, whereas uterine tissue samples from subjects with chronic pelvic pain are characterized by a proliferation of small-diameter nerve fibers, which can be asymmetric, throughout the myometrial stroma[3].

Previous studies of the innervation of the mammalian female reproductive system have revealed the great importance of the autonomic nervous system, especially adrenergic fibers[4,5]. For the primary identification of both myelinated and unmyelinated nerve fibers in the uterus, the protein gene product 9.5 (PGP 9.5) has been widely used; for

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example, in tissue samples from women affected by endometriosis[6] or uterine fibroids[7]. PGP 9.5 is a highly specific pan-neuronal marker used for the visualization of nerve cell bodies and fibers as well as their qualitative and quantitative assessment.

Adrenergic innervation is involved in myometrial contractility, blood flow, and endometrial secretory function[8]. The adrenergic contribution to uterine innervation was demonstrated by the presence of tyrosine hydroxylase (TH) immunoreactivity in myometrial tissue[5,8]. Several animal models have been used for observation of the sympathetic branch of the uterine autonomic nervous system; for example, rat, guinea pig, and equine models. TH-immunoreactive nerves were found in all regions of the equine uterus by Bae et al[5]. They were mostly located parallel to the muscle fibers and along blood vessels. Their density was higher in the myometrium than in the endometrium[4]. The same locational (myometrial) prevalence was found in the rat uterus. In its upper part, TH-immunoreactive nerves were observed in the longitudinal layer, whereas in the lower uterus, the circular layer was predominant[9]. Reproductive hormones also have an impact on sympathetic nerves. For instance, pregnancy-induced degeneration was reported in the adrenergic innervation of the guinea pig uterus[10]. In women with endometriosis, the prevalence of estrogens correlates with an increasing number of adrenergic nerves in myometrium and all endometrial layers (functional and basal)[11].

The human and animal uterus also contains abundant nitric oxide-synthesizing nerves that could be either autonomic and/or sensory. They are undoubtedly involved in the pathophysiology of common gynecological pathologies, including uterine fibroids, due to their participation in inflammatory reactions and oxidative stress[8,12,13].

Telocytes (TCs) are a newly discovered type of interstitial cell with unique morphology and functions, described in animals and humans. The TC has a small, oval-shaped cellular body, containing a nucleus surrounded by a small amount of cytoplasm[14–18]. TCs have a variable number of telopodes (Tps) (very long cellular extensions), which are probably the longest cellular prolongations in the human body. It is important to note that their form and amount could change during pregnancy or disease[19,20]. Ten morphological criteria named "the platinum standard" are currently used for their primary detection, which is always confirmed by immunohistochemical identification in tissues[14,19]. Making homo- and heterocellular contacts with myocytes, nerves, immune, and stem cells, these cells are involved in contractility and the immune response, and form a three-dimensional network that may function as a scaffold to define the correct organization of tissues and organs. TCs also take part in neurotransmission[21–24].

Despite progress in current gynecology, uterine leiomyoma (UL) is still the most widespread pathology that affects women of reproductive age. UL arises from uterine smooth muscle tissue and is characterized by the production of excessive quantities of extracellular matrix[25–27]. The aim of our study was to examine the autonomic nervous system and identify TCs in normal myometrium and ULs in order to reveal diversity in a gross structural organization between normal and affected myometrial tissue.

Materials and Methods

Subjects

Fifteen consecutive patients with symptomatic UL were scheduled for elective surgery (laparoscopic hysterectomy) and selected for the study group (15 women, mean age 54.7 $\pm$ 12.3 years). Patients with UL had detectable tumors in the uterus during gynecological examination before the operation. They presented with mild, recurrent episodes of vaginal bleeding and pain. The control group consisted of 15 consecutive patients (15 women, mean age 55.2 $\pm$ 13.5 years) who underwent elective surgery for other reasons and had no pre- or intraoperative signs of uterine fibroids. Hysterectomy was performed according to the standard procedure. Post-surgery histological examination did not reveal any signs of UL. Samples of tissue from the foci of fibrosis and adjacent myometrium were taken for further observation from the study group. Samples of unaffected myometrium were also prepared from the control group. All patients were surgically treated at the Institute of Gynecology Jagiellonian University Medical College in 2018.

Ethical Approval

The study was conducted in accordance with the moral, ethical, regulatory, and scientific principles governing clinical research. All surgical samples were retrieved with the approval of the Jagiellonian University Bioethical Committee using procedures that conformed to the Declaration of Helsinki guidelines (protocol number – 122.6120.40.2016).

Tissue Processing

Fresh hysterectomy specimens were collected and rinsed thoroughly with PBS (phosphate-buffered saline, 0.01 M, pH = 7.4), fixed in 4% phosphate-buffered paraformaldehyde, routinely processed and embedded in paraffin. Serial sections were cut and mounted on poly-L-lysine-coated glass slides.

Routine Histology

The sections were deparaffinized, rehydrated, and stained with either hematoxylin–eosin (H&E) to evaluate the gross tissue organization or Masson trichrome staining to detect collagen deposits.

Table 1. Type, Sources and Dilution of Antibodies.

Antibody	Catalog number and company	Dilution
Primary antibodies		
Polyclonal rabbit anti-PGP 9.5	Z5116, Dako, Denmark	1:200
Polyclonal goat anti-NOS	sc-49055, Santa Cruz, USA	1:100
Monoclonal mouse anti-ChAT	sc-55557, Santa Cruz, USA	1:100
Monoclonal mouse anti-TH	AB318, Millipore, USA	1:200
Polyclonal rabbit anti-c-kit	A4502, Dako, Denmark	1:100
Monoclonal mouse anti-CD34	M7165, Dako, Denmark	1:100
Polyclonal goat anti-PDGFR alpha	AF-307-NA, R&D Systems, USA	1:100
Monoclonal mouse anti-tryptase	M7052, Dako, Denmark	1:100
Secondary antibodies		
Biotinylated goat anti-mouse	115-065-146, Jackson ImmunoResearch, USA	1:500
Cy3-conjugated polyclonal goat anti-rabbit	111-165-144, Jackson ImmunoResearch, USA	1:500
FITC-conjugated streptavidin	016-010-084, Jackson ImmunoResearch, USA	1:500
FITC-conjugated polyclonal donkey anti-goat	J2609, Santa Cruz, USA	1:40
Alexa Fluor 488 Goat Anti-Mouse	115-545-146, Jackson ImmunoResearch, USA	1:400
Alexa Fluor 594 Goat Anti-Rabbit	111-585-144, Jackson ImmunoResearch, USA	1:400
Alexa Fluor 488 Rabbit Anti-Mouse	315-545-045, Jackson ImmunoResearch, USA	1:400
Alexa Fluor 488 Goat Anti-Rabbit	111-545-144, Jackson ImmunoResearch, USA	1:400
Alexa Fluor 594 Donkey Anti-Goat	705-585-003, Jackson ImmunoResearch, USA	1:400

Immunofluorescence

After deparaffinization and rehydration, the slides were incubated for 30 min in PBS with the appropriate normal serum and 0.3% Triton X-100 (Sigma, USA) at room temperature, followed by overnight incubation at 4°C in a solution of PBS with the appropriate normal serum containing a primary antibody (or a mixture of primary antibodies) and 0.3% Triton X-100. After 5 washes (10 min each) in PBS, the specimens were incubated for 1 h at room temperature with a secondary antibody (or a mixture of secondary antibodies) diluted in PBS with the appropriate normal serum and 0.3% Triton X-100. Finally, the slides were washed in two changes (10 min each) of PBS and cover-slipped with fluorescence mounting medium (Dako, Denmark). Label specimens were analyzed immediately. The following primary and secondary antisera were used (Table 1).

Microscopic Examination

Slides were examined using an MN800FL epifluorescence microscope (OptaTech, Warszawa, Poland) equipped with a Jenoptik Progress C15Plus color camera (Figs 1, 2, 4) and Olympus DP74 digital CCD camera (Figs 3, 6, 7). In turn, immunofluorescence in CD34/PDGFRα-positive cells (Fig. 5) was detected and analyzed using the scanning confocal microscopy (FV1200, Olympus). Digital images were collected at either 200× or 400× magnification. The qualitative analysis of cells and nerve fibers was provided in 10 consecutive high-power fields of vision (400×) using the computer-based image analysis system Multiscan 18.03 software (CSS, Warsaw, Poland). All samples were assessed by two independent specialists (each blinded to the other) without any knowledge of the clinical parameters or other prognostic factors to avoid bias. The presence and distribution of the pan-neuronal marker PGP 9.5 immunoreactivity was evaluated to assess the uterine autonomic innervation. Nerve cells and nerve fibers were evaluated on the basis of their morphology. TH, choline acetyltransferase (ChAT), and inducible nitric oxide synthase (iNOS) immunoreactivity were evaluated to assess the presence and distribution of different populations and subtypes of autonomic nerves. The use of mast cell tryptase staining enabled c-kit-positive mast cells to be distinguished from c-kit-positive TCs. TCs were considered as cells that were c-kit positive and tryptase negative concurrently, with the characteristic morphology and distribution[28–30] in tissue samples. In addition, cells positive for CD34 and PDGFRα with the characteristic morphology and localization were also recognized as TCs.

Results

Light microscopy of uterine fibroids, adjacent myometrium, and normal myometrium using Masson's trichrome staining for collagen revealed collagen to be abundant in the fibroid tissue, while the myometrium had sparse, well-aligned collagen bundles adjacent to smooth muscle cells (Fig. 1). Hematoxylin and eosin staining demonstrated that myomas were mainly composed of smooth muscle cells and fibrous connective tissue. Smooth muscle cells were uniformly sized and spindle shaped with rhabditiform nuclei. Cells were arranged in a swirl-type pattern.

In the foci of fibroids, nerve fibers immunoreactive for PGP 9.5 were particularly parallel to each other and formed bundles around myometrial nodules. Most of them were placed in the fibroid pseudocapsule (Fig. 2, images A, B, and C). However, single neurons and plexuses of nerve

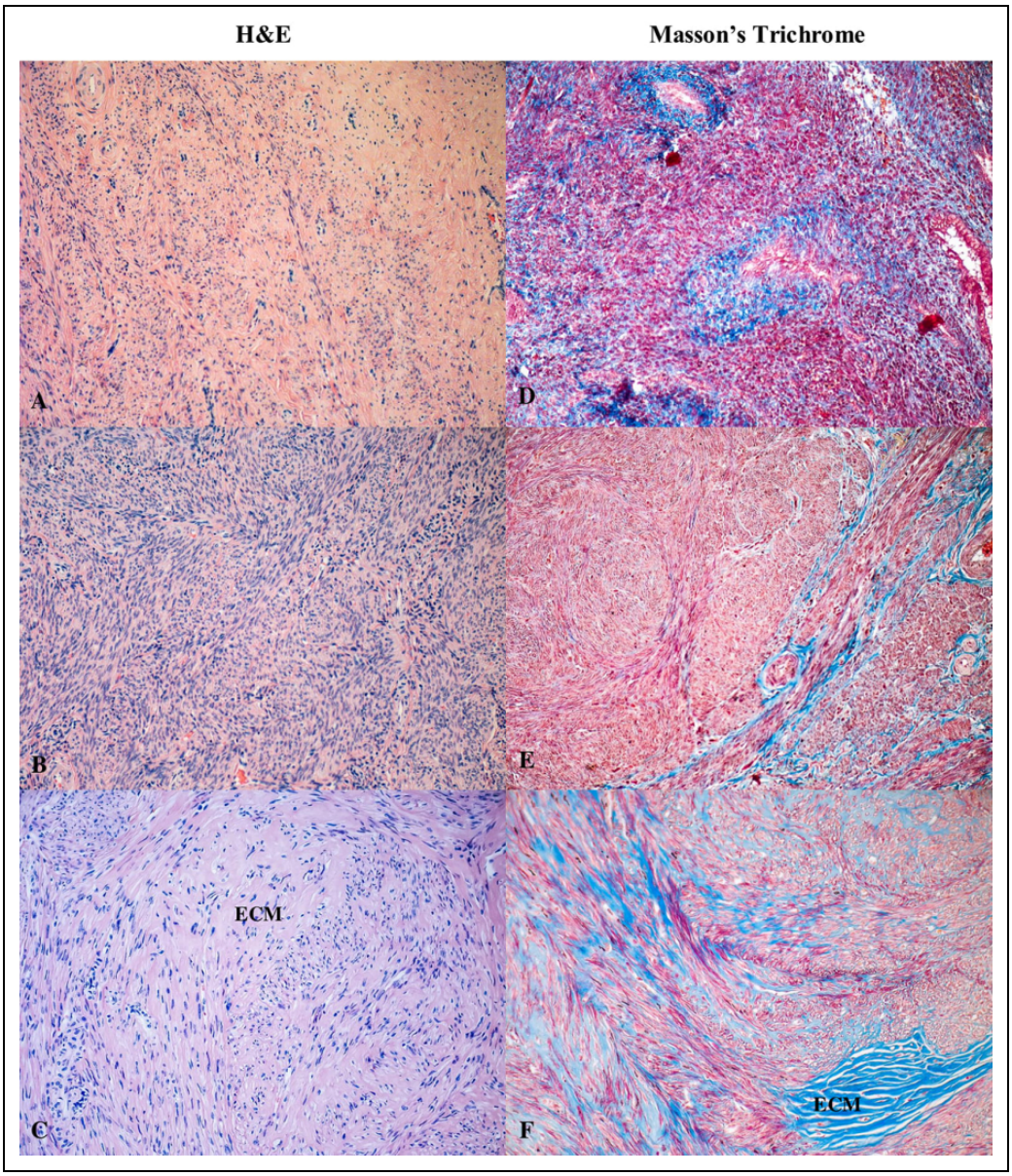

Figure 1. Hematoxylin–eosin and Masson's trichrome stained sections of human myometrium. The myometrium sections from the control group (**A**, **D**) compared with the foci of leiomyoma (**C**, **E**) and adjacent myometrium from the same uterus (**B**, **F**). With Masson's trichrome staining, collagen deposits were blue and muscle fibers were red. Fragments of disordered smooth muscle cells were separated by abundant extracellular matrix. Total magnification: × 200.

fibers were observed inside leiomyomata. Normal myometrium had individual nerve fibers positive for PGP 9.5. The surrounding myometrium from myomatous uteri showed the presence of nerve fibers immunopositive for PGP 9.5 whereas its number was lower than that in the foci of myoma but higher than in the normal myometrium.

Nerves with immunopositivity for TH were detected in the foci of leiomyoma, adjacent myometrium and normal myometrium (Fig. 2, images D, E and F). In unaffected myometrium single, thin fibers, without strict orientation in space manifested themselves as TH-positive neurons. In contrast, in fibroids, spindle-shaped nerve fibers were densely located parallel to the muscular bundles. In samples of adjacent myometrial tissue, its orientation had less strict character—individual fibers repeated the eccentric lines framing the muscle fibers.

A network of nerve bundles and fibers with ChAT-immunoreactivity was found in the uterine fibroids. They formed a net structure inside the myoma and were distributed around the perimeter of collagen deposits. Conversely, in the normal myometrium, single nerves were parallel to muscle fibers. Fewer ChAT-positive nerve fibers, which are in a longitudinal direction, were revealed in unaffected tissue (Fig. 2, images G, H and I).

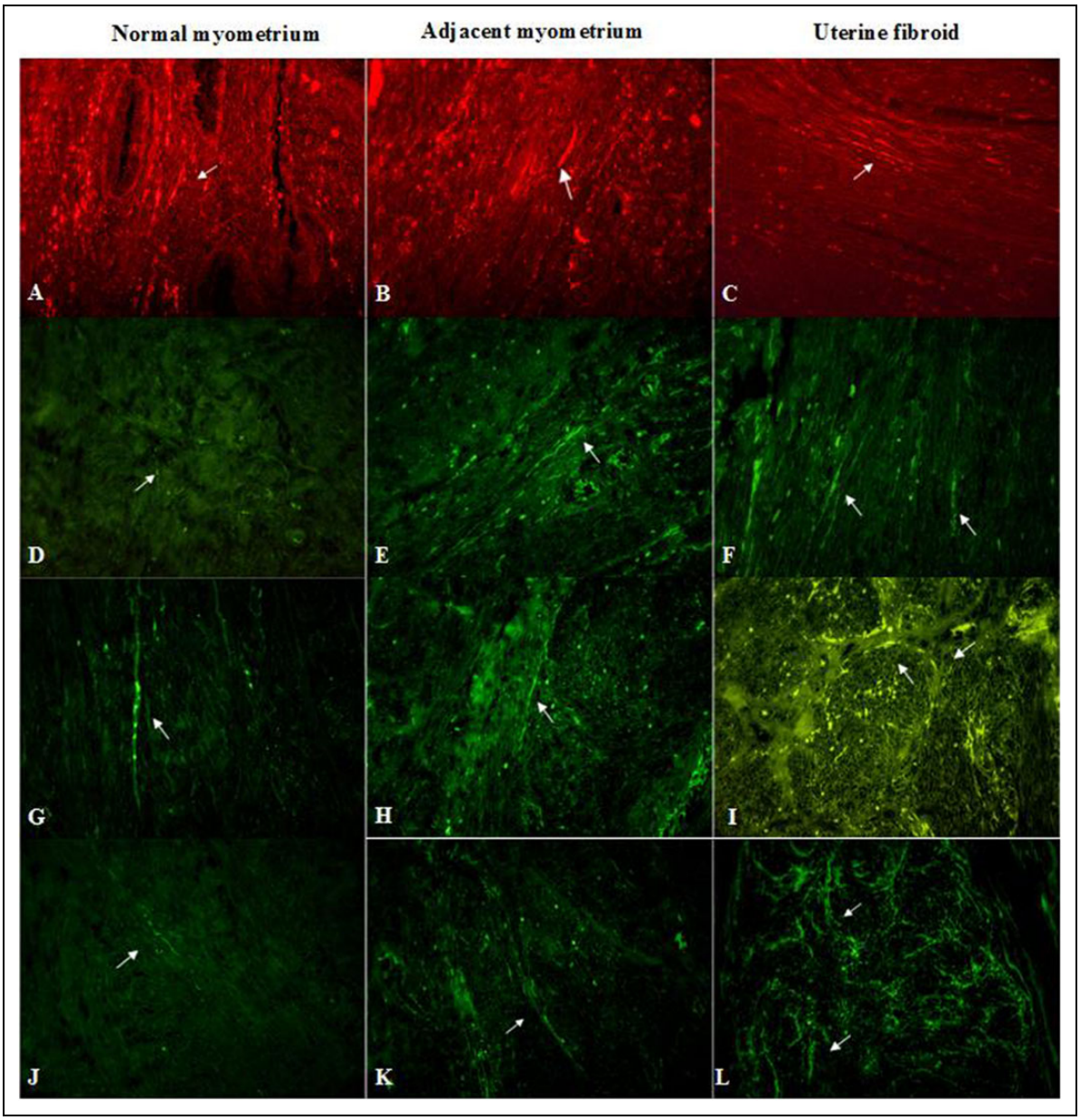

Figure 2. Myometrial samples stained for PGP 9.5 (red, Cy3) – A, B, C; TH (green, FITC) – D, E, F; ChAT (green, FITC) – G, H, I; and NOS (green, FITC) – J, K, L in an unaffected uterus (A, D, G, J), the foci of leiomyoma (C, F, I, L) and adjacent myometrium (B, E, H, K). Arrows indicate immunopositive nerve fibers in all samples. Total magnification: × 400.

Nerves immunoreactive for iNOS were found throughout all samples. Numerous iNOS-immunoreactive nerve bundles and fibers were found in the uterine myoma. They were distributed around vessels and muscle bundles, repeating its nodular structure. The density of immunoreactivity for iNOS was greater in the uterine fibroids than in the adjacent myometrium. In the normal myometrium from the control group, few linear nerves were observed between muscle layers (Fig. 2, images J, K and L).

Double immunolabeling for c-kit and tryptase was performed for the identification of mast cells. C-kit and tryptase double-positive mast cells were generally round or oval shaped, with a centrally located nucleus. The c-kit-positive/mast cell tryptase-negative cells were considered TCs (Fig. 3).

The CD34-positive cells had an elongated oval-shaped cellular body (Fig. 4). They were located among the intertwined myometrial fibers and in close vicinity to blood vessels. The general pattern of their localization resembled parallel eccentric lines. However, in some regions their localization reflected the direction of smooth muscle bundles. PDGFRα-positive cells were found throughout all samples.

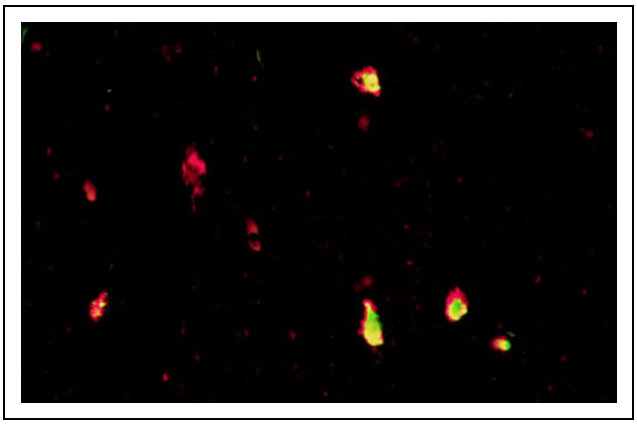

Figure 3. Tissue sample from the foci of leiomyoma stained for c-kit (red, Alexa Fluor 594) and tryptase (green, Alexa Fluor 488). C-kit-positive/tryptase-negative cells have red color and presented by telocytes, while c-kit-positive/tryptase-positive mast cells are yellow because of combination of both colors. Total magnification: × 400.

PDGFRα-positive cells were mostly present in normal myometrium close to blood vessels, whereas some single cells were separately observed in leiomyomas and the adjacent space (Fig. 5).

Double immunostaining for such neuronal markers as iNOS and PGP 9.5 combined with a telocyte marker CD34 revealed double-immunolabeled cells (Figs 6, 7).

Discussion

In this study, autonomic innervation combined with the detection of TCs was observed in normal myometrium and in leiomyomata. We expect that the interaction between TCs and nerves might be significant for the pathophysiological mechanisms of uterine fibroids, and thus we examined disease-affected and unaffected specimens of myometrial segments from patients with UL. In particular, we identified TCs by CD34 and PDGFRα immunolabeling. We also used

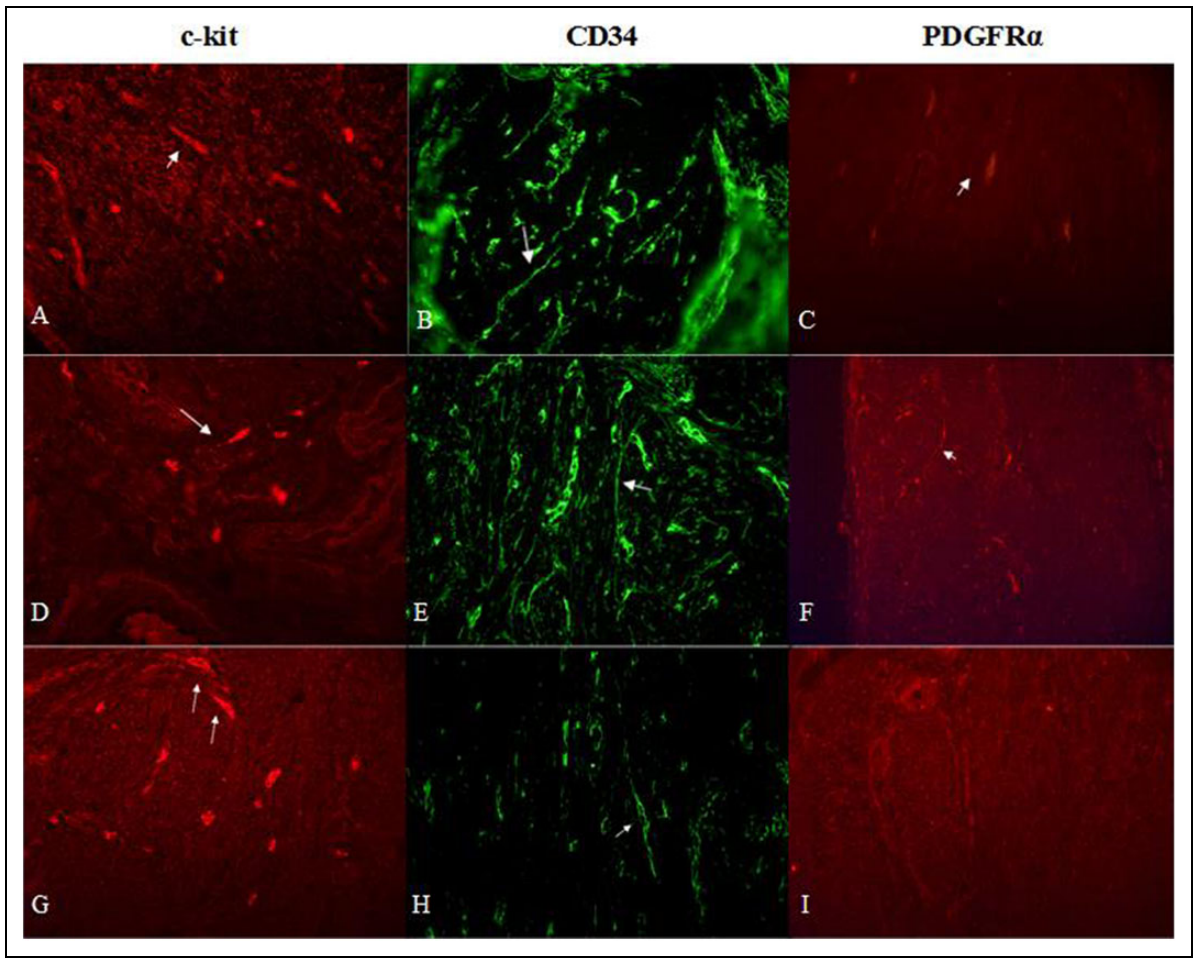

Figure 4. Myometrial samples stained for c-kit (red, Alexa Fluor 594), CD34 (green, Alexa Fluor 488) and PDGFRα (red, Alexa Fluor 594) in an unaffected uterus (A, B, C) and affected by leiomyoma (focus of fibroid (G, H, I) and adjacent myometrium (D, E, F)). Arrows indicate telocytes in all samples. Total magnification: × 400.

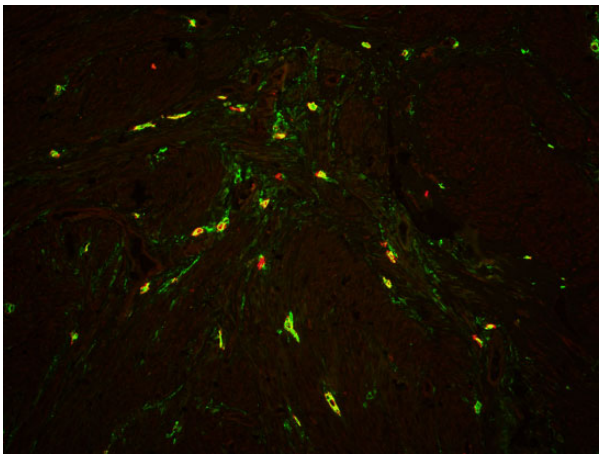

Figure 5. Sample of leiomyoma stained for PDGFR alpha (red, Alexa Fluor 594) and CD 34 (green, Alexa Fluor 488). Double-immunopositive cells with elongated bodies located between muscle fibers and close to blood vessels are identified as telocytes. Laser scanning confocal microscopy FV1200 (Olympus). Total magnification: × 400.

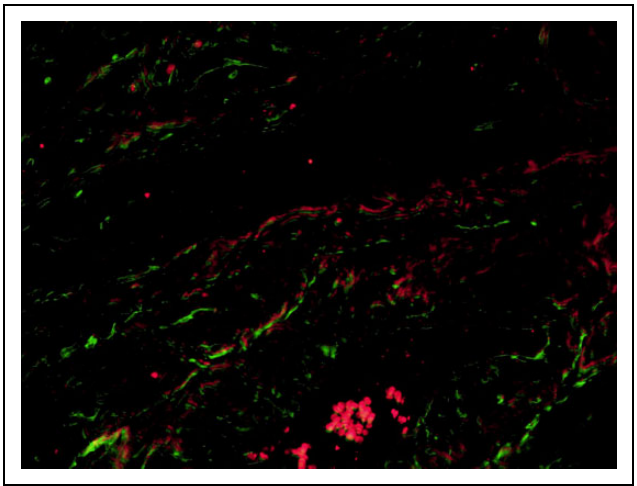

Figure 6. Double immunolabeling of uterine fibroid's tissue for CD34 (green, Alexa Fluor 488) and iNOS (red, Alexa Fluor 594). Nerves are presented as red filaments accompanied by green structures (telocytes and blood vessels) in the longitudinal direction. Total magnification: × 400.

CD34/c-kit and c-kit/tryptase double immunolabeling to clearly distinguish TCs from mast cells, respectively.

The density of TCs and its possible interplay with nerves have been described in a variety of muscular organs (for example, intestine, uterus, heart, Fallopian tubes). For instance, the human appendix has more TCs than do the other parts of the digestive system, which is explained by its complex innervation[20]. The myenteric plexus of the human appendix consists of several distinct networks, localized between and within the circular and longitudinal

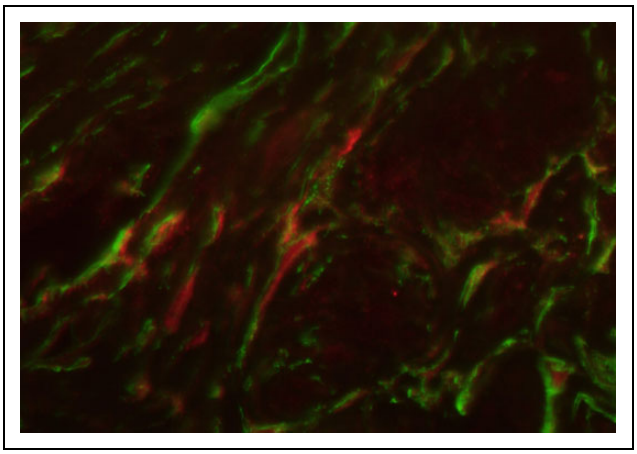

Figure 7. Myometrial tissue sample stained for CD34 (green, Alexa Fluor 488) and PGP 9.5 (red, Alexa Fluor 594). Nerve fibers (red network) are crossed by telocytes (marked by green) throughout or/and located in their vicinity. Total magnification: × 400.

muscle layers. Hanani noted this morphological feature as unique in comparison with other parts of the intestine[31]. According to the functional classification, enteric motor neurons are either inhibitory (e.g., NOS-positive neurons) or excitatory (e.g., ChAT-positive neurons)[32]. They both play an important role in the regulation of vascular tone and myometrial contractility as well. In Crohn's disease, for instance, the gut dysmotility is accompanied by a decrease in and disappearance of TCs[30]. The cholinergic excitatory output is suppressed in animal models of Crohn's colitis and ulcerative colitis[33]. Despite the decrease in TCs in UL, the density of iNOS and ChAT-positive neurons increased. We hypothesize that changes in the number of TCs lead to local myometrial misbalance.

UL usually has a highly vascularized pseudocapsule formed by compressed myometrium. Its architecture is similar to unaffected myometrium and contains nerve fibers and neuropeptides. The normal myometrium and pseudocapsule of fibroids exhibited similar immunoreactivity for PGP 9.5[34–36]. Díaz-Flores et al. stressed that TCs are present in neuromuscular spindles, mostly located in the inner and outermost layer of the capsule[37]. A pronounced tendency has been revealed by Zhang et al. during observation of patients with endometriosis, adenomyosis, and uterine fibroids accompanied by pain or without it[38]. Women with pain symptoms have PGP 9.5-immunoactive nerve fibers in the functional layer of the endometrium, while those without pain do not. Thus, PGP 9.5-immunoreactive nerves might be involved in mechanisms of pain generation in common gynecological diseases[38–41]. Our results showed the prevalence of PGP 9.5-positive nerves in the pseudocapsule.

Adrenoceptors might also be involved in leiomyoma growth. Receptors with noradrenaline as a neuromediator are divided in two groups with regional domination. Most α1-adrenergic receptors are present in circular bundles of the

uterus (cervico-isthmic area), whereas α2-adrenergic receptors are common in the body and have three subtypes (A, B, and C). Likewise, β2 adrenergic receptors are located in the uterine body. Generally, α-adrenergic receptors are involved in contraction, while β-adrenoreceptors play a role in tocolysis. However, the role of α2-, β1-, and β3-receptors in myometrial contractility is not clear[2]. Controversial data encourage attention to the dynamics of adrenergic receptors in uterine fibroids and their possible involvement in pathogenesis. Lee et al. described the expression of β-adrenergic receptor subtypes at different levels of UL cells and adjacent myometrium[42]. The distribution of β1-adrenergic receptor expression was the same in the two cell types, while β2-adrenergic receptors were more highly expressed in UL than in normal myometrium. No difference in β3-adrenergic receptor expression was found. These authors found that c-fos induction by *Scutellaria barbata D. Don* in uterine fibroid cells led to a regression of leiomyoma. At the same time, Adolfsson et al. found that the α2/β2-adrenoceptor ratio was increased in leiomyoma, due to a significant decrease in β2-adrenoceptor expression[43].

Uterine autonomic innervation is influenced by hormonal regulation. Estrogens enhance the growth of UL and depress the development of uterine innervation (especially the sympathetic branch of the autonomic nervous system)[44–46]. TCs also express estrogen and progesterone receptors, which are specific for their localization. These cells might function as affecters and/or effectors in the pathogenesis of UL. Gevaert et al. have already described their possible role in signal transduction between the urothelium and the underlying nerve endings and stressed the role of the regional expression of hormonal receptors in upper lamina propria TCs[47].

Nitric oxide (NO) is produced in neurons from L-arginine by the action of the enzyme nitric oxide synthase (NOS). This process usually passes upon stimulation by pro-inflammatory cytokines (Interleukin-1, tumor necrosis factor α, and interferon γ)[48]. NO is a potent dilatator of smooth muscle. In the uterus, myometrium contains NO-synthesizing nerves that could be autonomic and/or sensory. Some NOS-positive nerves in the uterus are parasympathetic and originate from neurons in the pelvic paracervical ganglia, and some are sensory and originate from neurons in thoracic, lumbar, and sacral dorsal root ganglia[49,50]. Papka et al. did not report any NOS-positive sympathetic nerves in the uterus. These authors described that in parasympathetic neurons, NOS-immunoreactivity coexists with acetylcholinesterase immunoreactivity in sensory nerves[49]. TH-positive neurons of the paracervical ganglia do not contain NOS-reactivity but some of them are apposed by NOS-varicosities[50]. iNOS expression is higher in leiomyoma than in normal myometrial cells[7,51]. Moreover, the uterus with leiomyoma or adenomyosis exhibited a higher expression of endothelial NOS, especially in cases associated with symptoms (menorrhagia and dysmenorrhea)[12]. This result illustrates that NOS may be involved in the pathomechanisms of invasion and excess growth of myometrium, similar to the process of vessel formation. Increased iNOS activity may decrease the tubal ciliary beat frequency and oviductal smooth muscle activity, and consequently could lead to tubal factor infertility[48–52]. On the other hand, in the uterus, NO plays a key role in mechanisms of uterine cyclicity[53], decidualization[54], and implantation[55]. Balance in the iNOS/NO system is essential for successful early implantation, pregnancy, and labor. In animal models, uterine TCs activated peritoneal macrophages and stimulated production of iNOS[48]. Consequently, TCs should be involved in all main pathomechanisms of uterine and tubal reproductive function. Double immunostaining for neuronal markers such as iNOS and PGP 9.5 combined with a TC marker CD34 in our specimens confirmed/demonstrated its interaction. Mostly cells that we considered as TCs were in parallel to nerve fibers in the myometrial tissue. We assume that partially CD34 immunopositive structures may represent the myometrial vessels. However, some of them were identified as TCs as well, that commonly form a network with smooth muscle bundles as well as nerve fibers.

Endometrial stem cells in culture differentiate into high-efficiency cholinergic neurons after stimulation with nerve growth factor and basic fibroblast growth factor. Moreover, ChAT activity increases[56]. Interaction between these growth factors and TCs is unclear, but TCs have immunopositivity for several growth factors, including vascular endothelial growth factor and plated-derived growth factor receptor alpha and beta, which merit further investigation in this area.

The alterations in the presence and location of adrenergic and cholinergic innervation in the human myomatous uterus indicate an important role for neural factors in the pathogenesis of the disease. The location of TCs, their immunopositivity to hormonal receptors, and their ability to induce NOS production give enough reasons to suggest their essential role in the regulation of myometrial proliferation. The close vicinity of TCs with nerve endings demonstrates the unique involvement of these cells in neuronal regulation in the uterus; however, the role of the cell–cell interaction with nerve fibers needs greater explanation. Further observation of TCs in the context of innervation in the healthy and myomatous uterus is needed as well.

Impact Statement

The current research has scientific value because of its primacy. There are no previous descriptions of the interplay between telocytes and autonomic innervation in leiomyomata. This study integrates modern knowledge of the pathological mechanisms of one of the oldest gynecological diseases, uterine leiomyoma. The presence of TCs in the foci of uterine fibroids and changes in density correlate with the myometrial structure. On the other hand, the difference in adrenergic and cholinergic innervation between affected and unaffected myometrium demonstrates the importance of the neuronal component in fibroid development. The correlation

of those components brings new features to the pathogenesis of leiomyoma.

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Author Contribution

Veronika Aleksandrovych, Krzysztof Gil: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis; study supervision; final approval of the manuscript. Magdalena Kurnik-Łucka; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; final approval of the manuscript. Tomasz Bereza: collection of data; critical revision of the manuscript for important intellectual content; final approval of the manuscript Magdalena Białas: histology; analysis and interpretation of data; critical revision of the manuscript for important intellectual content; final approval of the manuscript. Dragos Cretoiu: critical revision of the manuscript for important intellectual content; final approval of the manuscript. Artur Pasternak: searching bibliographic databases; editing and revising of the manuscript; analysis and interpretation of data; final acceptance of the manuscript. Jerzy A. Walocha: critical revision of the manuscript for important intellectual content; final approval of the manuscript.

Ethical Approval

The study was conducted in accordance with the moral, ethical, regulatory and scientific principles governing clinical research. All surgical samples were retrieved with the approval of the Jagiellonian University Bioethical Committee using procedures that conformed to the Declaration of Helsinki guidelines (protocol number – 122.6120.40.2016).

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Jagiellonian University Bioethical Committee (protocol number – 122.6120.40.2016) approved protocols.

Statement of Informed Consent

Written informed consent was obtained from the patients for their anonymized information to be published in this article.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Decision letter:
June 14, 2019
AMS-09271-2019-02
Identification of PDFGRα+ cells in uterine fibroids - link between angiogenesis and uterine telocytes.

Dear Dr Krzysztof Gil,

I am pleased to inform you that your manuscript, entitled: Identification of PDFGRα+ cells in uterine fibroids - link between angiogenesis and uterine telocytes., has been finally accepted for publication in Archives of Medical Science.

We would like to inform that your paper will be published after receiving publishing fee. In order to receive the invoice, please complete the form including data for invoicing, which is available in the payment bookmark. Failure to complete this form means that you choose not to obtain the invoice.

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Thank you for submitting your work to our journal.

Kindest regards,
Prof. Maciej Banach, MD, PhD, FAHA, FESC, FNLA
Editor-in-Chief,
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Review 1:
Most of my queries were properly addressed. In my opinion the manuscript is ready for publication.

Review 2:
This study may be publish in “ Archives of Medical Science” in present form.

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Archives of Medical Science

Identification of PDFGRα+ cells in uterine fibroids - link between angiogenesis and uterine telocytes.

Type

Research paper

Keywords

angiogenesis, hypoxia, leiomyoma, CD34, telocytes, interstitial Cajal-like cells

Abstract

Introduction
Telocytes (TCs), labelled also as Interstitial Cajal-like cells (ICLC), CD34□-cells or PDGFR□+-cells (platelet-derived growth factor receptor alpha positive cells), a new type of cells of mesenchymal origin, were described over one decade ago. The unique nature of these cells still deserves attention from the scientific community. Telocytes make homo- and heterocellular contact with myocytes, immunocytes and nerves, have their own immunohistochemical and secretome profiles and thus might regulate local regenerative processes including angiogenesis and fibrosis. The aim of our study was to observe the missing link between angiogenesis and telocytes in leiomyoma, the most common benign tumors affecting women of reproductive age.

Material and methods
We observed uterus tissue samples from leiomyoma, adjacent myometrium and unchanged tissue from patients with leiomyoma and control subjects using routine histology, histochemistry, immunofluorescence (CD117, CD31, CD34, PDGFRα, tryptase, sFlt-1) and image analysis methods.

Results
The declining of the telocytes density in the foci of fibroids correlated with a poor vascularization inside leiomyoma. Moreover, the expression of sFlt-1 (anti-angiogenic-related factor) significantly raised inside a fibroid. In leiomyoma the decrease of telocytes and blood micro-vessels density were accompanied by prevalence of collagen deposits, unlike the unaffected myometrium.

Conclusions
Our results provide the demonstration of TCs in human uterine fibroids and highlight their possible involvement in the pathogenesis of myometrial pathology in context of the angiogenesis.

Explanation letter

Editor-in-Chief of the Journal of Archives of Medical Science
Professor Maciej Banach

Dear Sir,
□

I am sending herewith a copy of revised manuscript.

We thank Editors and Reviewer for their opinions and remarks which helped us to improve our paper. We revised the manuscript according to all mentioned suggestions. Corrections have been applied to the text of the manuscript (marked red).

Yours sincerely,
Veronika Aleksandrovych Tomasz Bereza, Magdalena Ulatowska-Białas, Artur Pasternak, Jerzy A. Walocha, Kazimierz Pityński & Krzysztof Gil
Krakow, 2019/06/01

Response to Reviewer 1 comments:
Comments to the Author
The submitted manuscript reports an interesting, labour-intensive and time-consuming study. However, there are number of queries that have to be addressed.

Answer: We thank Reviewer 1 for his/her opinions and remarks which helped us to improve our paper.

Specific comments:
1. So-called uterus tissue or unaffected tissue do not exist.

Answer: The term “unaffected tissue” exists and is commonly used in medical articles. Moreover, this collocation had been accepted by highly qualified native English speaking editors at American Journal Experts (the certificate has been attached). Also, mentioned collocation is widely used even in titles of articles published by BMC Cancer (“Prevalence of HMTV in breast carcinomas and unaffected tissue from Mexican women”). “Uterus tissue” has been replaced with “uterine tissue” (marked red). We assume that some pathologies do not use this collocation, but it is still popular in oncological journals. As an example, we can demonstrate an article in British Journal of Cancer: L Chung, S Shibli, K Moore, E E Elder, F M Boyle, D J Marsh and R C Baxter. Tissue biomarkers of breast cancer and their association with conventional pathologic features. Br J Cancer. 2013 Feb 5; 108(2): 351–360.
However, according to reviewer suggestion we have replaced this term with: “unaffected tissue” has been replaced with “unchanged tissue” (marked red).

2. Origin, size, number and topographical characteristic of selected leiomyomas should be added. Any differences between cervical and the one from the fundus and body of the uterus or between subserosal and subendometiral or with and without calcification?

Answer: Detailed morphological characteristics of leiomyomas has been added (marked red). No cases of cervical leiomyoma and any kind of degeneration had not been included in the current research.

3. Most of the used pronouns (e.g., we, our) should be replaced by the passive voice.

Answer: The quality of the text is based on the revision by editors at American Journal Experts (the certificate has been attached).

4. In most cases authors evaluated incidence no frequency since time was fixed for all individuals.

Answer: Our research has mostly an observational character. We took histological samples after hysterectomy, without former deep analysis of patient’s histories. Our aim was primary observe telocytes and its possible correlation with angiogenesis. We intend to add more clinical data in our

future research and articles.

5. Statistical analysis was not performed.

Answer: This pilot study could present only semi-quantitative analysis of immunopositive cells content. We used a common scale for such describing (grading with an amount of pluses).

6. The discussion is interesting. However, limitations and clinical implications of the study should be added.

Answer: Clinical implications of the study has been added in the discussion (marked red). Limitations of the current study are not clear because of the morphological character of the research. Our selection of patients is based on the presence or absence of leiomyoma without any co-existence of different pathological conditions, affected human myometrium in patients.

7. The list of references should be uniformed according to instruction for authors.

Answer: The list of reference has been prepared according to instruction on AMS website ("instructions for authors").

Response to Reviewer 2 comments:
Comments to the Author
Dear Editor
I am writing about paper entitled as "Identification of PDFGRα+ cells in uterine fibroids – link between angiogenesis and uterine telocytes". Paper was evaluated and my report as following.
Sincerely

Report

Recently, telocytes (TCs) were described as a new cell type in the interstitial space of many organs, including myometrium. TCs are cells with very long, distinctive extensions named telopodes (Tps). It is suggested that TCs play a major role in intercellular signaling, as well as in morphogenesis, especially in morphogenetic bioelectrical signaling. Platelet-derived growth factor receptor alpha (PDGFRα) expression is frequently observed in many kinds of cancer and is a candidate for therapeutic targeting.

In this manner study is interesting and research has include new original findings.
This study may be publish in " Archives of Medical Science" in present form.

Answers: We thank Reviewer 2 for his/her opinions.

Response to Reviewer 3 comments:
Comments to the Author
The manuscript presents the preliminary results. The title of the study suggests more than it actually is in the manuscript. TCs cells were found and possible ways of further testing were given, TCs cells were found and possible ways of further testing were given, but these associations and regulations were not analyzed. Molecular research methodology is correct. Statistic analysis, understandably, lack. The work is simple but presented in a communicative way.

Answers: We thank Reviewer 3 for his/her opinions and remarks which helped us to improve our paper. We intend to expand the topic and add different biomedical techniques in further observation of telocytes with focusing on angiogenesis in human myometrium. This pilot study will be a good basis for our next paper. Statistical analysis has semi-quantitative analysis because of the simplicity of the research object. However, we will improve this part by adding other biomedical techniques to

our research, that will allow to have a sufficient statistical assessment.

Response_to_Reviewer_V_Alekasandrovych.docx
AJE_Aleksandrovych et al.pdf

AMS Archives of Medical Science

Abstract

Introduction: Telocytes (TCs), labelled also as Interstitial Cajal-like cells (ICLC), $CD34^{+}$-cells or $PDGFR\alpha^{+}$-cells (platelet-derived growth factor receptor alpha positive cells), a new type of cells of mesenchymal origin, were described over one decade ago. The unique nature of these cells still deserves attention from the scientific community. Telocytes make homo- and heterocellular contact with myocytes, immunocytes and nerves, have their own immunohistochemical and secretome profiles and thus might regulate local regenerative processes including angiogenesis and fibrosis. The aim of our study was to observe the missing link between angiogenesis and telocytes in leiomyoma, the most common benign tumors affecting women of reproductive age.

Material and methods: We observed uterine tissue samples from leiomyoma, adjacent myometrium and unchanged tissue from patients with leiomyoma and control subjects using routine histology, histochemistry, immunofluorescence (CD117, CD31, CD34, PDGFRα, tryptase, sFlt-1) and image analysis methods.

Results: The declining of the telocytes density in the foci of fibroids correlated with a poor vascularization inside leiomyoma. Moreover, the expression of sFlt-1 (anti-angiogenic-related factor) significantly raised inside a fibroid. In leiomyoma the decrease of telocytes and blood micro-vessels density were accompanied by prevalence of collagen deposits, unlike the unchanged myometrium.

Conclusions: Our results provide the demonstration of TCs in human uterine fibroids and highlight their possible involvement in the pathogenesis of myometrial pathology in context of the angiogenesis.

Keywords: angiogenesis; telocytes; interstitial Cajal-like cells; leiomyoma; hypoxia; CD34.

Introduction

Currently, the most common benign tumor affecting up to 80% of all women of reproductive age is still uterine leiomyoma (UL) [1]. This monoclonal myometrial diploid formation can be the source of considerable quality-of-life issues for approximately 25% of all women who suffer from clinically significant symptoms [2]. The majority of them can be divided into three groups: abnormal bleeding, pressure/pain, and reproductive problems [3]. Moreover, UL has effects on the endometrium and implantation [1]. Until now, risk factors for UL have been described by numerous publications, whereas not one theory exists explaining its pathogenesis. The majority of ULs (60%) are chromosomally normal, and the remainder share similar tumor-specific abnormalities [4]. Undoubtedly, growth factors, cytokines, chemokines and hormonal misbalance play a crucial role in the development of the pathomorphological features of UL (fibrosis, angiogenesis and immune response). The effector cells involved in these processes could be a key opening the nature of widespread gynecological pathology [5]. The following growth factors are among those most involved in the pathogenesis of UL: insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), heparin-binding epidermal growth factor (HB-EGF), tumor necrosis factor-α (TGF-α), transforming growth factor beta (TGF-β), acidic fibroblast growth factor (aFGF) and adrenomedullin (ADM) [6-8]. The impact of sex steroids (estrogen and progesterone) on cell proliferation is well known, while the interaction between them and growth factors is still quite debatable.

Uterine leiomyoma is strongly associated with local hypoxia, stimulated in turn production of extracellular matrix as well as angiogenic response in the myometrial tissue [9]. The presence of broad avascular areas within leiomyoma [10] could be an enhancing factor in formation of its own "vascular capsule" at the border between the tumor and the surrounding myometrium [11]. Hypoxia stimulates production of soluble fms-like tyrosine kinase 1 (sFlt-1) or VEGFR-1 (VEGF receptor-1), anti-angiogenic-related factor. Moreover, it declines an apoptosis in myometrial cells with no further effect on leiomyoma cells [12]. Among cells, having receptors to PDGF and VEGF and characterized by high sensitivity ho hypoxia, we can mark such cells as telocytes. They also like hematopoietic stem cells (HSC) [13] have CD34-immunopositivity and involve in a local angiogenesis, that were proved in cardiovascular system and lungs [14-16].

Telocytes (TCs) are a novel type of interstitial cell population. Telocytes were first described by the Popescu group in 2005 and characterized by a small cell body and extremely long prolongations named telopodes (Tps) with alternating thin segments (podomers) and dilated segments (podoms). Currently, these cells are identified by immunohistochemistry, immunofluorescence and, occasionally, by transmission electron microscopy (TEM). The presence of TCs has been reported in a variety of anatomical units in human and animal bodies. These cells have their own unique morphology, demonstrate specific direct (homocellular and heterocellular junctions) and/or indirect (chemical, paracrine/juxtacrine signaling, microvesicles and exosomes, sex hormone and microRNAs) contact with various surrounding cells, and have gene expression and immunohistochemical profiles [17-22]. Primarily, only eight basic ultrastructural identification criteria were proposed by Huizinga *et al.* in 1997 and unified as the "gold standard" [23]. Later, the addition of two more precise criteria regarding quantitative estimation of cell organelles and accurate description of cell prolongations by Popescu and his group yielded the "**platinum standard**" of diagnosis for

TCs [20; 21; 24]. The most useful markers for general identification are CD34 and PDGFRα [25; 26].

Dynamics and possible involvement of TCs in pathogenesis were observed in such diseases as systemic sclerosis [27; 28], Crohn's disease [29], myocardial infarction [30], gallstone disease [18; 31; 32], psoriasis [33], endometriosis and acute salpingitis [34-36], liver fibrosis [37], primary Sjögren's syndrome [38], keratoconic human cornea [39] and pathologies of the urinary bladder [40; 41]. In addition, the difference in the number of TCs in the myometrium and endometrium during pregnant and nonpregnant states has been described [42-44]. Cretoiu *et al.* focused on the distribution and role of TCs in the female reproductive system [45; 46].

The interaction of TCs and local angiogenesis in a human leiomyoma has not been clearly verified yet. Several studies have described TCs in UL, while no one marked its interplay with myometrial vascular structure. The aim of our study was to determine the link between myometrial TCs and vascularization in a human leiomyoma, and to clarify their possible role in the angiogenesis. We suggest that the interplay of TCs and adjacent cells could reveal new aspects in the pathophysiology of myometrial changes.

Materials and methods

Subjects

Twenty patients with symptomatic intramuscular solid UL (mostly located in the fundus of the uterus) were scheduled for elective surgery (laparoscopic hysterectomy) and selected for the study group (20 women, mean age 56.8 ± 10.0 years). The control group consisted of 15 patients (15 women, mean age 57.6 ± 12.7 years), who underwent elective surgery for other reasons and had no pre- or intraoperative signs of uterine fibroids. Hysterectomy was performed according to the standard procedure. Samples of tissue from the

foci of fibrosis and adjacent myometrium were taken for further observation from the study group. Samples of unchanged myometrium were also prepared from the control group. All patients were surgically treated at the Institute of Gynecology Jagiellonian University Medical College in 2018. The study was conducted in accordance with the moral, ethical, regulatory and scientific principles governing clinical research. All surgical samples were retrieved with the approval of the Jagiellonian University Bioethical Committee (protocol number – 122.6120.40.2016) using procedures that conformed to the Declaration of Helsinki guidelines.

Tissue processing

Tissue samples from fresh hysterectomy specimens were collected and rinsed thoroughly with PBS (phosphate-buffered saline, 0.01 M, pH = 7.4), fixed in 4% phosphate-buffered paraformaldehyde, routinely processed and embedded in paraffin. Serial sections were cut and mounted on poly-L-lysine-coated glass slides.

Routine histology

The sections were deparaffinized, rehydrated and stained with either hematoxylin–eosin (H&E) to evaluate the gross tissue organization or Masson's trichrome staining to detect collagen deposits.

Immunofluorescence

Indirect double immunofluorescence after heat-induced epitope retrieval was used to allow the simultaneous visualization of two antigens. After deparaffinization and rehydration, the slides were incubated for 30 min in PBS with appropriate normal serum at room temperature, followed by overnight incubation at 4°C in a solution of PBS with appropriate normal serum containing primary antibodies. After 5 washes (10 min each) in PBS, the specimens were then incubated for 1 h at room temperature with secondary antibodies diluted in PBS. Finally, the slides were washed in two changes (10 min each) of

PBS and cover-slipped with fluorescence mounting medium (Dako, Denmark) and covered glasses Menzel-Gläser. Labeled specimens were analyzed immediately. The primary antisera and secondary antibodies used are listed in Table I.

Table I. Type, sources and dilution of antibodies

Antibody	Catalog number , company, country	Dilution
Primary antibodies		
Polyclonal rabbit anti-c-kit	A4502, Dako, Denmark	1:100
Monoclonal mouse anti-CD34	M7165, Dako, Denmark	1:100
Polyclonal rabbit anti-CD34	orb44647, Biorbyt, UK	1:200
Monoclonal mouse anti-CD31	M082301-2, Dako, Denmark	1:100
Polyclonal goat anti-PDGFR alpha	AF-307-NA, R&D Systems, USA	1:100
Monoclonal mouse anti-tryptase	M7052, Dako, Denmark	1:100
Polyclonal rabbit anti-VEGFR-1	orb127531, Biorbyt, UK	1:100
Secondary antibodies		
Alexa Fluor 488 Goat Anti-Mouse	115-545-146, Jackson ImmunoResearch, USA	1:400
Alexa Fluor 594 Goat Anti-Rabbit	111-585-144, Jackson ImmunoResearch, USA	1:400
Alexa Fluor 594 Donkey Anti-Goat	705-585-003, Jackson ImmunoResearch, USA	1:400
Alexa Fluor 488 Rabbit Anti-Mouse	315-545-045, Jackson ImmunoResearch, USA	1:400
Alexa Fluor 488 Goat Anti-Rabbit	111-545-144, Jackson ImmunoResearch, USA	1:400
Alexa Fluor 594 Goat Anti-Mouse	115-585-146, Jackson ImmunoResearch, USA	1:400

Microscopic examination of telocytes, collagen deposits and vascular parameters

Slides were examined using an MN800FL epifluorescence microscope (OptaTech, Warszawa, Poland) equipped with Olympus DP74 digital CCD camera. Digital images were collected at either 200×, 400× and 600× magnification. The qualitative analysis of cells was provided in 10 consecutive high-power fields of vision (600×) using the computer-based image analysis system Multiscan 18.03 software (CSS, Warszawa, Poland). All samples were assessed by two independent specialists (each blinded to the other) without any knowledge of the clinical parameters or other prognostic factors to avoid bias.

The use of mast cell tryptase staining enabled c-kit-positive mast cells to be distinguished from c-kit-positive TCs. TCs were considered cells that were c-kit positive and tryptase negative concurrently, with the characteristic morphology in tissue samples. Additionally, cells double positive for CD34 and PDGFRα with the characteristic morphology and localization were also recognized as TCs. In order to distinguish populations of CD34-positive cells in the myometrial tissue (vessels and telocytes), we used double immunolabelling for CD34/CD31. Double positive structures were marked as vessels, whereas CD43-positive cells with elongated oval-shape body and long extensions – as telocytes. The vascular density was evaluated by the analysis of CD31 and sFlt-1 (VEGFR-1) immunopositivity in all myometrial samples. In all sections the immunoreactive cells found were evaluated with respect to the relative frequency (arbitrarily graded as very few = (+), few = +, moderate density = + +, multiple density = + + +). The percentage of collagen deposits and muscle tissue have been analyzed in specimens, stained with Masson trichrome. The collagen and muscle fibers volume ration were performed in ten consecutive fields of view of each sample.

Results

The histopathological changes in all uterine samples in this study were determined by hematoxylin–eosin and Masson's trichrome staining. Leiomyoma was composed of interlacing fascicles of uniform spindle cells. The nuclei were elongated, and the cytoplasm was abundant, eosinophilic and fibrillar. The adjacent myometrium and the foci of fibroids were cytologically identical, but the last one had a circumscription, nodularity and denser cellularity (Fig. 1). Light microscopy of uterine fibroids, adjacent myometrium and normal myometrium using Masson's trichrome staining for collagen revealed collagen to be abundant in the fibroid tissue, while the myometrium had sparse, well-aligned collagen

bundles adjacent to smooth muscle cells. The study group was characterized by the

	Affected uterus		Unchanged uterus
	Fibroid	**Adjacent myometrium**	**Normal myometrium**
Collagen (%)	44 ± 15	18 ± 6	24 ± 10
Muscle fibers (%)	33 ± 13	45 ± 13	43 ± 10

prevalence of collagen deposits in compare with the control group. The amount of muscle fibers was lower in compare with the adjacent myometrium. Conversely, the percentage of muscle fibers was higher in the normal myometrium, while collagen was scanty presented in this type of tissue. In addition, the more significant difference in contents of both components was revealed in the myometrium, surrounded myometrial fibroids. The prevalence of muscle fibers correlates with poor organization of collagen deposits, that was approximately in 2.5 times lower in compare with muscles (Table II).

Table II. The percentage of the collagen and muscle fibers in different types of myometrium

Immunofluorescent labeling was performed for the primary identification of TCs on uterine tissue affected and unchanged by UL. We used current, mostly proven markers, including CD34, PDGFRα and the canonic c-kit. Double immunolabeling for c-kit and tryptase was performed for the identification of mast cells and signs of consequent inflammation. In immunostained slides, c-kit and tryptase double-positive mast cells were generally round or oval shaped, with a centrally located nucleus. The c-kit-positive/mast cell tryptase-negative cells were considered TCs (Fig. 2). These cells were mostly fusiform in shape with small branches. They have been detected in UL and adjacent myometrium as well as in normal myometrium from healthy uterus.

We found also that double immunopositive cells for CD34 and PDGFRα (putative uterine TCs) are present in leiomyomas, adjacent myometrium and normal myometrium. Mostly they had elongated oval-shaped cellular bodies and were distributed among the intertwined myometrial fibers and in close vicinity to blood vessels. The general pattern of their localization resembled parallel eccentric lines. However, in some parts, they reflected directional smooth muscle bundles. CD34/PDGFRα-positive cells were scantily presented in the foci of leiomyoma (Fig. 3).

Immunolabeling for CD31 was performed for assessment of vascular density in myometrial tissue from affected and unchanged by leiomyoma samples. The CD31 labelling showed lower expression in the foci of leiomyoma. The normal as well as the adjacent to the pathology myometrium had a plenty of CD31-positive cells, mostly formed circular and longitudinal lines (Fig. 4). In addition, double immunostaining for CD34/CD31 revealed cells, which were only CD34-positive and had long extensions. They have been detected between vessels and myometrial fibers, not rare in close vicinity to small vessels. Its expression was higher in the normal myometrial and lower in affected by myoma. We assume that CD34-positive cells were uterine telocytes (Fig. 5).

The opposite tendency was observed in samples labelled by VEGFR-1 (sFlt-1). The expression of this marker was higher in the foci of leiomyoma, while normal and adjacent myometrium had less sFlt-1 positive cells (Fig. 6).

- Figure 1 –
- Figure 2 –
- Figure 3 –
- Figure 4 –
- Figure 5 –
- Figure 6 -

In all sections the immunoreactive cells found were evaluated with respect to the relative frequency. The subjective qualitative analyses showed the decreasing in density of TCs in fibroids in compare with both types of unchanged myometrium (adjacent and from healthy uterus). The expression of CD31 was higher in the normal myometrium and significantly lower in the foci of leiomyoma. The opposite effect was typical for sFlt-1 expression. Leiomyoma was characterized by increasing of sFlt-1 expression. Results of semiquantitative analysis of CD34 immunoreactivity in TCs from human fetal skeletal muscle samples are summarized in Table III.

Table III. Relative frequency of c-kit-positive/tryptase-negative, CD34–positive and PRGFRα-positive, CD31-positive and sFlt-1-positive cells in different parts of human unchanged and affected by leiomyoma uterus. 0 = absence, (+)=very few, + =few, + + =moderate density, + + + =multiple density.

	c-kit+/ trypatse-	CD34+/PDGFRα+	CD31	VEGFR-1 (sFlt-1)
Normal Uterus				
Unchanged myometrium	+++	+++	+++	+
Myomatous Uterus				
Adjacent myometrium	++	++	++ +	+
Focus of fibroid	+	+	+	++

Discussion

Since TCs were reported for the first time, a number of researches worldwide have described these cells in different organs. Their primary identification is frequently sought. The c-kit receptor was the most attractive target in immunohistochemical observation. However, duplex immunoreactivity gave a reason to discuss a few populations of TCs in the human myometrium and the human small intestine [47-49]. In 2005, Ciontea *et al.* suggested

that at least two subpopulations of TCs could exist in the human myometrium: c-kit-positive and c-kit-negative cells [48]. In addition, consistent with previous studies, Went *et al.* provided a list of c-kit-negative structures including the endometrium, myometrium and uterine cervix [50]. Moreover, Duquette *et al.* separately revealed vimentin (+)/c-kit (-) TCs in the human myometrium [47]. Later, in 2014, Yang *et al.* revealed that TCs were negative for c-kit in the rat oviduct [35]. First, Faussone-Pellegrini et al. concluded in 2011 that CD34-labeling "remains the best available choice for TC identification, possibly in combination with c-kit and vimentin labeling", as c-kit-negative cells were described not only in the human uterus but also in the animal uterus [51]. Two years later, Vannucchi *et al.* proposed the double immunofluorescent staining for CD34 and PDGFR-α as a specific marker for TCs in the gastrointestinal tract [52], which was successful in the heart and others organs [53]. Finally, in 2016, Faussone-Pellegrini *et al.* emphasized that "CD34 is the most reliable TC marker" [26]. Recently, some authors published the results of TC identification in the uterus based only on c-kit immunolabeling, which, in our opinion, is an omission. Moreover, some of them stressed that leiomyoma does not have any c-kit-positive cells, while an excess number of mast cells, common for studied pathology, is strongly c-kit-positive. Therefore, we stressed a significance of used double immunofluorescent staining with anti-tryptase antibodies for separating mast cells from TCs. We found CD34- and PDGFR-α-positive cells in myometrial tissue from the foci of fibroids, the surrounding walls of the same uterus and healthy samples without UL. We emphasized TCs exist in all samples of observed tissue.

Several studies have been devoted to explaining a possible role of new cells in diseases. In addition, all authors marked some quantitative and qualitative features common to TCs in pathological conditions: a reduction in numbers and/or damage of cells (up to absence) as well as shrinkage and shortening of Tps [26; 28; 54; 55]. Most likely, they are more sensitive to local ischemia than other stromal cell types, such as fibroblasts,

myofibroblasts and mast cells. Manetti *et al*. observed the dynamics of TCs in tissues (the gastric wall- submucosa and muscle layers, the myocardium and the lung) from patients affected by systematic sclerosis and suggested that the reduction of TCs can lead to changing of the three-dimensional organization of the extracellular matrix and as a result – the development of fibrosis [28]. In addition, the similar declining of TCs has been documented in myocardial infarction [30], aging of the human heart [56], liver fibrosis [37], ulcerative colitis [57], Crohn's disease [29], gallstone disease [19], endometriosis [35], psoriasis [34], renal ischemia/reperfusion injury [40; 41] and different lung diseases [56]. Regardless of location, staging and character of pathology, the reduction of TCs is correlated with subsequent fibrosis, has a cellular origin and is common for many injuries and pathologies [58; 59]. We intend to provide further quantitative analysis of TC number in the foci of myoma and healthy myometrium, although we could hypothesized that the density of TCs declines in leiomyoma.

The interaction between TCs and the pathophysiological mechanisms common for the selected diseases is unclear. From one point of view, the amount of TCs declined under changes in the microenvironment associated with pathology. From the opposite side, the decreasing of TCs could be a key point in the pathogenesis. These cells reflect damage, or they could be involved in the background of the diseases; the question remains open. We revealed the presence of TCs in uterine fibroids. Before, only comparisons of the density and the distribution in pregnant and nonpregnant uteruses in rats and humans had been provided. TCs constituted approximately 7% of the total cell number in nonpregnant myometrial cell culture and approximately 3% of the entire cell population in the myometrium of adult nonpregnant humans [60; 61]. The TC interstitial system is composed of cells that by either homocellular or heterocellular contact integrates overall information from the vascular, the nervous and the immune systems, the interstitium and stem cells. In the myometrium, TCs

can influence the contractile activity of smooth muscle cells. Of note, they differed in telopodal width and podomic thickness with pregnancy states, which can be related to their function [44; 45]. Current studies showed that the podomers are thicker in nonpregnant myometrium than in pregnant myometrium (~82 versus 75 nm), and the podoms were thicker in pregnant myometrium (~316 versus 269 nm) [21; 43].

Richter *et al.* demonstrated that the numbers of TCs and Tps correlate negatively with the amount of mature fibrillar collagens and correlate positively with degraded collagens in the human heart [54]. We marked the same trend that required further investigation.

The growth factors have an influence on myometrial cellular transformation and turnover. For instance, PDGF modulates the rate of cell proliferation in myometrium and leiomyoma cells and likely plays a role in smooth muscle cell (SMC) hypertrophy as its expression is increased in the myometrium during gestation. It is upregulated by estrogen in uterine SMC and might interact with other growth factors such as TGF-β and EGF to enhance proliferation [5]. TCs are immunohistochemically positive for platelet-derived growth factor receptor alpha and beta (PDGFRα and-β) and VEGF. Of note, telocytes might be involved in the regulation of excessive cellular matrix production inside the foci of myoma [62].

Myometrial changes relate to three basic pathophysiological links: fibrosis, angiogenesis and the immune response. Growth factors involved in the pathogenesis of UL have receptors on TCs. Recent data showed that TCs are immunopositive to TGF-β, which is also involved in myocardial physiopathology. We suggest that the same could occur in myometrial contractility. One of the essential elements of leiomyomata is the vascular capsule. Neovascularization correlates with hypoxia and misbalance of vascular and coagulation factors. We observed the decreasing of vascularization in the foci of leiomyoma, accompanied by rising of the expression of VEGF receptor-1 (sFlt-1) amount. Hypoxia could be a leading factor leads to this changing. However, the common feature for both observed

subjects was that in the foci of leiomyoma we saw declining of telocytes and of vascularization too. Uterine telocytes are sensitive for angiogenic factors (PDGF and VEGF) and ischemia, declined and even disappeared during fibrosis, observed in close vicinity to blood vessels. They might play a role in the angiogenic response, universal for the human body. In clinical practice it might clarify the pathogenesis of oxidative response in the myometrium and help in selection of further appropriate therapy as a consequence. We intend to observe this correlation in our future scientific work.

In conclusion, our data provide the demonstration that TCs are present in human uterine fibroids and highlight their possible involvement in the pathogenesis of myometrial pathology in context of the angiogenesis. Further studies will allow clarification of the details of their putative roles in angiogenesis and the principles of their interaction in the myometrium. We hypothesize that deep observation of TCs in the human uterus brings additional value to reproductive medicine.

Conflict of interest: The authors declared no conflict of interest.

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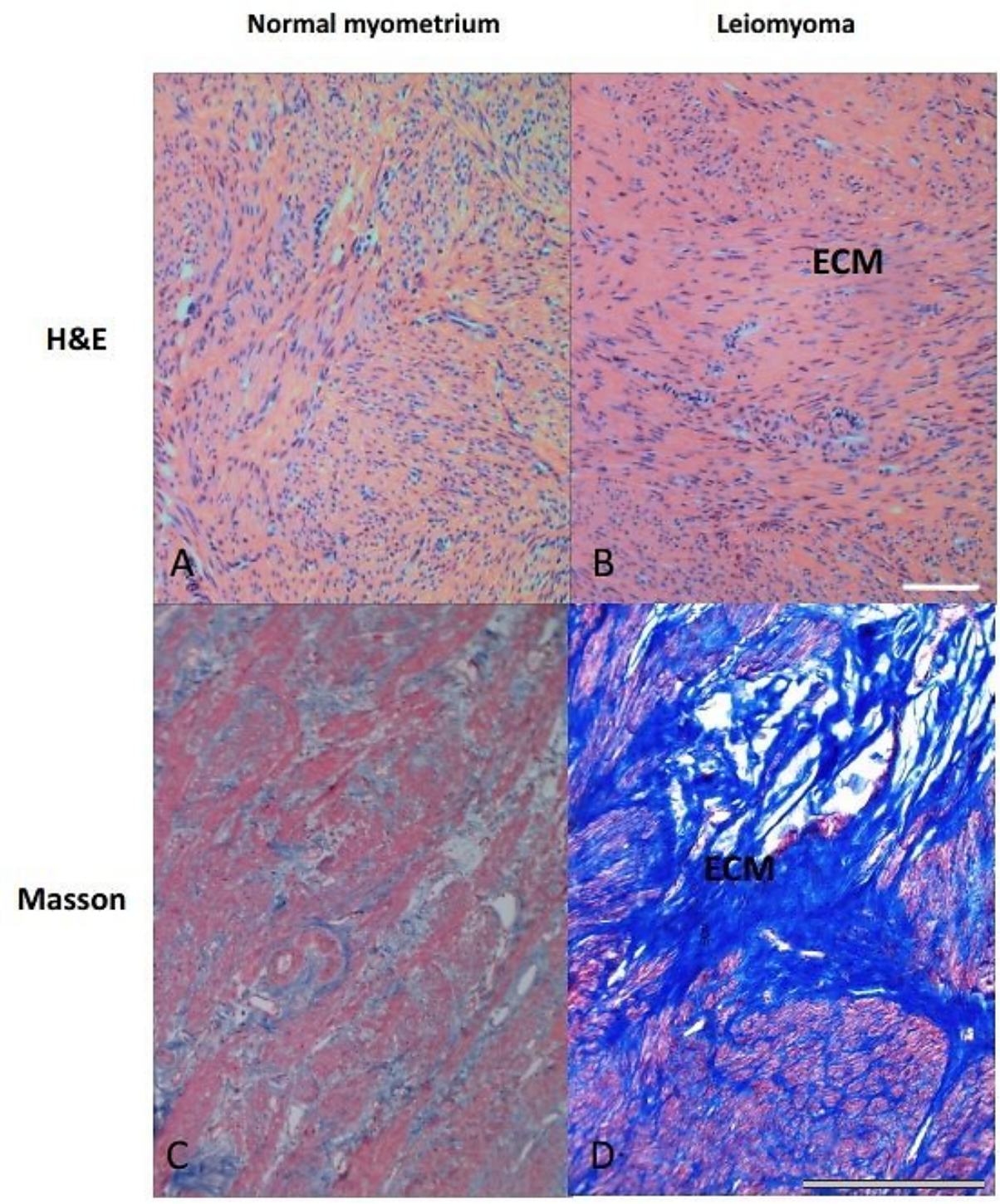

Fig. 1 Hematoxylin–eosin and Masson's trichrome-stained sections of human myometrium . The myometrium sections from the control group (A, C) compared with the foci of leiomyoma (B, D). The myometrium sections from the control group (A) compared with the foci of leiomyoma (B). With Masson's trichrome staining, collagen deposits were blue in color; muscle fibers, red in color. Fragments of disordered smooth muscle cells were separated by abundant extracellular matrix (ECM). Scale magnification bar: 100□m.

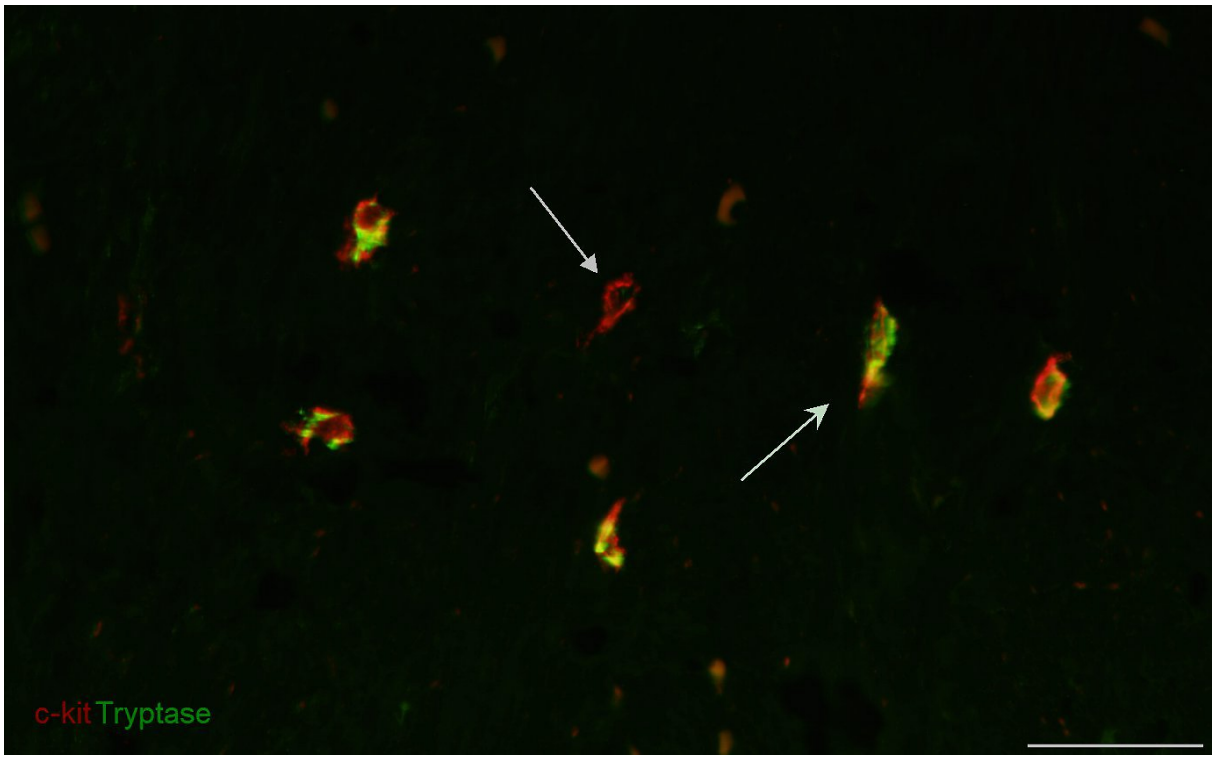

Fig. 2 Sample of leiomyoma stained for c-kit (red, Alexa Fluor 594) and tryptase (green, Alexa Fluor 488). Double immunopositive cells with round-shape bodies marked by the bullet arrow are mast cells. Cell with strong immunopositivity only for c-kit and marked by the field arrow is identified as telocyte. Scale magnification bar: 50□m.

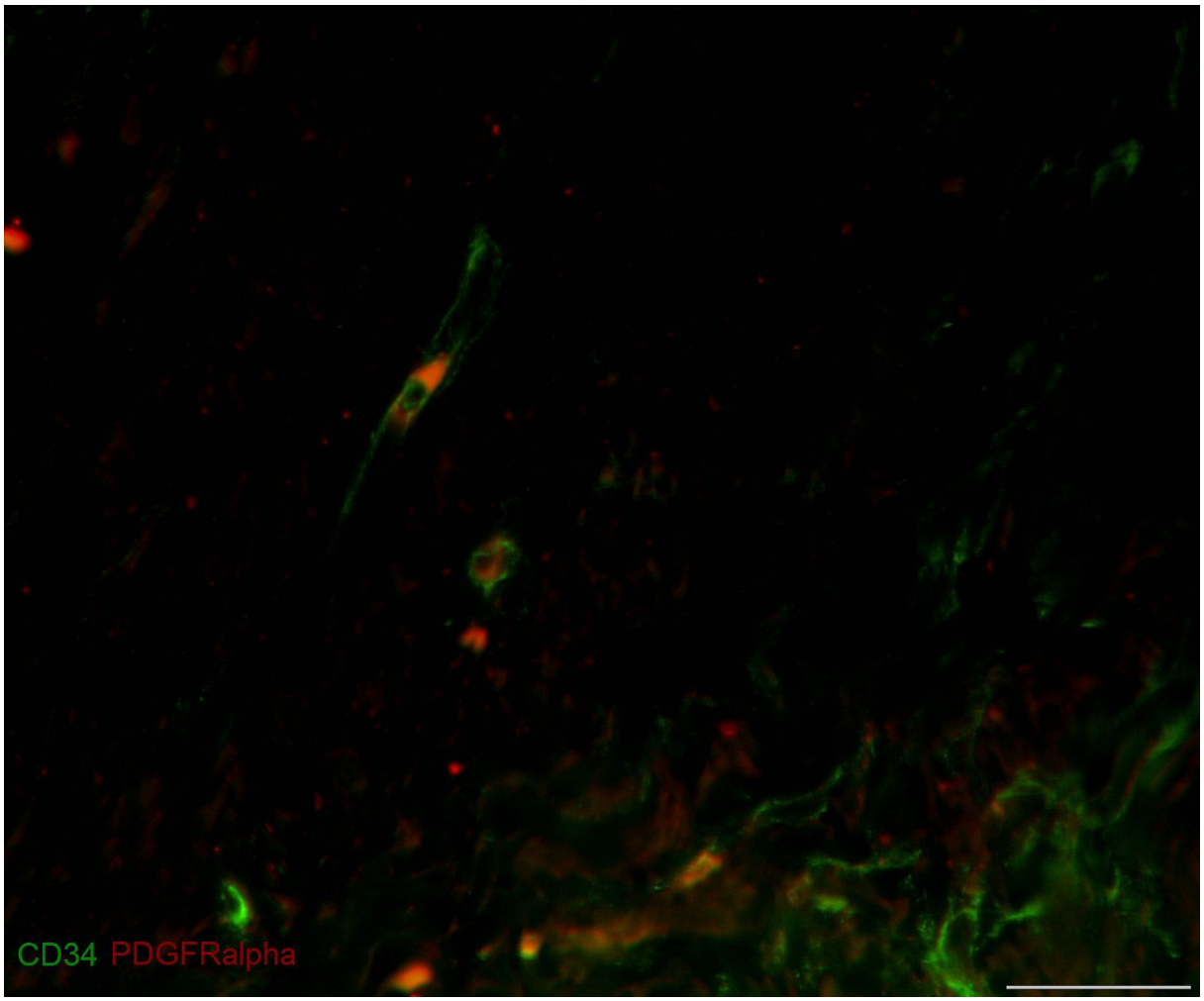

Fig. 3 Sample of leiomyoma stained for PDGFR alpha (red, Alexa Fluor 594) and CD 34 (green, Alexa Fluor 488). Double immunopositive cells with elongated bodies located between muscle fibers and close to blood vessels are identified as telocytes. Scale magnification bar: 20□m.

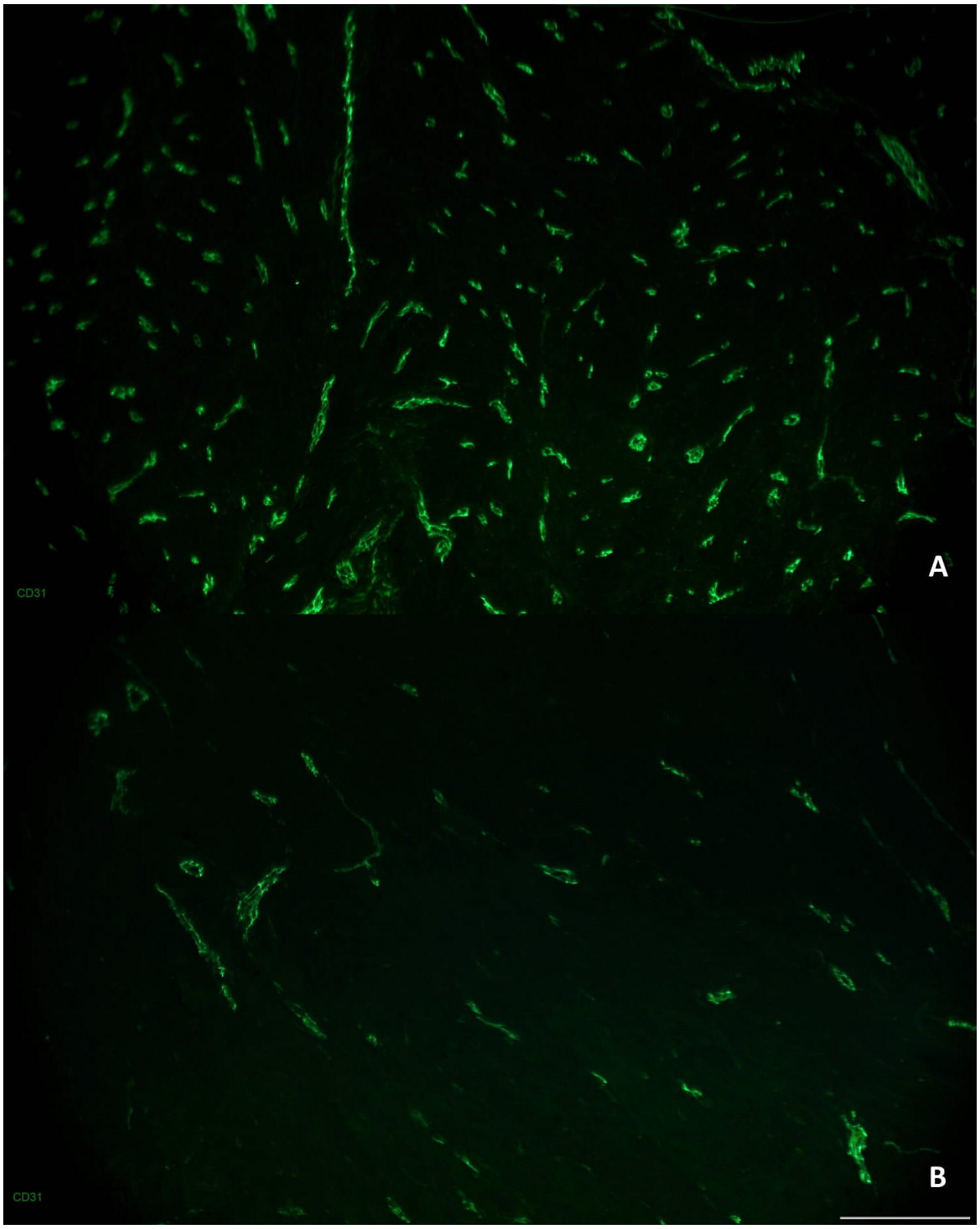

Fig. 4 Vascular density in leiomyoma (B) and unaffected myometrium (A), assessed by staining for CD31 (green, Alexa Fluor 488). A rich expression of CD31 cells was common for the healthy myometrial tissue, whereas samples affected by leiomyoma had a poor vascularization within the pathological focus. Scale magnification bar: 50□m.

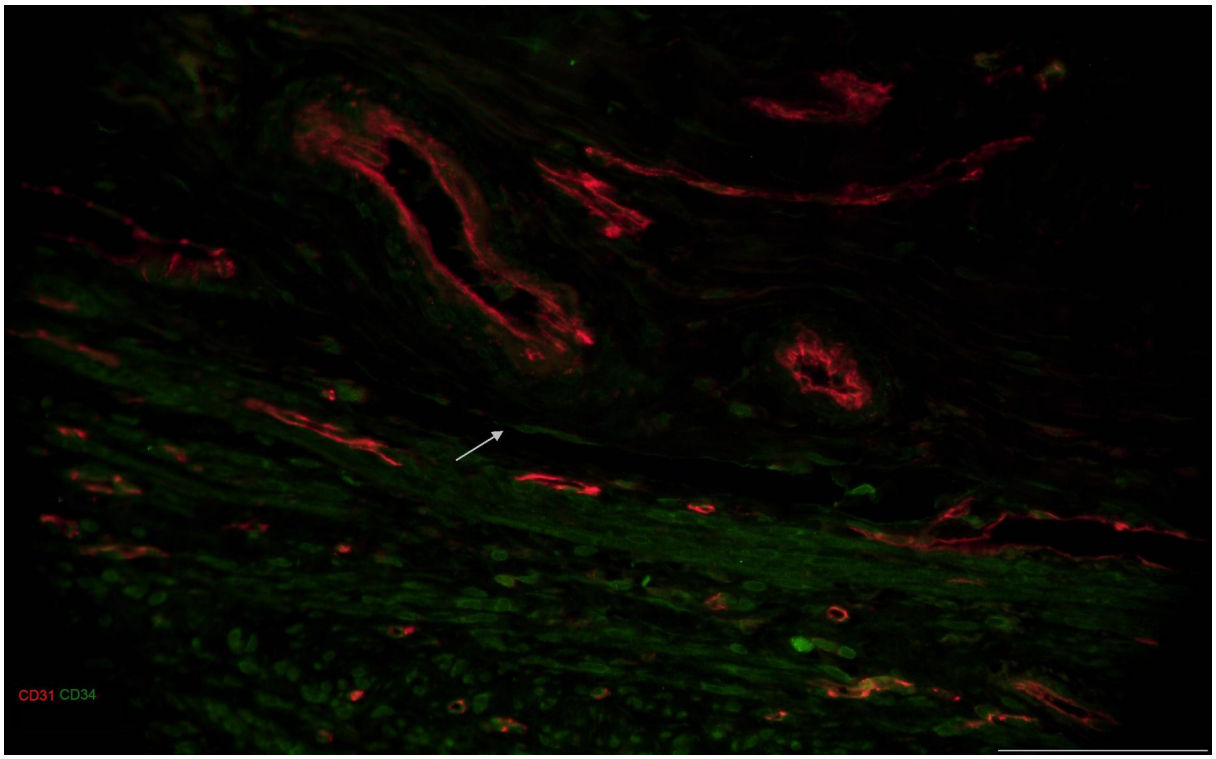

Fig. 5 Sample of leiomyoma stained for CD31 (red, Alexa Fluor 594) and CD 34 (green, Alexa Fluor 488). Double immunopositive structures were identified as vessels of different caliber, while cells with elongated bodies located between muscle fibers and close to blood vessels are identified as telocytes (one of them is marked by arrow on the image). Scale magnification bar: 50□m.

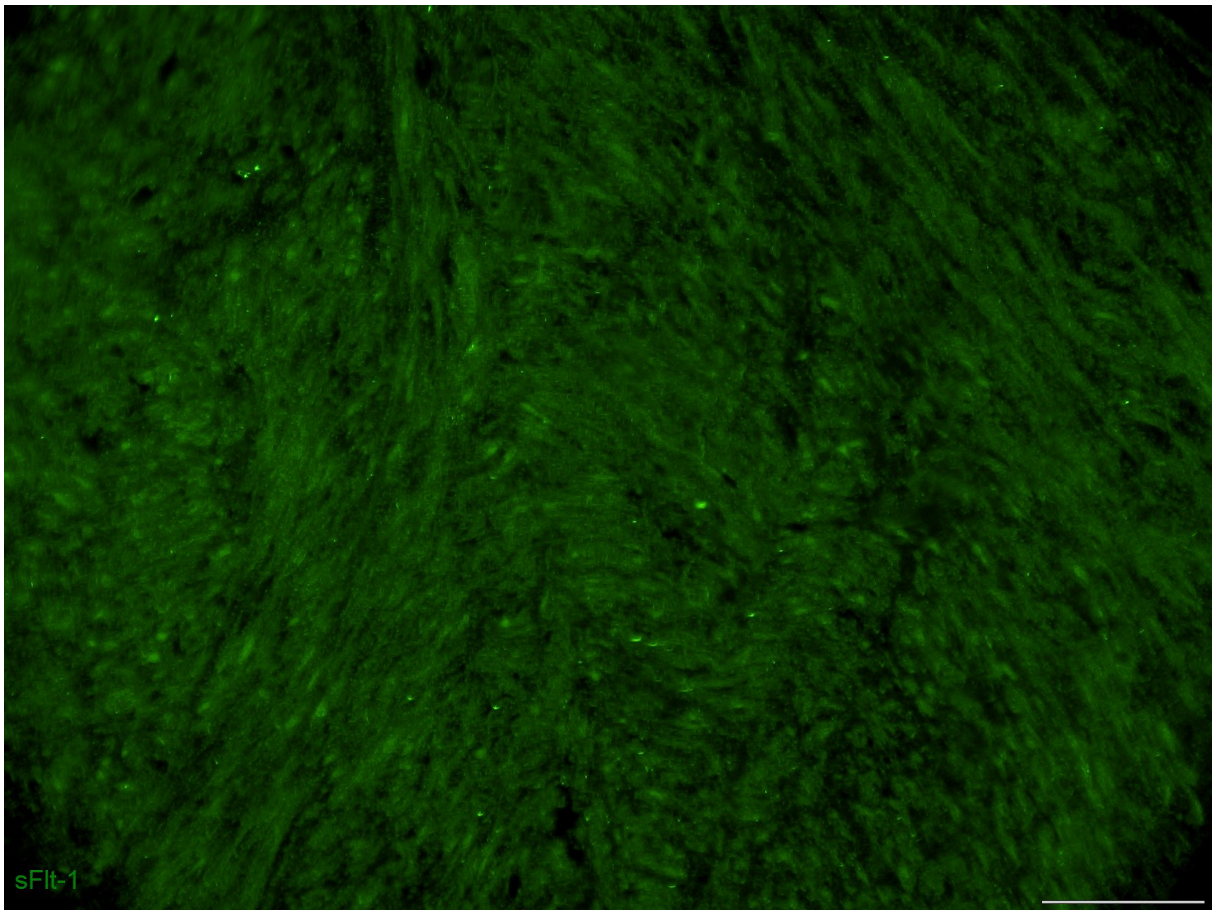

Fig. 6 Sample of leiomyoma stained for sFlt-1 (VEGFR-1) (green, Alexa Fluor 488). Immunopositive cells are located mostly close to vessels within leiomyoma, some of them are observed between myometrial fibers. Scale magnification bar: 50□m.

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Figures

Figure 1 - Download source file (112.46 kB)
Fig. 1 Hematoxylin–eosin and Masson's trichrome-stained sections of human myometrium . The myometrium sections from the control group (A, C) compared with the foci of leiomyoma (B, D). The myometrium sections from the control group (A) compared with the foci of leiomyoma (B). With Masson's trichrome staining, collagen deposits were blue in color; muscle fibers, red in color. Fragments of disordered smooth muscle cells were separated by abundant extracellular matrix (ECM). Scale magnification bar: 100□m.

Figure 2 - Download source file (53.06 kB)
Fig. 2 Sample of leiomyoma stained for c-kit (red, Alexa Fluor 594) and tryptase (green, Alexa Fluor 488). Double immunopositive cells with round-shape bodies marked by the bullet arrow are mast cells. Cell with strong immunopositivity only for c-kit and marked by the field arrow is identified as telocyte. Scale magnification bar: 50□m.

Figure 3 - Download source file (75.81 kB)
Fig. 3 Sample of leiomyoma stained for PDGFR alpha (red, Alexa Fluor 594) and CD 34 (green, Alexa Fluor 488). Double immunopositive cells with elongated bodies located between muscle fibers and close to blood vessels are identified as telocytes. Scale magnification bar: 20□m.

Figure 4 - Download source file (141.75 kB)
Fig. 4 Vascular density in leiomyoma (B) and unaffected myometrium (A), assessed by staining for CD31 (green, Alexa Fluor 488). A rich expression of CD31 cells was common for the healthy myometrial tissue, whereas samples affected by leiomyoma had a poor vascularization within the pathological focus. Scale magnification bar: 50□m.

Figure 5 - Download source file (320.69 kB)
Fig. 5 Sample of leiomyoma stained for CD31 (red, Alexa Fluor 594) and CD 34 (green, Alexa Fluor 488). Double immunopositive structures were identified as vessels of different caliber, while cells with elongated bodies located between muscle fibers and close to blood vessels are identified as telocytes (one of them is marked by arrow on the image). Scale magnification bar: 50□m.

Figure 6 - Download source file (295.47 kB)
Fig. 6 Sample of leiomyoma stained for sFlt-1 (VEGFR-1) (green, Alexa Fluor 488). Immunopositive cells are located mostly close to vessels within leiomyoma, some of them are observed between myometrial fibers. Scale magnification bar: 50□m.

7. CONCLUSIONS

In order to conclude all results of our published work in main statements, we want to emphasize next:

1. Telocytes have been revealed in all parts of the human uterus (cervix, corpus, foci of the fibroid) in unaffected tissues and affected by leiomyoma.
2. Uterine fibroids are characterized by decreasing number of telocytes in comparison to normal myometrium.
3. In leiomyoma the amount of extracellular matrix (collagen) has exceeded, which was revealed by H&E and Masson trichrome stainings.
4. The density of iNOS and ChAT-immunopositive neurons in the uterine fibroids increases in comparison to control samples.
5. Telocytes and PGP-immunopositive neurons form specific “cooperation” in the foci of leiomyoma, that were revealed by double immunostaining for PDGFRα and PGP 9.5 markers. The tandem of two different populations of such cells is mostly located close to the border areas between myoma and adjacent myometrium (in areas of pseudocapsules).
6. Autonomic innervation and telocytes are involved in the microenvironment misbalance, which is characteristic for uterine leiomyoma. Reduction in number of telocytes could leads to fibrosis and local immune response in myometrium.
7. The declining of the telocytes density in the foci of fibroids correlates with a poor vascularization inside leiomyoma.

8. DISCUSSION

The interplay of TCs and the constituents of UF are no less important in the framework of its pathogenesis (Fig. 3).

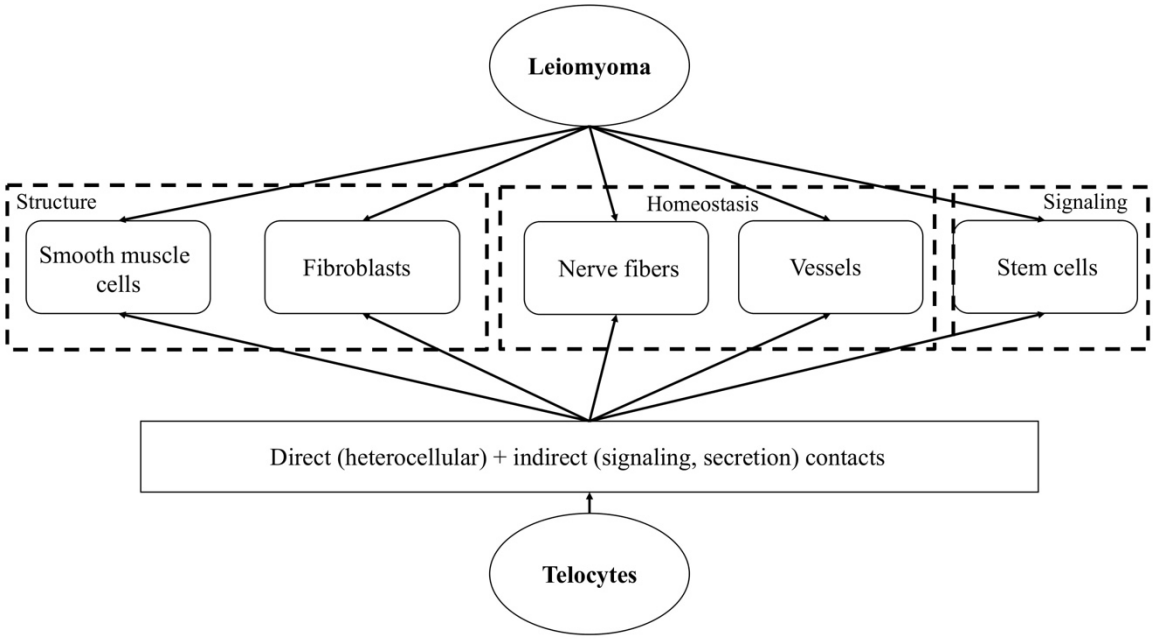

Fig. 3 Interactions between telocytes and different components of uterine fibroid.

3-Dimensional role of telocytes in uterine fibroid

When the myometrium is affected by fibroid, there is a loss of normal gross tissue organization. The prevalence of collagen in comparison with muscle fibers is sometimes accompanied by dysfunctional uterine spontaneous contractions and responsiveness [131]. Homo- and heterocellular contacts with smooth muscle cells and fibroblasts allow TCs to form networks with a 3-D organization (**Article 1**). Sometimes, TCs may even form 2-D networks [7; 91]. The physiological balance between cellular components and the interstitium is destroyed by the prevalence of fibrosis in various diseases (hepatic fibrosis, gallstone disease, systemic sclerosis, primary Sjögren's syndrome, psoriasis, myocardial

infarction), notably in uterine leiomyoma [34; 39; 55; 132-135], which is accompanied by the decline, or even disappearance, of TCs in tissues and organs [39]. Ultrastructural alterations of TCs, including swollen mitochondria, cytoplasmic vacuolization and the presence of lipofuscinic bodies, with their reduction in the skin, are common in systemic sclerosis, often correlating with the subsets and stages of the process. Moreover, the same changes have been described in the gastric wall (submucosa and muscle layers), the myocardium and the lung [133]. Damage and loss of telocytes might be caused by ischemic injury, as TCs appear to be more sensitive to ischemia than other stromal cell types, such as fibroblasts, myofibroblasts, and mast cells [132]. Ischemia can lead to an alteration of the three-dimensional organization of the extracellular matrix and, as a result, development of fibrosis (**Article 2**). The uncontrolled activity of fibroblasts/myofibroblasts might be a consequent of impact of TCs in the foci of uterine fibroid.

Another key aspect of UF is the regulation of matrix production. Undoubtedly, this process correlates with local secretion of growth factors [136], some of which are under hormonal regulation, especially by progesterone [137]. Sex steroid receptors are dependent on TC localization. In the human gallbladder, these cells are negative for the progesterone receptor, while in normal cells and those affected by UF in the myometrium, they are strongly positive [89]. Scant data are available on the immunosensitivity of TCs to different growth factors, but VEGF and PDGFR alpha are always expressed in the aforementioned cells. In our opinion, the immunological properties of TCs, along with their local sensitivity to sex steroid hormones and contacts with smooth muscle cells, allow us to predict their intermediate role in the production and regulation of the ECM (**Article 2**).

Uterine fibroid results in the abnormal orientation of muscle fibers and contractive disturbances [131]. Normally, smooth muscle cells and nerve fibers provide the contractive

function of the myometrium. As TCs express K^+ channels and make close contacts with myocytes [94], they also take part in myometrium contraction. However, we still lack much information about the electrical activity of TCs isolated from the human myometrium. Several experiments demonstrated opposite results [16; 30; 131]. We know that telopodes in the non-pregnant myometrium are longer, the podomers are thicker and the podoms are thinner compared to those in the pregnant myometrium [1; 4; 138]. These morphological transformations lead to altered exo- and ectosome secretion, as well as excitation of the myometrium, most notably during pregnancy. The fluctuation of sex steroid hormones, as well as additional hormonal therapy, certainly affects myometrial TCs [138; 139]. Similarly, the pathogenesis of myoma partly depends on the hormonal background. The decline or disappearance of TCs in the foci of fibroids might be a factor of local electrophysiological disorder (**Article 1, 2**).

Homeostatic role of telocytes in uterine fibroid

Vascularization and innervation of fibroids are essential for their expansion, which generally provides a symptomatic components of this pathology, namely, bleeding and pain. The formation of a pseudocapsule, including vessels and nerve fibers, is a specific feature of UF that is hypothetically connected with prognosis [129; 140]. Pro-angiogenic factors are a top priority among the pathomechanisms of vascular capsule formation. Moreover, some angiogenic factors are overexpressed in UF, including VEGF and basic fibroblast growth factor [140]. Telocytes have been detected in close proximity to blood vessels (on the endothelial surface) [141; 142]. Telocytes are positive for VEGF immunolabelling and secrete this growth factor as well [143], which may induce the proliferation of pulmonary endothelial cells [105]. Moreover, TCs are sensitive to hypoxia and participate in neo-angiogenesis [55]. Estrogens regulate the secretion of VEGF and

have receptors on myometrial telocytes [140], suggesting the indirect involvement of myometrial TCs in the formation of the vascular capsule in leiomyoma (**Article 4**).

The density of TCs has been the focus of observations, providing an exciting study of various tissues, including the intestine, ureter, myometrium, myocardium, and fallopian tube. UL is no exception from this list; however, its innervation still has wide gaps, and knowledge about it is limited. We know that TCs are located close to nerve endings and that some of them make connective structures using Tps between smooth muscle cells and nerves [1; 4; 91]. Interestingly, the appendix has the highest numbers of TCs compared to other parts of the intestine, which is explained by the need for its complex innervation [144]. The opposite situation is common in Crohn's disease, wherein gut dismotility is accompanied by the decline and disappearance of TCs [93]. Hence, the number of TCs correlates with the function of excitable tissue and its damage. The decline of TCs in UF and their detection in the pseudocapsule might might be responsible for pain and electrophysiological disturbance in myometrial tissue (**Article 3**).

Importantly, the iNOS/nitric oxide system is fundamental not only in the female reproductive system. Uterine TCs activate peritoneal macrophages and stimulate the production of iNOS [86]. UF is characterized by a prevalence of NOS-positive nerve fibers. We hypothesized that myometrial TCs participate in this pathophysiological unit of fibroids by regulating iNOS production (**Article 3**).

Integrative role of telocytes in uterine fibroid

Mirancea defined telocytes as "nurse" cells because they collect information from nervous, vascular, and stem cells, as well as from the immune system [145]. Albulescu *et al*. hypothesized that the TC secretome plays a modulatory role in stem cell proliferation

and differentiation [143]. Is TC function over-estimated, or it could have value in the pathogenesis of UF?

The reported presence of close contacts between TCs and lymphocytes or plasma cells might indicate an immunomodulatory role for TCs. Mast cell synapses with TCs have been found in the human myometrium. Close contacts with TCs suggest that mast cells might take part in this cellular cross talk. It is possible that mast cells and myometrial TCs assist, or even function, as pacemaker cells in myometrial contractions. In addition, such close cell connections suggest juxtactrine cell-to-cell signaling (chemical synapse), and the (micro)vesicles found in the synaptic cleft may correspond to an exosome-based mechanism [29; 30]. Myometrial mast cells have receptors for stem cell factor (SCF), which is produced by myometrial cells [146]. Therefore, under the control of smooth muscle cells, mast cells secret mediators that affect tissue remodeling and growth. TCs make contacts with smooth muscle cells, as well as with mast cells. TCs can be associated with immunological response in UL, as well as with tissue growth.

The latest area of interest is the relationship between stem cells and TCs. The stem cell niche's involvement with TCs has been described in skeletal muscle, heart, lung and skin [13; 68; 147; 148]. This interaction is always discussed in the context of tissue remodeling, regenerative medicine, and targeted therapy. Stem cells unaffected and affected by uterine fibroid myometrium are not similar. The latter possesses mutated MED12 and reduced levels of DNA repair [149; 150]. A trigger factor may affect the microenvironment (niche), consequently causing further UF development. As a component of the stem cell niche, TCs might be involved in this process.

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Telocytes: facts, speculations and myths

(Review article)

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Abstract: Telocyte (TC) is an interstitial cell type with a small cellular body and extremely long tentacle-like extensions. TCs were discovered a decade ago and have specific morphological characteristics, immunohistochemical and secretome profiles, electrophysiological properties, microRNA expression. Moreover, they are different in gene expression from other cells. TCs play an important role in plenty of processes. Apparently, they are involved in homeostasis, remodelling, regeneration, repair, embryogenesis, angiogenesis and even tumorigenesis. "Telocytes need the world", was emphasized by Professor Popescu and it will be actual at any time. This review summarizes particular features of TCs in different organs and systems, emphasizing their involvement in physiological and pathophysiological processes.

Key words: Interstitial Cajal-like cells (ICLC), telocytes, telopodes, fibroblast-like cells, CD34.

History

About one decade ago, there has been discovered a novel cell type with unique morphology and functions. L.M. Popescu's group from Bucharest, Romania, focused on interstitial (stromal) cells in the connective tissue of many organs of humans and laboratory mammals, which named interstitial Cajal-like cells (ICLC) in 2005. A few years later, in 2008, M.S. Faussone-Pellegrini and her team from Florence, Italy, described ICLC in the muscle coat of the human gut and noticed they consistently differed from the canonical gastrointestinal cells of Cajal (ICC) in both ultrastructure and immunophenotype. In 2010 the acronym ICLC was replaced with a more appropriate name one and introduced to scientific world for the first time in the paper "*TELOCYTES – a case of serendipity: the winding way from Interstitial Cells of Cajal (ICC), via Interstitial Cajal-Like Cells (ICLC) to TELOCYTES*" in the Journal of Cellular and Molecular Medicine. From that time, this novel cell type became known as the TCs (using the Greek affix "Telos"). Nowadays, all cell name's synonyms (ICLC, fibroblast-like cells, telocytes) are widely used in publications [1–4].

Morphology of telocytes

The TC has a small, oval-shaped cellular body, containing nucleus, surrounded by a small amount of cytoplasm. The cellular body average dimensions are, as measured on TEM images: 9.39 μm ± 3.26 μm (min = 6.31 μm; max = 16.42 μm). The nucleus occupies about 25% of the cell volume and contains clusters of heterochromatin attached to the nuclear envelope.

The perinuclear cytoplasm is rich in mitochondria (which occupy about 5% of the cell body) particularly in podoms, which contain a small Golgi complex, as well as the elements of rough and smooth endoplasmic reticulum and cytoskeletal elements (thin and intermediate filaments). The cell periphery is represented by the usual plasmalemma, with no (or thin and discontinuous) basal lamina, and some caveolae (about 2–3% of cytoplasmatic volume; ~0.5 caveolae/μm of cell membrane length) [1–7].

Telocytes have a variable number of telopodes (Tps) (very long cellular extensions), which are probably the longest cellular prolongations in the human body. Tps are made by an alternation of dilated portions, named podoms (250–300 nm), containing mitochondria and endoplasmic reticulum and podomers (~80 nm) with thin segments. The podomers are thicker in nonpregnant myometrium than in pregnant one (~82 versus 75 nm), and the podoms were thicker in pregnant myometrium (~316 versus 269 nm) [1, 2, 5]. The shape of the TCs depends on the number of their telopodes (Tps): piriform for one prolongation, spindle for two Tps, triangular for three, stellate, etc. Their spatial appearance is that of a polyhedron with a different number of vertices, depending on their Tps number [8].

Tps form a three-dimensional network that may function as a scaffold to define the correct organization of tissues and organs [9]. Mandache *et al.* mentioned that telopodes develop a wrapping activity gathering masses of amyloid fibrils, partially or totally surrounding them. These cellular 'bags' made by telopodes have sometimes inner cytoplasmic processes with honeycomb-like appearance which fragments in bunches the amyloid fibrils [10].

Huizinga *et al.* proposed eight basic ultrastructural criteria for TC identification in 1997 ("**gold standard**"). Later Popescu and his group added two more criteria and formed "**platinum standard**" of diagnosis for TCs [3, 11, 12].

1. Location: among tubulo-alveolar structures, in the non-epithelial space
2. Close contact with target: nerve bundles, and/or epithelia, and/or smooth muscle cells, and/or capillaries, immunoreactive cells by "stromal synapses"
3. Characteristic cytoplasmic processes
   a. Number: (1–5, frequently: 2–3)
   b. Length: tens to hundreds of μm
   c. Thickness: uneven caliber, <0.5 μm with dilations, but very thin from the emerging point
   d. Aspect: moniliform, usually with mitochondria in dilations
   e. "Ca^{2+} release units": present
   f. Branching: dichotomous pattern
   g. Organization in network: labyrinthine system of overlapping cytoplasmic processes
4. Gap junctions: with smooth muscle cells or with each other
5. Basal lamina: occasionally present
6. Caveolae: 2–4% of cytoplasmic volume; ~0.5 caveolae/μm of cell membrane length
7. Mitochondria: 5–10% of cytoplasmic volume
8. Endoplasmic reticulum: about 1–2% of cytoplasmic volume, either smooth or rough
9. Cytoskeleton: intermediate and thin filaments, as well as microtubules
10. Myosin thick filaments: undetectable

Some characteristics (morphology and density) of telocytes change with aging and some conditions. For instance, amount of TCs is decreasing in liver fibrosis, mirroring the recent findings as described in the colonic wall in ulcerative colitis, the terminal ileum of patients affected by small bowel Crohn's disease, and skin, gastric wall, lung and myocardium in systemic sclerosis [13, 14]. In pregnant uteri endometrial TCs increase, compared with non-pregnant, in spite of a significant decline in the number of myometrial TCs. Postpartum uteri show the highest significant count of myometrial TCs and non-significant difference in endometrial TC count, as compared with the adult non-pregnant group [15, 16]. Alunna *et al.* revealed that telocytes were markedly reduced in minor salivary glands from primary Sjögren's syndrome patients compared to normal and

non-specific chronic sialadenitis of minor salivary glands. Such a decrease was associated with both worsening of glandular inflammation and progression of ectopic lymphoid neogenesis [17]. Li *et al.* mentioned that TCs in vasculature could appear with slightly modified morphology, with more spherical, shorter and thicker prolongations [18].

Cantarero *et al.* have identified in TCs the presence of a single non-motile cilium called primary cilium. Primary cilia contain a 9+0 axoneme, consisting of nine outer doublet microtubules but lacking the central pair of microtubules. Except for nodal cilia, primary cilia are thought to lack axonemal dyneins and be immotile. Primary cilia in TCs might play a role in signalling processes within the vascular niche [19]. Moreover, in arterioles, TCs often send Tps bordering the tunica adventitia, while in venules and capillaries, they were located parallel with the longitudinal axis of the smooth muscle cells of the vessel wall [19]. The density of TCs in blood vessels is different, by region [18].

Distribution of telocytes

Telocytes have been found in a large variety of organs and are distributed in vertebrates (fish, reptiles, birds, mammals, including human) (Table 1) [5, 9, 15].

Table 1. Localization of telocytes in various organs.

Organ	Localization of telocytes
Blood vessels (coronary arteries, internal thoracic arteries and carotid arteries)	on the endothelial surface [19, 20]
Bone marrow	in close spatial relationships with small blood vessels and/or capillaries [20]
Canine dura mater	closed to capillary and surrounded by a great deal of collagen fibers [9]
Duodenum	in the lamina propria, immediately below mucosal crypts [19]
Endocardium	in the subendothelial layer, between the endocardial endothelium and the cardiomyocytes bundles [21–23]
Endometrium	in the human endometrial stroma of the stratum functionalis and in the basal endometrium after menstruation [24–27]
Epicardium	in human subepicardial area, in between collagen fascicles, in the neighbourhood of a cardiomyocyte [28]
Esopagus	in lamina propria of human oesophageal mucosa, submucosa, as well as in muscular layer, in the adventitia [29, 30]
Exocrine pancreas	in close proximity with both secretory acini and exocrine epithelial ducts and regulatory nerves and blood vessel apparatuses [31–34]
Eye	in limbus, sclera and uvea of eye [35]

Organ	Localization of telocytes
Fallopian tube	in mucosa and muscular layer among smooth muscle fibres [3, 11, 36]
Fascia lata	between collagen fibers [37]
Gallbladder	in the muscularis propria and in the bile ducts [38–40]
Heart valves	in the interstitial layer of human cardiac valves in all three valve types (mitral, tricuspid and aortic), in both apex and base of heart valves [41]
Ileum	in the in the muscularis and the lamina propria [42]
Jejunum	in the lamina propria of jejunum just beneath the epithelial layer of the mucosal crypts and in between the smooth muscle cells of muscularis mucosae [43]
Kidney	around renal tubules and vessels in the kidney cortex interstitium (in sub-capsular space) [44–46]
Liver	in the Disse space of the liver [47]
Lungs	in interstitial space of a intralobular bronchiole, in terminal and respiratory bronchioles, in alveolar ducts [48, 49]
Mammary gland	in non-epithelial tissue compartments [50–52]
Meninges and choroid plexus	in the vicinity of putative stem cells [53]
Mesentary	in the vicinity of and intermingled with capillaries, nerve bundles, adipocytes and other interstitial cells, mainly macrophages and fibroblasts [54]
Minor salivary glands	formed an almost continuous layer encircling both the excretory ducts and the secretory units [17]
Myocardium	TCs represent a small fraction of human cardiac interstitial cells [55–60]
Myometrium	in the myometrial interstitium [15, 61, 62]
Neuromuscular spindles	form the innermost and (partially) the outermost layers of the NMS capsule, and the internal capsule [63]
Parotid glands	around ducts of various calibers [64]
Placenta	in the large stem villi, with their long, slender process surrounding the blood vessel wall, or interposed between arterioles and the trophoblast basement membrane in small stem villi [65–67]
Pleura	in human parietal pleura, the sub-mesothelial space contained numerous telocytes [68]
Prostate	in prostatic stroma, especially in the adjacent epithelial area [69]
Pulmonary vein	at the internal limit of the myocardial sleeves, parallel with the long axis of the pulmonary vein [70]

Table 1. Cont.

Organ	Localization of telocytes
Renal pelvis	in the lamina propria [71]
Skeletal muscles	in interstitium: [72, 73]
Skin	in dermis [74]
Spleen	in red pulp [75]
Temporomandibular joint disc	closed to collagen bundles [76]
Testis	in the outer layer around peritubular cells [77]
Thoracic duct	subendothelial region of the wall as well as in intimate association with smooth muscle bundles throughout the media [78]
Trachea	among smooth muscle fibers and endothelium [48, 79]
Trigeminal ganglion	in close vicinity to microvessels and nerve fibers around the neuronal-glial units (NGUs) [80]
Urethra	in the lamina propria [71]
Ureters	in the lamina propria, mainly exist in between smooth muscle bundles [45, 81, 82]
Urinary bladder	in the lamina propria [45, 83]

Vicinity of telocytes and its secretomes

TCs demonstrate specific direct (homocellular and heterocellular junctions) and/or indirect (chemical, paracrine/juxtacrine signalling, microvesicles and exosomes, sex hormone and microRNAs) contacts with various surrounding cells. Homocellular junctions allow TCs to keep an architecture of tissue, generating 3D (three-dimensional) networks. Moreover, they contain elements of the cytoskeleton such as microfilaments, microtubules and vimentin [3]. Connections between TCs-exosomes-intercellular junctions cytoskeleton form the equivalent of a primitive nervous system [84]. In the heart TCs make contacts with different morphology (puncta adhaerentia minima, processus adhaerentes and manubria adhaerentia) [22, 23]. In the TCs the most represented are the nexuses (gap junctions), that are known to allow the exchanges of metabolites and signals [2, 7].

Heterocellular contacts TCs make with a variety of cells: smooth muscle cells, nerves, immunocytes (macrophages, mast cells and lymphocytes), stem cells, melanocytes in the eye [35], erythrocytes in the spleen [75] and with Schwann cells in the heart [23]. Gherhiceanu *et al.* reported that TCs make contact with virtually all types of cells in the human heart. His team suggested that heterocellular contacts occur by means of minute junctions (*point contacts*, *nanocontacts* and *planar contacts*)

and the mean intermembrane distance is within the macromolecular interaction range (10–30 nm) [23]. Moreover, TCs establish close contacts, stromal synapses (connective connections), with tracheal mast cells and in the trigeminal ganglion [79, 80].

Telocytes release at least three types of extracellular vesicles: exosomes (45 ± 8 nm), ectosomes (128 ± 28 nm) and multivesicular cargos (1 ± 0.4 μm) from their Tps and, occasionally, from the cell body [6, 85]. Yang *et al.* observed that the vascular TCs secreted more vesicles and bands in the Tps than the TCs that were located within other structures. The presence of a large number of vesicles appears to be a conserved feature of TCs regardless of their location [77]. These cells secrete interleukins (IL-2, IL-6, IL-10 and IL-13), growth factors (VEGF and EGF), nitric oxide, macrophage inflammatory protein 1α and 2 (MIP-1α and MIP-2), Monocyte Chemoattractant Protein 1 (MCP-1), Growth-Related Oncogene/Keratinocyte-derived Chemokine (GRO-KC). Three major classes of elements in telocyte secretomes include growth factors, chemoattractants, and cytokines/chemokines, indicating that telocytes may regulate stem cell growth and differentiation, microenvironmental formations [86–89]. Yang *et al.* revealed the presence of TCs that were directly connected to Leydig cells, which suggests that TCs are indirectly involved in the secretion of testosterone, rostenedione and dehydropiandrosterone [77].

Telocytes and its genes, proteins and microRNAs

The four different studies were conducted on gene expression profile of TCs in the last two years. Researchers focused on TCs-specific or TCs-dominated gene profiles in chromosome 1, 2, 3, 17 and 18 using global comparison between TCs and other cell types found in the mouse lung tissue [90]. TCs had a strong number of up- and down-regulated genes in all patterns (Table 2). Important to note that amount of down-regulated genes was 2–3 times higher than up-regulated in all observed chromosomes.

Table 2. Number of up-regulated and down-regulated genes in chromosomes of telocytes.

Chromosome	Number of up-regulated genes	Number of down-regulated-genes
1	14	39
2	26	80
3	13	59
4	17	56
17	16	68
18	10	22

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After analysis of up-regulated genes functions in TCs, it had been mostly suggested that these cells are involved in cellular signaling, cell expansion and movement (migration, adhesion, migration and division), embryogenesis, morphogenesis and tissue homoeostasis (including immune homeostasis), tissue remodelling and repair, maintenance of oxidative microenvironment preventing tumorigenesis and anti-inflammatory responses [90–96].

Zheng *et al.* provided the first proteomic analysis on TCs and showed these cells are exactly different from the protein expression point of view. In TCs proteins were mainly located in the cytoplasmic compartment and involved in cell signalling, energy and metabolic pathways. Myosin-14, periplakin and envoplakin, SOD2 (SODM), acid ceramidase were up-regulated in TCs. Several proteins up-regulated in TCs were found among the top 100 vesicular proteins that are present most frequently in mammalian extracellular vesicles proteome [92, 96].

TCs express significant amount of pro-angiogenic microRNAs (miR126, miR130a, let-7-family, miR-10, miR-155, miR-503, miR-126, miR-27b, miR-503, and miR-100), also miR-21, miR-22, miR-29 and miR-199a, both stromal specific and vascular smooth muscle specific (miR-143/145). These cells do not express miR-193 and have lack of expression of cardyomyocyte-specific miRs (miR-1 and miR-133a or miR-208) [2, 60, 97, 98].

Immunohistochemical features

Nowadays immunohistochemistry combined with TEM is the most applicable method to identify TCs (Table 3). Despite the fact that has not yet been found a specific marker for TCs, usually for primary identification scientists use CD34 [99]. Important to note that CD117/c-kit has been excluded for some organs or its parts [99] and differs between TCs populations (possible site dependant) [2, 6, 100]. For instance, rat uterus tissue contains different types of immune positive TCs: c-kit (–)/vimentin (+), c-kit (+)/vimentin (+), c-kit (+)/CD34 (+), while in human dermal tissue TCs were c-kit (–)/CD34 (+)/CD31 (–) [101, 102]. This range might be the basis of region-specific TCs roles [6, 103].

Table 3. Immunohistochemical profile of telocytes.

Positive	Negative
CD34, CD117/c-Kit, plated-derived growth factor receptor alpha and beta (PDGFRα and-β), VEGF, inducible nitric oxide synthase (iNOS), calveolin-1, vimentin, connexin 43, oestrogen and progesterone receptors (PRs), CD44, desmin, nestin, cadherin-11, CD29, CD10	Procollagen 1, CD31/PECAM-1, α-smooth muscle actin (α-SMA), CD11c, CD90/Thy-1, CD68, CD1a, CD62-P, CD45

The best available choice is a combination of four immunohistochemical markers: CD34, c-kit, vimentin and PDGFRα [4, 14, 103]. However, for differential diagnosis between TCs and other cells is often used a double immunolabelling [100, 104]. Compare cardiac TCs with cardiac fibroblasts and pericytes, Bei *et al.* demonstrated that cardiac TCs are CD34/c-kit, CD34/vimentin and CD34/PDGFR-β positive and α-SMA weak positive, while cardiac fibroblasts are only vimentin and PDGFR-β positive and pericytes are CD34 negative, α-SMA and PDGFR-β positive [104, 105]. Endoneurial fibroblasts are CD34 positive [7, 106].

Zhou *et al.* experimentally showed a high expression level for PDGFR-α compare with PDGFR-β in cardiac TCs [102]. The double immunofluorescent staining for CD34 and PDGFR-α is considered to be a specific immunohistochemical marker for TCs in gastrointestinal tract [100, 107].

TCs inconstantly express stem cell markers such as Sca-1 (Stem cell antigen-1) and Oct4 (octamer-binding transcription factor 4) [2]. Chang *et al.* depicted that splenic TCs express nanog (a transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells) and Sca-1, while c-kit negative [44]. Using flow cytometry analysis, Bei *et al.* showed that TCs were homogenously positive for mesenchymal marker CD29 but negative for hematopoietic marker CD45, which is similar to bone marrow-derived mesenchymal stem cells [105]. In addition, as CD34+ cells may lose CD34 expression and acquire other marker expressions "in vivo" and "in vitro" [105, 108]. Petre *et al.* found that TCs in the mammary gland stroma were CD10±/c-kit-/vimentin+ [109, 110].

Electrophysiological characteristics

Recently, studies on the electrophysiological properties of TCs have shown various types of ionic channels in different organs (transient outward and inward currents). In different organs TCs have been shown to possess different types of potassium, chloride and calcium channels.

Lee *et al.* found that TCs in murine detrusor muscle express small-conductance Ca^{2+}-activated K^+ channels, most prominently the SK3 isoform, whereas expression of SK channels was low in smooth muscle cells [111]. It followed that SK channel regulation of bladder excitability was likely mediated through TCs rather than through SMCs. Moreover, SK3 channels have been identified in the myometrium and in the glandular and luminal epithelium of the endometrium [112]. Kim *et al.* showed the presence of calcium-activated potassium channels in stomach [3, 113, 114]. Cretoiu *et al.* suggested that rhythmical intracellular calcium discharges originating in TCs contribute to the pacemaker activity [3].

Sheng *et al.* firstly demonstrated that cardiac atrial and ventricular TCs expressed large conductance Ca^{2+}-activated K^+ current (BK_{Ca}) and inwardly rectifying

K^+ current (IK_{ir}), but not transient outward K^+ current (I_{to}) and ATP-sensitive potassium current (K_{ATP}) [115].

In human myometrium, patch-clamp recordings of TCs revealed a calcium-dependent hyperpolarization-activated chloride inward current, but absence of L-type calcium channels, which was postulated to modulate myometrial smooth muscle contractions [3, 115]. Rosenbaum *et al.* observed small-conductance calcium-activated potassium currents in human myometrium and concluded that its expression is higher in non-pregnant compared to pregnant tissue [116]. A similar situation with SK3 expression in vascular endothelium is found during pregnancy. These are also expressed in TCs and are down-regulated during pregnancy when they reduce contractility [6, 117].

T-type calcium channels are present in TCs from human myometrium, which in pregnancy and labour participate in the generation of endogenous bioelectric signals responsible for the regulation of the surrounding cell behaviour. It might be the missing link for describing the molecular mechanisms by which TCs are involved in mechanical stretching during uterine enlargement in pregnancy. The expression of α-subunit of T-type calcium channels in TCs is less intense in the case of non-pregnant myometrium [6, 118]. Steroid hormones and oxytocin might mediate the higher expression of T-type calcium channels in TCs derived from pregnant myometrium. As TCs have steroid hormone receptors, this might lead to frequent and sustained contractions that are able to trigger birth [6]. In fetal cardiac myocytes, T-type Ca^{2+} channels were suggested to play role in the regulation of cardiomyocyte size [118].

TCs have differences in reactivity to the low-level laser stimulation (LLLS). In pregnant myometrium primary cultures a growth rate of lateral telopodal extension of TCs is higher than in non-pregnant ones. Twenty-five percent of TCs from pregnant uterus present a local thickening of the TP upon LLLS. The local thickening phenomenon was directly correlated with a delayed telopodal response to stimulation [119]. C-kit inhibition by imatinib (receptor antagonist) led to a reduction in both the amplitude and frequency of myometrial contraction in a dosedependent manner. TCs might be players in the coordination of uterine activity in a kit-independent manner [25, 120].

Myometrial TCs have large input resistance, ranging between 1.2 and 12 GΩ. They failed to produce the regular slow waves of depolarization described in classical ICCs, although some irregular excursions of membrane potential ranging from 10 to 35 mV have been observed by Duquette *et al.* TCs did not generate action potentials in response to depolarizing current. Only passive electric potentials were recorded when current pulses were applied [105].

Possible role of TCs

Nowadays, more researchers focus on intercellular communication of TCs and its roles in cells niche. The number of publications is gradually rising, reflecting the importance of these cells. Sometimes at the beginning, data might be slightly speculative, but later they can be empiric proved. Likewise, more attention to attract connection TCs with smooth muscle cells, nerve endings, vessels and stem cells. They play a key role in a variety of pathological processes (myocardial infarction, heart failure, renal ischemia-reperfusion injury, liver fibrosis and others) and adaptive responses [121–125].

TCs might behave as an immune system modulator interrelating immune cells in interstitium context and providing functional support [30]. For instance, TCs are major cell type of the human thoracic duct [78]. The importance of TCs in normal and pathological immune response is faceted, proved by different point of view. Ardeleanu *et al.* proposed that TCs could be the common cells of origin for both perivascular epithelioid cell tumours (PEComas) and gastro-intestinal stromal tumours (GISTs) [34, 121]. Mou *et al.* proposed that stromal cells containing TCs might influence the self-assembly of reconstituted breast cancer tissue [51]. Mandache *et al.* considered that TCs might play an important role in amyloid deposits formation [10]. Important to note, TCs can be a structural and functional unit of main immunological barriers in the human organism. Yang *et al.* suggested that TCs play important roles in the blood-testis barrier [77], whereas Gherghiceanu *et al.* proposed involving TCs in "blood–myocardium barrier" as they the main population in the sub-epithelial layer of endocardium [21].

The tandem telocytes-stem cells has been found in stem-cell niches in various organs (e.g. epicardium, lungs, skeletal muscle, choroid plexus, skin) [35, 122, 123]. Cantarero *et al.* proposed TCs with nerve fibers and blood vessels form such functional unit as "'mesenchymal cell niche" [19], while Luesma *et al.* suggested that there are two different types of stem-cell niches into the eye: epithelial niches (basal cells in cornea and conjunctiva) and stromal niches (iris, corneoscleral junction). The TCs network could even be a scaffold for stem cells migration between different layers of the eye [35]. Moreover, a study, made by Gherghiceanu and Popescu in 2009, has suggested that TCs are involved in mesothelial renewal and might guide the migration of mesenchymal cells into the mesothelial layer of the epicardium [28]. According to Petre *et al.* TCs "could be actors in the mammary stem niche" [43, 109]. Splentic TCs could take part in formation of splenic hematopoietic niche and play important role in transmitting the signals [75]. Alunna *et al.* suggested that a loss of minor salivary glands telocytes might have important pathophysiological implications in primary Sjögren's syndrome [17].

Telocytes are located in the neuromuscular spindles and participate in the control of muscle tone and motor activity. They produce electric slow waves that trigger and

coordinate smooth muscle contractions in the uterus. The decreasing in TCs caused dysregulation of oviduct motility, suggesting that tubal TCs impairment leads to the infertility of tubal origin and even tubal ectopic pregnancy [103, 126, 127]. Matyja *et al.* showed that a reduction in TC number may be a consequence of the toxicity of the supersaturated bile, while some other bile components (glycocholic and taurocholic acids) may exert protective effects on TCs and thus possibly influence the mechanisms regulating gallbladder and extrahepatic bile duct motility [39, 128–130]. Fu *et al.* found that hepatic TCs were significantly decreased by 27%–60% in human liver fibrosis, suggesting that loss of TCs might lead to the altered organization of extracellular matrix [13].

Telocytes have a powerful potential in tissue repair and regeneration (in heart, lung, skeletal muscle, skin, meninges and choroid plexus, eye, liver, uterus and urinary system) [131, 132]. It might be a future target for therapeutic value in preventing of diseases. In conclusion, it is not superfluous to emphasize the importance of new studies, that allow us better understanding the nature of Telocytes.

Conflict of interest

None declared.

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DECLARATION OF CO-AUTHORSHIP

As a co-author of the article entitled: "**Telocytes: facts, speculations and myths (Review article)"** I declare that my own substantial contribution to the preparation, implementation and conducting of research and presentation of the work's results in this publication included: drafting of the manuscript; critical revision of the manuscript for important intellectual content; final approval of the manuscript.

At the same time, I agree to submit the above-mentioned article by Veronika Aleksandrovych as a part of her doctoral thesis in the form of a coherent thematic collection of articles published in scientific journals.

I declare that an independent and identifiable part of the mentioned article shows the individual contribution of Veronika Aleksandrovych in acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; final approval of the manuscript.

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DECLARATION OF CO-AUTHORSHIP

As a co-author of the article entitled: "**Telocytes: facts, speculations and myths (Review article)"** I declare that my own substantial contribution to the preparation, implementation and conducting of research and presentation of the work's results in this publication included: drafting of the manuscript; critical revision of the manuscript for important intellectual content; final approval of the manuscript.

At the same time, I agree to submit the above-mentioned article by Veronika Aleksandrovych as a part of her doctoral thesis in the form of a coherent thematic collection of articles published in scientific journals.

I declare that an independent and identifiable part of the mentioned article shows the individual contribution of Veronika Aleksandrovych in acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; study supervision, final approval of the manuscript.

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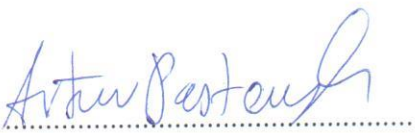
DECLARATION OF CO-AUTHORSHIP

As a co-author of the article entitled: **"Telocytes: facts, speculations and myths (Review article)"** I declare that my own substantial contribution to the preparation, implementation and conducting of research and presentation of the work's results in this publication included: drafting of the manuscript; critical revision of the manuscript for important intellectual content; final approval of the manuscript.

At the same time, I agree to submit the above-mentioned article by Veronika Aleksandrovych as a part of her doctoral thesis in the form of a coherent thematic collection of articles published in scientific journals.

I declare that an independent and identifiable part of the mentioned article shows the individual contribution of Veronika Aleksandrovych in acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; study supervision, final approval of the manuscript.



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March 6, 2019, Krakow

DECLARATION

Dr Marek Sajewicz, who was a co-author of the further articles entitled:

1. **"Telocytes: facts, speculations and myths (Review article)"**
2. **"Identification of uterine telocytes and their architecture in leiomyoma"**

has died in 2018.

Veronika Aleksandrovych

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DECLARATION OF CO-AUTHORSHIP

As a co-author of the article entitled: **"Identification of uterine telocytes and their architecture in leiomyoma"** I declare that my own substantial contribution to the preparation, implementation and conducting of research and presentation of the work's results in this publication included: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, study supervision, and final approval of the manuscript.

At the same time, I agree to submit the above-mentioned article by Veronika Aleksandrovych as a part of her doctoral thesis in the form of a coherent thematic collection of articles published in scientific journals.

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Katedra Patofizjologii UJ CM
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March 6, 2019, Krakow

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